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AUTOIMMUNITY TO HEAT SHOCK PROTEIN 60 (HSP60): ANALYSIS OF ANTIGENICITY OF HSP60 AND ANTIGEN-SPECIFIC CYTOKINE PRODUCTION

by

YAJUN YI

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABBREVIATIONS

aa	:	Amino acid residue
APC	:	Antigen presenting cells
ConA	:	Concanavalin A
CO_2	:	Carbon dioxide
CTL	:	Cytotoxic T cell
d	:	Day
dpm	:	disintegrations per minute
DNA	:	Deoxyribonucleic acid
DTH	:	Delayed type hypersensitivity
EAE	:	Experimental autoimmune encephalomyelitis
EB	:	Elementary body
ELISA	:	Enzyme linked immunosorbent assay
FCS	:	Fetal calf serum
Fig	:	Figure
GAGs	:	Glycosaminoglycans
GPIC	:	Guinea pig inclusion conjunctivitis
GST	:	Glutathione S-transferase
h	:	Hour
hsp60	:	Heat shock protein 60
^3H	:	Tritiated

HBSS : Hank's balanced salt solution

IDDM : Insulin-dependent diabetes mellitus

IFA : Incomplete Freund's adjuvant

IFN : Interferon

IFU : Inclusion forming units

Ig : Immunoglobulin

IL : Interleukin

IL-2R : Interleukin-2 receptor

i.p. : Intraperitoneal

kDa : Kilodalton

log : Logarithm

LPS : lipopolysaccharide

LGV : Lymphogranuloma venereum

LNCs : Lymph node cells

M : Molar

mAb : Monoclonal antibody

mg : Milligram

MHC : Major histocompatibility complex

min : Minute

ml : Milliliter

mM : Millimolar

MOMP : Major outer membrane protein

mRNA	:	Messenger RNA
NCM	:	Nitrocellulose
ng	:	Nanogram
Ova	:	Ovalbumin
O.D.	:	Optical density
PBLs	:	Peripheral blood lymphocytes
PBS	:	Phosphate buffer saline
PID	:	Pelvic inflammatory disease
RB	:	Reticulate body
rpm	:	rotations per minute
S.D	:	Standard deviation
sIg	:	Surface immunoglobulin
TCR	:	T cell receptor
TGF-ß	:	Transforming growth factor-ß
Th	:	T helper
TNF	:	Tumour-necrosis factor
U	:	Unit
μCi	:	Microcurie
μg	:	Microgram
vs	:	Versus

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ABSTRACT

Heat shock protein 60s (hsp60s) are a family of related proteins which are inducible and/or constitutively expressed by prokaryotic and eukaryotic cells in response to stressful stimuli and appear to protect the cell against damage due to stress induced protein misfolding. The family of hsp60 proteins has a high degree of amino acid sequence identity (over 40%) from over 50 different prokaryotic and eucaryotic species. Immune response to *Chlamydia trachomatis* heat shock protein 60 (hsp60) may determine chlamydial disease pathology. Native and recombinant chlamydial hsp60 elicits delayed mononuclear cell inflammation when applied to the conjunctivae of immunologically primed experimental animals. High-titre antibodies to the chlamydial hsp60 have been consistently found to be associated with infertility related to tubal occlusion and ectopic pregnancy in women with chlamydial pelvic inflammatory disease.

Our hypothesis is that the autoimmune responses to self hsp60 elicited during infection are possibly due to the stressful conditions at an inflammatory site inducing expression of hsp60 in both microbial and host cells. The objective of the present study was to examine the possibility that hsp60 may induce autoimmunity and determine the mechanism of the autoimmune responses.

Initially I characterized the antibody responses to specific hsp60 peptides with sera engendered in humans by natural infections and in rabbits by immunization with whole *C. trachomatis* elementary bodies (EBs). Thirteen major epitopes in chlamydial hsp60 were identified with the human sera, 10 of which were also detected with rabbit antisera. Seven of the 13 epitopes recognized by human

antisera exhibited cross-reactive antibody binding to homologous peptide sequences in human hsp60. Then using a mouse immunization model I found that mice are normally tolerant to mouse hsp60 and that immunization with chlamydial hsp60 alone does not induce strong autoimmune responses to mouse hsp60. Under conditions of concurrent immunization with chlamydial and mouse hsp60, however, autoimmune B and T cell responses to self hsp60 are readily generated. The ability of chlamydial hsp60 plus mouse hsp60 to induce autoimmunity critically depended on the amount of mouse hsp60 and chlamydial hsp60 used for immunization. Moreover, tolerance to mouse hsp60 was found to be mainly due to T cell anergy and was associated with a predominant antigen-specific Th2-like IL-10 cytokine secretion pattern. Autoimmune responses to self hsp60 induced by co-immunization with chlamydial and mouse hsp60 were associated with a marked decrease in IL-10 production and an increase in IFN-gamma production in response to mouse hsp60. Finally, at least two T helper sites located at regions (aa1-150, aa268-409) on chlamydial hsp60 were identified as responsible for high levels of autoantibody induced by the co-immunization protocol.

These observations are helpful for understanding the immunologic properties of chlamydial/host hsp60 and their relationship to disease pathogenesis. Moreover, these observations may ultimately be useful for antigen-specific immunotherapy in the treatment of autoimmunity induced by chlamydial hsp60.

INTRODUCTION

I. LITERATURE REVIEW

1.1.1. Taxonomy

Chlamydiae are a family of obligate intracellular bacterial parasites with a tropism for columnar epithelial and lymphoid cells. It became clear that chlamydiae had all the requisite properties of bacteria (Stephens, 1993). Like other bacteria, they (1) possess a cell envelope similar to that of gram-negative bacteria, (2) contain both DNA and RNA, (3) possess prokaryotic ribosomes and synthesize their own proteins, nucleic acids, and lipids, and (4) are susceptible to a wide range of antibiotics.

The genus *Chlamydia* consists of four species: *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae* (Page, 1968; Grayston, 1989) and *Chlamydia pecorum* (Fukushi, 1992). *C. trachomatis* was later subdivided into three biovars: trachoma, lymphogranuloma venereum (LGV), and mouse (Moulder, 1984). The degree of DNA relatedness between *C. trachomatis* and *C. psittaci* has been reported to range from less than 10% to almost 30% (Cox, 1988; Fukushi, 1989; Kingsbury, 1968), and *C. pneumoniae* DNA exhibits less than 10% relatedness to the DNA of the other two species (Cox, 1988). However, the monophyletic origin of the genus is unquestionable. Molecular evaluation of 16S rRNA sequences confirms that chlamydiae are eubacteria, but with only very distant relationships to other eubacterial orders (Weisburg, 1986). The 16S rRNA sequences in *C. trachomatis* biovar LGV and *C. psittaci* 6BC differ from each other by only 5% while at the same time being deeply separated from all other known eubacterial 16S rRNA sequences (Weisburg, 1989). The 16S rRNA of *C. pneumoniae*

closely resembles that of the other two chlamydial species. The 16S rRNA genes of the trachoma and LGV biovars are closely related, and the corresponding genes in *C. trachomatis* biovar mouse and *C. psittaci* strain guinea pig inclusion conjunctivitis (GPIC) share partial but not complete nucleotide sequence homology with the *C. trachomatis* biovar LGV gene (Palmer, 1986). All three species have proteins with extensively shared sequences (Campbell, 1990; Campbell, 1989; Herring, 1989; Zhang, 1989; Zhang, 1990). Of the three biovars of *C. trachomatis,* LGV and trachoma exhibit almost 100% DNA homology, but the DNA relatedness of the mouse biovar to the other two biovars is 30 to 60% (Weiss, 1970; Fukushi, 1989). There is a very high level of sequence homology between the proteins of the trachoma and LGV biovars (Hamilton, 1989; Peterson, 1988; Zhang, 1989).

Of the three biovars of *C. trachomatis,* only biovars of trachoma and LGV have been well characterized regarding their interactions with host cells in vitro. The two biovars have been further separated by indirect microimmunofluorescence into 15 often closely related serovars (Wang, 1982; Wang, 1973; Grayston, 1975). LGV, which infects lymphatic cells to produce the disease, lymphogranuloma venereum, for which it is named, exists in three serovars, L1, L2, and L3. The trachoma biovar is a parasite of the squamocolumnar cells of mucous membranes, which it infects by two different modes of transmission (Schachter, 1978). Serovars A, B, Ba, and C are associated with trachoma, a follicular conjunctivitis spread by close person-to-person contact, whereas serovars D through K are commonly associated with sexually transmitted infections. Except for genus-specific antigens on the chlamydial lipopolysaccharide (LPS), the major serovar-, subspecies-, and species-specific antigenic epitopes are all attributable to a single, quantitatively predominant, major outer membrane protein (MOMP) (Caldwell, 1981;

Stephens, 1982). There are no nonhuman reservoirs for any of these chlamydial infections. Compared with the trachoma biovar, LGV has relatively few well-characterized strains. By far the most frequently used are strains 434L and 440L, isolated from patients with classical LGV (Schachter, 1969). More than a score of trachoma isolates belonging to both the A through C and the D through K groups of serovars have been employed in cell culture investigations. Biovars trachoma and LGV often differ sharply in their behaviour in cell culture, but within each biovar, little variation among the different strains examined has been recorded.

C. psittaci is genetically (Fukushi, 1989) and phenotypically (Perez-Martinez, 1985; Spears, 1979a; Spears, 1979b) more diverse than *C. trachomatis*. Comparison of 29 strains of *C. psittaci* growing in mouse fibroblasts (L cells) revealed numerous differences in growth rate and other characteristics (Spears, 1979a). Strains designated as *C. psittaci* have been isolated from humans and a very large number of avian and mammalian species, in which they produce a broad spectrum of disease (Schachter, 1969; Storz, 1971).

The interaction of chlamydiae with host cells in culture has been studied with only a limited number of established cell lines and primary cell cultures. For *C. psittaci*, the L-929 line of mouse fibroblasts (Sanford, 1948) has been by far the most frequently used. For *C. trachomatis*, cell lines Hela 229 (a line derived from a human cervical carcinoma [Scherer, 1953]), McCoy (a mouse fibroblast line of uncertain origin [Gordon, 1972]), and BHK-1 (a line of diploid hamster fibroblasts [Stoker, 1964]) have been widely employed.

Most strains of *C. psittaci* and the LGV and mouse biovars of *C. trachomatis* may be titrated for infectivity by the formation of plaques in L-cell monolayers, but the trachoma biovar can not, probably because it does not initiate secondary cycles of infection (Furness, 1962).

However, the infectivity of chlamydiae, the plaque formers included, is most often titrated in terms of inclusion-forming units, i.e., the number of inclusions (number of infected cells) produced under standard conditions (Furness, 1960; Kuo, 1976). It is probable, but not proven, that one inclusion-forming unit equals one infectious elementary body (EB).

1.1.2. Growth cycle.

The chlamydial infectious cycle is initiated by the EB binding to receptor(s) on the host cell. Polycations (Kuo, 1973) and divalent cations (Hatch, 1981; Sneddon, 1985) enhance chlamydial binding to eukaryotic cells, suggesting that electrostatic interactions function in the initial phase of chlamydial attachment. Subsequent steps in chlamydial attachment likely involve specific ligand-receptor interactions or other high-affinity binding mechanisms which are saturable. The adherence of any *C. trachomatis* strain is competitively inhibited by heterologous strains, suggesting that this specific binding involves a common host cell receptor (Vretou, 1989). Chlamydial proteins that bind to host cells have been identified by separating chlamydial polypeptides by electrophoresis and reacting them with surface-iodinated host cell extracts or isolated host cell membranes. In one investigation, a number of biovar LGV and trachoma strains of *C. trachomatis* displayed two proteins with apparent molecular masses of 18 and 32 kDa that bound to extracts of HeLa cells (Wenman, 1986a), whereas two *C. psittaci* strains had only a single binding component of 17- to 19-kDa (Hackstadt, 1986). A second study revealed that single biovar LGV and trachoma strains have 18- and 31-kDa proteins that bind to membranes from either HeLa or McCoy cells (Wenman, 1986a) and that one *C. psittaci* strain

(MN/Cal 10) has 16- and 30-kDa proteins that are bound by HeLa cells (Wenman, 1986b). The gene encoding the 31-kDa protein has been cloned and expressed in *E. coli* (Kaul, 1987). These putative chlamydial adhesins have many of the properties to be expected of ligands that attach chlamydiae to their host cells. They are found in isolated cell wall fractions (Wenman, 1986a). Heparin, already identified as an inhibitor of attachment, is also bound by the 18- and 32- kDa proteins (Hackstadt, 1986). A heparin sulfate-like glycosaminoglycans (GAGs) present on the surface of chlamydia organisms is required for attachment to host cells. Zhang and Stephens (1992) reported that chlamydiae synthesize a sulfated oligosaccharide that is a molecular mimic of eukaryotic heparin sulfate and use this for binding to a heparin sulfate receptor (Zhang, 1992). It has also been suggested that the MOMP facilitates a chlamydial attachment by promoting electrostatic and hydrophobic bonding with host cells (Su, 1990). Anti-MOMP monoclonal antibodies can efficiently inhibit attachment to host cells (Su, 1990). However, to date there are no direct data implicating MOMP as having a specific role as an adhesin. MOMP and MOMP peptides have not been shown to competitively inhibit EB attachment, and MOMP expressed on the surface of *E. coli* does not induce specific attachment of *E. coli* recombinants to host cells (Stephens, 1993). Therefore, neither the chlamydial adhesin nor its host cell receptor have been definitively identified.

There are two indisputable common characteristics of the initial interaction of the chlamydial cell with host cells. First, chlamydiae enter host cells that usually are not actively phagocytic (nonprofessional phagocytes [Rabinovitch,1968]); and second, entry terminates in the appearance of the chlamydial cell in a membrane-bound vacuole (inclusion) in the host cell cytoplasm. Even the incompletely characterized chlamydia like organisms that parasitize the cells of invertebrates

have these hallmarks of chlamydial entry. Studies concerning the process of entry have produced conflicting results. Part of the confusion occurs because chlamydiae may use different modes of cell entry (Stephens, 1993). Entry of chlamydiae involves active phagocytosis that is induced by EB binding to host cells (Byrne, 1978), and it occurs through a microfilament-dependent mechanism (Ward, 1984). As well, evidence for entry via receptor-mediated endocytosis has also been described (Hodinka, 1988). Internalized endocytic vesicles containing EBs do not fuse with host lysosomes (Friis, 1972). The mechanism by which chlamydiae prevent phagolysosomal fusion is not known, but it is likely a key virulence property that promotes intracellular survival. Shortly after internalization, the EB differentiates into a larger (800 nm) cell type termed the reticulate body (RB). DNA, RNA, and protein synthesis is initiated, and the RB multiplies by binary fission (Becker, 1978). The endocytic vesicle expands dramatically to accommodate the increasing number of RBs, and the resulting intracellular inclusion body displaces much of the host cell cytoplasm and organelles. Chlamydial-specific antigens have not been convincingly demonstrated in association with the plasma membrane of infected cells.

Chlamydiae reproduce by means of a developmental cycle (Ward, 1988) that consists of the alternation of two cell types, EBs and reticulate bodies (RBs). EBs never divide. They are released from infected host cells and enter uninfected ones, where they reorganize into RBs. RBs never infect new host cells (Tamura, 1967). Their role is to multiply and eventually transform themselves into a new generation of EBs.

Chlamydiae have evolved distinct infectious and reproductive forms in order to meet the often conflicting demands of extracelluar survival and intracellular multiplication. On entering a host cell, the chlamydia EB promptly begins to reorganize into an RB. This simple statement

generates several important questions. What component of the intracellular environment provides the signal for the EB-RB transformation? How is this signal transmitted to the phagosome-enclosed EB? What are the first steps in reorganization of an EB into an RB? How are these events regulated? Presently, none of these questions can be answered completely. It is of interest that *C. trachomatis* can inhibit fusion of its inclusion with lysosomes yet promote fusion of its inclusion with other *C. trachomatis*-containing inclusions. *C. psittaci* inclusions do not fuse with each other or with inclusions containing *C. trachomatis* (Matsumoto, 1991). RBs differ from EBs in many ways, both biologically and chemically. The RB has an osmotically and mechanically fragile cell wall, probably because the MOMP is no longer cross-linked with disulfide bonds (Hackstadt, 1985), It also has a fibrillar nucleoid in contrast to the highly compacted nucleoid of the EB (Costerton, 1976); the RB is noninfectious, and metabolically active. The early biochemical events are the synthesis of protein and reduction of the MOMP disulfide bonds to initiate the EB to RB reorganization (Hatch, 1986; Plaunt, 1988). Protein synthesis in RBs depend on the transport of ATP and ADP possibly via the porin channels provided by reduction of the MOMP (Hatch, 1982; Bavoil, 1984; Peeling and Brunham, 1989).

Once started, reorganization of EBs into RBs proceeds rapidly via numerous morphologically intermediate stages. By 8 to 12 h after infection almost pure RB populations are seen. The major outer membrane protein (MOMP) is constitutively expressed throughout the chlamydial growth cycle and functions as a porin in the metabolically active stage of the chlamydial growth cycle (Bavoil, 1984). Synthesis and incorporation of monomeric MOMP into outer membrane complexes is first detected 12 h after infection and continues throughout the developmental cycle (Hatch, 1986). In contrast, 60-and 12-kD outer-membrane proteins are only expressed late in

the developmental cycle (Newhall, 1987; Hatch, 1986), and their expression coincides with the asynchronous differentiation of noninfectious RBs into infectious EBs. This secondary differentiation process involves (a) disulfide bonding among the three outer-membrane proteins (60kd, 12kd and MOMP) and (b) the reformation of the rigid outer-membrane structure characteristic of the EB. The growth cycle is completed when the cell lyses or the intact inclusion is expelled from the host cell, thereby freeing EBs to reinitiate the infectious process (Todd, 1985). By 48 h, the intracellular chlamydial population consists mainly of EBs with outer membrane complexes containing MOMP still in the monomeric from. When the cells lyse at 46 h, the MOMP is rapidly cross-linked and infectious EBs are released.

1.1.3. The growth related persistence or latency of chlamydial infection.

Several in vitro models of persistence (Stephens, 1993) have been presented: 1) penicillin-induced persistence 2) essential metabolite (e.g. folic acid) deprivation, 3) interferon-gamma-mediated persistence, and 4) persistently infected cells selected following inoculation of high multiplicities of infection. Each is mediated by a partial blockade of chlamydial growth and /or development. The mechanism of interferon-gamma inhibition of chlamydial-infected cells differs depending upon the host cell employed. Using murine cells interferon-gamma induces nitric oxide production that inhibits chlamydial growth probably by limiting access to iron (Mayer, 1993). In contrast, interferon-gamma treatment of human cell lines inhibits chlamydial growth by limiting tryptophan availability (Thomas, 1993). In each of the first three systems chlamydial growth results in the ability to detect inclusions in infected cells and the inclusions usually

contain atypical and often large RB-like forms. The potential utility of these models to in vivo mechanisms depends upon the envisioned duration of the metabolic starvation and whether or not chlamydia are in a metabolic state that would make them refractory to immune mechanism for resolution of infection or eradication by antibiotic treatment. Significantly, the proposed model of IFN-gamma persistence of infection additionally is handicapped in that three serovars are autotrophic for tryptophan (Allan, 1983); thus this model does not represent a generalized mechanism. If these organisms were metabolically inactive they may be refractory to resolution by immune mechanisms or antibiotics. It will be of interest to apply modern protein and nucleic acid detection techniques to decipher the so called "cryptic" state of this model of persistence. Unfortunately there is little compelling epidemiological or clinical data or in vivo or in situ biological data, such as the detection of atypical RB-like forms in ultrastructural examinations of infected tissues, to support the relevance of persistence in vivo. Nothing about the "cryptic" state has been defined in biochemical or molecular terms. Thus such fundamental questions involving the natural history of infection remain open (Stephens, 1993).

1.1.4. Structure and Composition of *Chlamydia*

Chlamydial cell walls resemble the walls of gram-negative host-independent bacteria which possess two trilaminar membranes, an outer membrane and an inner cytoplasmic membrane (Matsumoto, 1988). The lipopolysaccharide in the outer membrane is rough and the cell wall lacks peptidoglycan. Both chlamydial cell types contain large and approximately equal amounts of MOMP with an apparent molecular mass of about 40 kDa. EBs also have lesser amounts of

12-, 59-, and 62-kDa outer membrane proteins exceptionally rich in cysteine residues and occurring in much smaller amounts in RBs (Hatch, 1984; Newhall, 1987; Zhang, 1987). The surfaces of chlamydial EBs are hydrophobic and negatively charged at neutral pH, with isoelectric points of about pH 5 (Batteiger, 1985; Schiefer, 1982; Soderlund, 1982). The MOMPs of both *C. trachomatis* biovars have identical isoelectric points (pI = 5.3 to 5.5). EB surfaces have patches of regularly spaced hemispheric projections that are specializations of the plasma membrane (Nichols, 1985; Matsumoto, 1975; Gregory, 1979). Provocative ultrastructural data suggest that chlamydial surface projections appear to pass through the inclusion membrane, thereby providing a direct link between the chlamydial and eukaryotic cytoplasmic compartments (Matsumoto, 1988). Throughout the developmental cycle, chlamydiae remain in a membrane-bound vesicle within the host cell which include the host membrane, the inclusion membrane , and the chlamydial inner and outer membranes. Whether this structure also provides the opportunity for processing and presentation of chlamydial antigens by endogenous MHC class I pathways is unknown. If antigen presentation does occur, the immunological consequence would be the opportunity for the host immune system to recognize and kill infected host cells (Stephens, 1993).

The only outer membrane complex (Caldwell, 1981) components defined at the molecular level are LPS, MOMP, OMP2, and OMP3. Both LPS and MOMP are surface-accessible to antibodies on RBs, but the immuno accessibility of LPS on EBs appears to be modified. OMP2 and OMP3 are developmental stage-specific cysteine-rich proteins present only in EBs. The current belief is that MOMP, OMP2 and OMP3 are extensively disulfide cross-linked, mediating the structural rigidity and osmotic resistance of the EB (Stephens, 1993,).

There are seven major chlamydial antigens recognized during natural human infection as seen by immunoblot analysis of sera from women with *C. trachomatis* cervical infection (Brunham, 1987). They are two heat shock proteins, hsp60 and hsp70; two membrane proteins, OMP-1 (40 kD, MOMP) and OMP-2 (57 kD, cysteine-rich outer membrane protein); LPS (10 kD); 32 kD (possibly a histone protein) and 29 kD (possibly the peptidyl cis/trans isomerase) antigens (Brunham, 1994).

The DNA is compactly organized in a central nucleoid and is a closed circular chromosome consisting of 1,045-kbase pairs (Stephens, 1993) with a molecular weight of 660 kDa. A molecule of this size could provide information for about 600 different proteins, which is approximately one fourth the amount provided by the *Escherichia coli* genome. Most strains of *C. trachomatis* also have a 7.4-kbase pair plasmid. It has recently been shown that this plasmid is not required for *C. trachomatis* growth or disease as a *C. trachomatis* strain isolated from a patient lacks the plasmid (Peterson, 1990). Interestingly, in vitro growth of this strain appears to be slower and less virulent; however, quantitative investigations of its biology have not been conducted (Stephens, 1993).

1.2. *C. trachomatis* Diseases

Infections caused by serovars A through K primarily localize to ocular and genital tract mucosal surfaces, where they commonly produce asymptomatic infections or acute self-limiting infections such as uncomplicated conjunctivitis, urethritis, or cervicitis. However, these may progress to chronic infections that provoke severe inflammatory responses and may lead to

blindness and infertility. Trachoma, a chronic inflammatory disease of the eye caused by serovars A, B, Ba, or C, is the leading cause of preventable blindness in the world. It is estimated that 500 million people in the world have trachoma, 7 million of whom are blind or have severely impaired vision (Morrison et al, 1992). Serovars D through K primarily infect the urogenital tract and are currently the leading cause of sexually transmitted infections in the United States and Europe. In women, *C. trachomatis* infections of the lower genital tract can ascend to the fallopian tubes, in which the immunopathological responses ultimately result in chronic inflammation which produces tissues fibrosis and organ damage (Grayston, 1985) such as chronic salpingitis,infertility or ectopic pregnancy. An estimated 200,000 women per year in the United States develop chronic chlamydial salpingitis, with approximately 20,000 becoming infertile as a result (Washington, 1987). Serovars L1, L2, and L3 cause lymphogranuloma venereum (LGV), a systemic chlamydial infection involving lymphatic tissues. LGV is not common in the United States, but it constitutes a significant public health problem in Africa and the Far East. A new chlamydial agent, TWAR, has recently been characterized as a common cause of acute respiratory infection (Stephens, 1993).

1.2.1. Virulence and Pathogenesis

Virulence differences between biovariants such as LGV and trachoma strain can broadly be considered from two points of view. First, virulence differences might be mediated at the interface of the organism and the host cell at attachment or uptake and inhibition of lysosomal fusion. Alternatively, this difference might reflect the ability of the LGV biovar to resist

cellular defense and/or grow more prolifically once inside the host cell. The mechanism is not clear; the rates of attachment and invasion appear similar, at least at high multiplicity of inoculum, but LGV is apparently better at resisting host cell defense as it survives better in macrophages and polymorphonuclear cells (Yong, 1987). It has been observed in vitro that LGV biovar strains produce earlier and larger inclusions than do trachoma biovar strains. The molecular basis for this "growth advantage" is unknown, although the trachoma biovars produce larger inclusions and greater infectious yields by the addition of cycloheximide to cultures. This fact suggests that the LGV biovar is a more efficient competitor with the host cell for required metabolites and raises the likelihood that host range and virulence may be determined by heterogeneity in biochemical capabilities. Virulence differences are probably not caused by a single determinant. Nevertheless, we have an inadequate understanding of the fundamental bases of virulence and host restriction, let alone an understanding of their molecular mechanisms.

On the basis of trachoma vaccine studies in monkeys and humans, Grayston and colleagues proposed that the host immune response during reinfection or chronic infection was the most important component in the production of severe inflammatory responses that led to conjunctival scarring (Grayston, 1985). The current model is that frequent reinfection and/or persistent infection leads to a cascade of inflammation and consequent scarring, with the significant sequelae of blindness, infertility and ectopic pregnancy (Stephens, 1993).

While immune pathogenesis is fundamentally the mechanism of chlamydial diseases, it is not clear if specific antigens and consequent immune responses are responsible, as in classical delayed-type hypersensitivity (DTH) responses, or if other inflammatory or immune regulatory processes such as autoimmunity cause disease (Stephens, 1993).

1.2.2. Immunity

In general our understanding of the immune responses to human chlamydial infections is limited, and the precise role of antibodies, T cells, phagocytes, and other immune effector mechanisms in host immunity are poorly defined. However, it is clear that both immunoprotective and immunopathologic responses are provoked by chlamydial infection.

The immune mechanisms primarily responsible for resistance to infection and clearance of an established infection are probably distinct, and little is understood about them. Monkey studies and human trials using intact EBs as a vaccine demonstrate that immunity to infection can be achieved. Significantly, protection in these studies was found to be serovar-specific at least within the context of the serovars used. The serologically variant antigen that defines the serovars is the MOMP, as determined by monoclonal antibodies and concordance between the serological relationships and those derived from MOMP gene (omp1) sequences (Yuan, 1990). Antibodies to surface exposed variant epitopes of MOMP play an important role in immune protection from infection by human strains of *C. trachomatis* (Stephens, 1993). The observation that this immunity is relatively short-lived suggests further that mucosal IgA is the primary effector. If sterilizing immunity is not achieved, then antibody-mediated clearance of an intracellular infection would appear to be a significant challenge for the immune system-even without considering subversive mechanisms for persistence. A role for MHC class I antigen presentation and cytotoxic T cell killing of *chlamydia*-infected cells has been reported by several groups (Beatty, 1994; Starnbach, 1994) with fundamental importance to understanding immunity to chlamydial infections. Besides the role of cytotoxic T lymphocytes (CTL), the control of

infection within the cell also relies on the inhibitory and potential cytotoxic effects of interferon-gamma or other cytokines suggesting an important role for CD4 lymphocytes in immunity (Byrne, 1988).

1.2.3. Immunopathogenesis

The chronic inflammatory sequelae that follow *Chlamydia* infection have been considered as due to immunopathogenic mechanisms (Brunham, 1994). Data from animal models of infection and human epidemiological studies demonstrate that chronic inflammation of trachoma and chlamydial salpingitis is not part of primary chlamydial infections, and only develops with reinfection. Repeated infections in monkeys, guinea pigs, or mice cause chronic inflammation, tissue damage, and scarring of the mucosal epithelium-pathologic changes that are not apparent in a primary infection (Taylor, 1982; Monnickendam, 1980; Patton, 1985; Patton, 1989a; Patton, 1987; Patton, 1989b; Quinn, 1986; Tuffrey, 1990). The concept that prior sensitization to chlamydiae causes more severe disease upon reinfection is also supported by results from early trachoma vaccine trials. Monkeys vaccinated parenterally with *C. trachomatis* developed more severe disease upon infection (Wang, 1967; Grayston, 1985; Grayston, 1962; Woolridge, 1967; Bell, 1969; Collier, 1967). The histopathological changes observed in these animal models resemble those of trachoma and chronic salpingitis, with (a) development of follicles whose germinal centres contain primarily B cells, (b) infiltration of the submucosal epithelium with macrophages and T cells, and (c) mucosal scarring (Patton, 1985; Patton, 1989a; Hogan, 1962; Moller, 1979; Abu, 1989; Whittum-Hudson, 1986; Burd, 1988).

Thus the immune response to chlamydial infection is somewhat paradoxical. Prior infection confers a degree of resistance to reinfection, as demonstrated by decreased shedding of infectious chlamydiae, but at the same time it sensitizes the host for the development of immunopathological changes.

A soluble noninfectious detergent extract of chlamydiae elicited severe ocular inflammation when applied to conjunctivae of previously infected animals (Watkins, 1986; Taylor, 1987). The inflammatory response was delayed (peaked at 24 hr), and it was characterized by follicular hyperplasia and a mononuclear macrophage and lymphocytic infiltrate of the submucosal epithelium. That histopathologic picture is typical of a delayed hypersensitivity (DH) response and resembles that found in conjunctivae from humans with chronic trachoma (Hogan, 1962; Abu, 1989). Most remarkable a single chlamydial antigen, the 60 kd heat-shock protein (hsp60), has been shown to be responsible for the delayed hypersensitivity response in previously infected animals (Morrison, 1989). Human data also suggest that hsp60 may elicit the immunopathologic responses of chlamydial infection. Thus high-titre antibodies to the chlamydial hsp60 have been consistently found to be associated with infertility related to tubal occlusion or ectopic pregnancy in women with chlamydial pelvic inflammatory disease (Morrison, 1989, 1992; Brunham, 1985; Toye, 1993). Wagar and Stephens (Wagar, 1990) detected strong serological responses to chlamydial hsp60 in patients with severe pathology, such as ectopic pregnancy caused by chlamydia, and in trachoma patients. In contrast, in the face of strong serological responses to other chlamydial proteins, no serological response to hsp60 was observed from most chlamydia-infected patients without severe pathology. Using the same patient sera, the reactivity to hsp60 was confirmed using protein obtained from the chlamydial gene expressed in *E. coli*

(Cerrone, 1991). In the presence of purified recombinant *C. trachomatis* 57-kDa hsp60, peripheral blood lymphocytes (PBLs) from 9 of 18 (50%) women with salpingitis, none of 10 women with a lower genital tract cervicitis, and 3 of 42 (7.1%) healthy women proliferated (Witkin, 1993). Furthermore, it appeared that among the patients with salpingitis, those women with a previous history of salpingitis or ectopic pregnancy had the highest prevalence of sensitization to hsp60. The T cell epitopes that PBLs of patients responded to was mapped to conserved hsp60 epitopes shared between the *C. trachomatis* and human hsp60 (Witkin, 1994). As hsp60s are highly conserved antigenically between bacteria and humans, eliciting immune responses to hsp60 in human hosts could precipitate enduring pathological events, even following resolution of infection by the induction of autoimmunity. Interestingly, other microbial hsp60s have also been documented to initiate chronic inflammatory diseases and in these diseases, autoimmune responses to hsp60 are also thought to be central to pathogenesis (Elias, 1991; van Eden, 1988). Thus, study of immune recognition of microbial hsp60 may help to elucidate the pathogenesis of a variety of chronic inflammatory diseases.

1.3. Heat Shock Protein 60 (hsp60)

Ritossa's early observations of the puffing of the *Drosophila* chromosomes in response to elevated temperatures resulted in the quest to characterize the changes produced in organisms following the stress of heat shock (Ritossa, 1962). These early observations resulted in the identification of a specific subset of proteins that were induced after heat shock and conferred protection to the cell during heat shock. These proteins were first named the heat shock proteins,

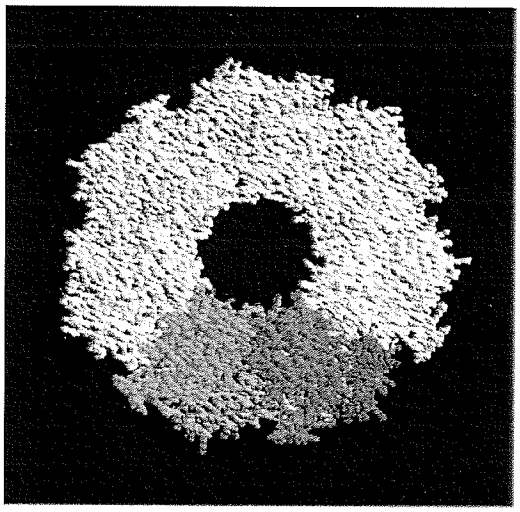
but as their induction to a variety of stress signals has now been well documented, they also are known synonymously as the stress proteins (Lindquist, 1988). The nomenclature of the hsps has been designated such that the molecular weight of the hsp identifies it as belonging to a particular group, with the known groups ranging in size from the 10-kDa family to the 100-kDa family. Proteins among family groups are highly conserved in species ranging from *Escherichia coli* to humans. While certain hsps are greatly induced by stress signals, it is now quite clear that cells have large quantities of hsps constitutively expressed and that these proteins are involved in all aspects of protein folding and oligomerization. In addition, hsps function in the intracellular transport to appropriate subcellular destinations of folding intermediates, the disassembly of oligomeric structures, and the facilitation of the removal of aggregated and/or improperly folded polypeptides (Gething, 1992). The term "molecular chaperon" also has been coined to describe proteins involved in these process (Ellis, 1990), e.g. protein-protein interactions. Hsp90 interacts with steroid receptors and with the virus encoded transforming protein, src; hsp70 and related proteins interact with clathrin baskets, DNA replication complexes, ER proteins , and the cellular tumour antigen p53 (Lindquist, 1988). Thus, hsps are inducible and/or constitutively expressed by prokaryotic and eukaryotic cells in response to stressful stimuli and appear to help protect cells from the adverse consequences of stress induced protein misfolding. (Kaufmann, 1990a; Jindal, 1989; Langer, 1991).

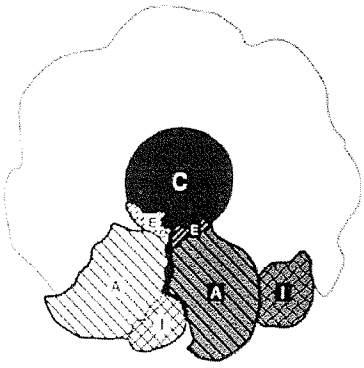
Genes that encode hsp60 have been cloned, sequenced, and expressed from strains of *C. trachomatis, C. psittaci,* and *C. pneumoniae.* (Morrison, 1989; 1990; Cerrone, 1991). Sequence analysis reveals an operon (termed hyp) which contains two open reading frames: hypA encodes

a polypeptide with a calculated molecular weight of about 11 kD, and hypB encodes a polypeptide of 58 kD. Comparative amino acid sequence analyses demonstrate that the hyp operon is an analogue of the *Escherichia coli* groE operon (Morrison, 1989; Hemmingsen, 1988). The groE genes of *E. coli* were originally identified as genes necessary for productive growth of bacteriophage lambda and T4 (Georgopoulos, 1973). The two genes groEL and groES comprise an operon under heat shock control (Tilly, 1983) located at 93.5 minutes on the *E. coli* chromosome (Guest, 1978). They encode abundant proteins: groEL a 58 kd protein and groES a 15 kd protein. GroEL is a porous cylinder of 14 subunits, each of relative molecular mass 58,000 daltons. The cylinder compose of two heptameric toroids stacked back-to-back with dyad symmetry as determined by electron microscopy (Langer, 1992; Chen, 1994). The subunits of GroEL are folded into three distinct domains (Braig, 1994), the largest being the well-ordered equatorial domain, containing residues 6-133 and 409-523 which form seven tightly packed α-helices. The equatorial domain provides most of the intratoroidal side-to-side contacts and all the contacts between the two rings. It also contains the ATP-binding site. A small intermediate domain (residues 134-190 and 377-408) connects the equatorial with the apical domain through short antiparallel polypeptide segments, which probably serve as hinges allowing allosteric domain movement. The intermediate domain also forms a diagonal projection that contacts the neighbouring apical domain on the right and with its base flanks the ATP-binding site. The apical domain (residues 191-376) forms the opening of the central cavity (fig.1). A number of segments from this domain face the central channel and the top surface are structurally ill-defined (Braig, 1994). Site-specific mutational analysis (Fenton, 1994) shows that the flexible regions are involved in the binding of polypeptide substrate and GroES. The same mutations in

the apical domain of GroEL that abolished polypeptide binding also prevented the association of the co-chaperoning GroES, a heptameric ring with subunits of molecular mass 10 kD. GroES binding to one end surface of GroEL reduces the affinity of the opposite end for a second GroES (Martin, 1993; Langer, 1992; Chen, 1994). GroES is required for the release of tightly bound polypeptide from its attachments sites at GroEL by coordinating the ATPase activity of the chaperonin (Hendrick, 1993). Upon ATP-binding or hydrolysis, conformational changes in the equatorial domains may be conducted by the intermediate segments to the apical domains, causing them to bury their polypeptide-binding regions (Hartl, 1994).

The hsp60 family has retained a high degree of amino acid sequence identity with hsp60 protein sequences from more than 50 different bacterial and eucaryotic cells having over 40% sequence identity (Viale, 1994). As an example , the *C. trachomatis* hsp60 has 48% amino acid sequence identity with human hsp60 or mouse hsp60 (Table 1). The high degree of amino acid sequence identity between a prokaryotic pathogen and a mammalian host protein suggests a structural basis for the initiation of an autoimmune response in which molecular mimicry could play a dominant role. This immune response to hsp60 may be associated with host chronic inflammatory disease because of autoimmune reactions that are perpetuated by self hsp60 (Kiessling, 1991; Jones, 1993).

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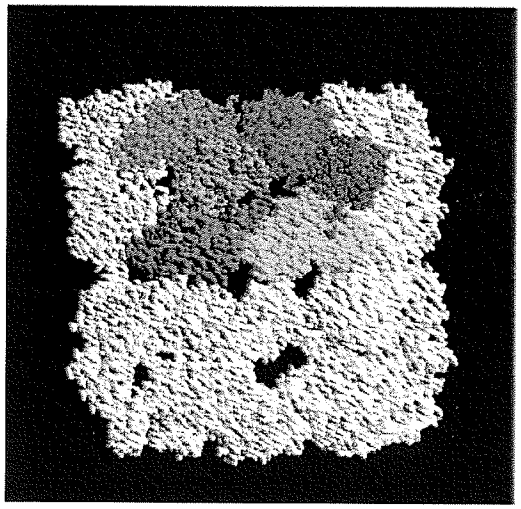

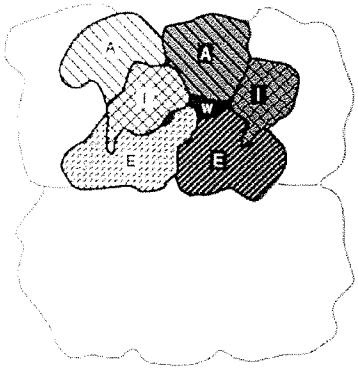
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Figure 1. Architecture of GroEL. Van der Waals space-filling model of the entire 14-mer shown in grey except for two adjacent subunits in the 'top' ring seen facing into the channel (top panel) and viewed from the outside (bottom panel). Domains within the left subunit are colour-coded as follows: equatorial, green; intermediate, gold; and apical, purple; the right subunit's equatorial, intermediate and apical domains are colour-coded yellow, red and blue, respectively. c, Drawing depicting diagrammatically the structure in b. E, I and A, equatorial, intermediate and apical domains, respectively; C, central channel; W, external opening of a side window. Figure is from reference: Brag, K. et al. 1994.

Table 1.

Amino acid sequence identity for hsp60 proteins from different sources

	Percent HSP60 amino acid sequence identity
C. trachomatis	
Serovar L2	100
Serovar A	98.9
Mouse pneumonitis	97.8
C. psittaci	93
C. pneumoniae	80
Coxiella burnetti	61
E. coli	60
Mycobacterium tuberculosis	57
Plant (Rub BP)	48
Human (Hu Cha 60)	48
Mouse	48

Data from reference: Brunham, 1994.

1.4. Mechanism of Immunopathogensis

There are several mechanisms by which chlamydial hsp60 might contribute to disease pathogenesis, resolution of infection, and modulation of the immune response to other chlamydial antigens.

1.4.1. Nonspecific tissue damage

The immunopathological consequence of mucosal chlamydial infection is scar formation. The mechanisms involved in mucosal scarring are not understood, but prolonged stimulation of a mononuclear inflammatory infiltrate might contribute. Repeated mucosal infection with chlamydiae, or the prolonged presence of chlamydial antigen in the mucosal epithelium, may be sources of antigen for chronic inflammation . The accompanying release of soluble factors from inflammatory cells and constituents (such as enzymes or cytokines) from chlamydial-lysed epithelial cells may cause fibroblast activation, collagen synthesis and epithelial scarring (Morrison, 1992).

1.4.2. Molecular Mimicry

The term "molecular mimicry" was initially used to explain persistent viral infection. Oldstone suggested that the major histocompatibility complex and viruses, especially retroviruses, encode similar antigens, which allow the host to regard an infecting virus as "self" and hence forego a protective immune response (Oldstone, 1987). Supportive evidence for the

hypothesis that molecular mimicry causes autoimmune disease in humans comes from two recent studies: one on the pathogenesis of celiac disease, the other on the pathogenesis of the nonrheumatoid arthritis of ankylosing spondylitis (AS) and Reiter's syndrome (RS). AS and RS appear to be genuine autoimmune diseases. Ninety-five percent of patients with AS and more than 80% with RS carry the HLA-B27 allele, compared with less than 7% of the general population. Six consecutive amino acids (QTDRED) are identical between the hypervariable domain of HLA-B27 and *K. pneumoniae* nitrogenase. Sera from a significant proportion of HLA-B27 individuals with RS or AS, but not from appropriate controls, react with a synthetic peptide containing the homologous region of HLA-B27.1. (Schwimmbeck et al, 1987).

Because of the ubiquitous occurrence of hsp60 among prokaryotes and eukaryotes, immune responses could be elicited that cross-react with host cells, and an autoimmune mechanism of tissue damage could be evoked. Although there is little evidence that such phenomena participate in disease pathogenesis of ocular and urogenital chlamydial infections, such a mechanism may be involved in the pathogenesis of some autoimmune diseases related to other bacterial hsp60. T cells involved in insulin-dependent diabetes mellitus (IDDM) of non-obese diabetic mice (NOD) are specific for an epitope shared by mycobacterial and mammalian hsp60 (Elias, 1990; 1991; Van Eden, 1988). A T-cell response to hsp60 and in particular, to aa337-460 in mycobacterial hsp60 has also been demonstrated in NOD mice developing diabetes mellitus. These T cells also respond to the membrane of ß cell secretory granules and hsp60 may be targeted to these granules. Immunization with the 337-460 peptide was reported also to induce diabetes mellitus. Hermann et al (1991) found that T cells from the synovial fluid of juvenile patients suffering from arthritis responded strongly to both mycobacterial and human hsp60.

These findings imply hsp60 cross-reactivity could play a role in some autoimmune diseases.

The humoral response to heat shock proteins (hsp) can be viewed from two aspects: 1) an antibody response against a foreign protein, for example from a microorganism, and 2) an antibody response against epitopes crossreactive between bacterium and host. The latter can be interpreted as the autoimmune response. Both anti-human hsp60 antibody and anti-mycobacterial hsp65 antibody were demonstrable in patients with ulcerative colitis (Elsaghier, 1992), juvenile chronic arthritis, diabetes mellitus, cystic fibrosis (Graeff-meeder, 1993) and arteriosclerosis (Xu, 1993). In Crohn's disease and nontuberculous mycobacterial pulmonary disease patients had high titre of antibody to mycobacterial hsp65 without detectable anti-human hsp60 antibody (Elsaghier, 1992). After immunization of mice with the mycobacterial hsp65, anti-hsp65 antibodies can cross-react with hsp60 from other prokaryotes (e.g. *E. coli* GroEL) both in ELISA and Western blot experiments, but consistently failed to give positive results in Western blot and only showed weak binding to the human hsp60 in ELISA (Barrios, 1994). The pattern of specificities of anti-chlamydial hsp60 antibodies has been defined by a panel of monoclonal antibodies (mAbs). The nine mAbs were chlamydial species specific which reacted with the hsp60 of all 15 *C. trachomatis* serovars. Six of the mAbs cross-reacted with hsp60 from other procaryotes. None of the mAbs reacted with eucaryotic hsp60 (Hela 229 cells) (Yuan, 1992). More examples of immune responses to hsp60 and chronic inflammatory disease are summarized in table 2. These data suggest that antibodies induced by hsp60 from microorganisms can cross react with human hsp60 only under selected condition. In general T cell clones with self hsp60 reactivity have also been commonly identified in a variety of chronic inflammatory diseases and appears to be more commonly detected than self hsp60 reactive antibodies.

Table 2. Association between immunity to hsp60 and autoimmune disease

Diseases in which hsp60 may have a role	Species	Response described
Adjuvant arthritis	Lewis rat	-T cell response to Mb-hsp65 -T cell clone specific for Mb-hsp65 induces arthritis
Streptococcal cell wall-induced arthritis	Lewis rat	-T cell response to Mb-hsp65
Rheumatoid arthritis	Human	-T cell response to Mb-hsp65 -antibodies against Mb-hsp65 -expression of hsp60 in synovial tissue -within a panel of 15 T cell clones obtained from 4 patients 12 different antigenic specificities for mycobacterial antigens were observed
Reactive arthritis	Human	-T cell response to Mb-hsp65 and h-hsp60
Juvenile arthritis	Human	-T cell response to Mb-hsp65, h-hsp60 -antibodies against both Mb-hsp65 and h-hsp60
Diabetes mellitus	NOD mouse	-T cell and antibody response to m-hsp60 and Mb-hsp65 -T cell clones (e.g. C9) specific for m-hsp60 induce diabetes
	Human	-antibody response h-hsp60

Note: Mb-hsp65 represent Mycobacterium bovis hsp65; h-hsp60 stand for human hsp60.

1.4.3. Autoimmunity induced by hsp60

Although T cells or B cells reactive with mycobacterial hsp60 can be demonstrated easily, evidence is lacking for either crossreactivity with eukaryotic hsp60 or molecular mimicry in many cases. This suggests that there is another mechanism involved in the induction of autoimmunity. At all stages of infection, there appears to be an involvement of hsps in both the pathogen and the host. Thus the response of hsps are involved in infections, both on the part of the pathogen and the host. Evidence for increased hsp60 expression in autoimmune lesions has been presented (Jones, 1990; Karlsson-Parra, 1990; Rajagopalan, 1990). Thus, heightened hsp60 levels have been found in the synovial lining and other cells present in rheumatoid arthritis lesions, oligodendrocytes of multiple sclerosis patients, B lymphocytes of patients with active lupus nephritis, and pancreatic cells of IDDM patients. It may be that autoimmune responses to self hsp60 are elicited during infection because the stressful condition of an inflammatory site induces expression of hsp60 in both microbial and host cells and the responding immune system is faced with the difficulty of accurately discriminating between two highly homologous proteins. In a particularly convincingly example, Boog et al (1992) demonstrated increased levels of self-hsp60 in the lining cells of inflamed synovial membranes from humans with juvenile chronic arthritis, a condition that is also associated with immunopathological response to microbial hsp60. Peetermans et al (1995) also found that it is the human hsp60 that is strongly expressed in antigen-presenting mononuclear cells in the mucosa of patients with inflammatory bowel disease and speculated that this could play a role in the initiation or maintenance of the inflammatory process.

Break down in the accurate immunological discrimination between highly homologous proteins one of which is in an invading pathogen and the other of which is a common host cognate may be a mechanism through which autoimmune pathogenic processes are initiated and perpetuated. In support of this speculation, Lin et al (1991) reported that when a homologous protein such as cytochrome c is used to induce an immune response, B and T cells can be induced to respond to self cytochrome c under conditions where the host immune system is simultaneously challenged with both foreign and self cytochrome c but not under conditions where the host is challenged with either antigen individually. This important observation has served as the impetus for the majority of my experimental analysis of autoimmune responses to hsp60.

1.5. Cytokines and Immune Tolerance

The paradigm of CD4 T cell differentiation from naive precursors into type 1 helper T cells (Th1) and type 2 helper T cells (Th2) has proven useful in understanding the outcome of host responses in infection immunity and in autoimmunity (Powrie, 1993a). Th1 cells produce interleukin 2 (IL-2), tumour necrosis factor-ß (TNF-ß), and interferon-gamma (IFN-gamma). These cytokines activate macrophages and induce delayed type hypersensitivity (DTH) responses. Th2 cells produced IL-4, IL-5, and IL-10, stimulate the production of mast cells, eosinophils and immunoglobulin E (IgE), and down regulate cell-mediated immunity (Mosmann, 1989).

Cytokines are one of the major mechanisms that regulate autoreactive T cells in the

peripheral immune compartment. For example, autoreactive T cells that induce organ-specific autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) generally display a Th1 phenotype (Miller, 1994). On the other hand, regulatory T cells that suppress the development of EAE produce cytokines that correspond to the Th2 profile (Karpus, 1992; Chen, 1994). These two cell populations cross-regulate one another because their respective cytokines act antagonistically (Swain, 1993; Street, 1994). For example, IL-10 inhibits the production of IFN-gamma and other Th1 cytokines by interfering with antigen presentation by macrophages and thereby down regulate Th1 cell function. (Paul, 1994).

The induction of peripheral tolerance to an alloantigen during organ transplantation is accompanied in many cases by a decrease in the production of cytokines such as IL-2 and IFN-gamma, while the production of cytokines such as IL-10 and IL-4 is sustained. As well IL-2 is able to reverse or prevent the induction of T cell tolerance to autoantigens (Jenkins, 1987; Essery, 1988). This phenomena can be explained by the "two signal model" where by the engagement of the T cell receptor by an antigen presenting cell in the absence of costimulatory molecules results in the induction of anergy (Schwartz, 1990; Umlaue, 1993).

Previous studies have demonstrated expression of a number of cytokines during chlamydial infection, IL-1, IL-2, IFN-gamma, IL-6, tumour necrosis factor (TNF) and colony-stimulating factors (CSFs) (Igietseme, 1993; Starnbach, 1995; Magee, 1991; 1992; Williams, 1990; 1993). More recently, it was reported that intravaginal infection of mouse pneumonitis biovar of *C. trachomatis* induced a local Th1 response (Cain, 1995). However, the potential relationship between tolerance versus autoimmunity induced by hsp60 and its relationship to antigen-specific cytokine expression has not been examined to date.

II. SCOPE OF THE PRESENT STUDY

To determine whether autoimmune responses are detectable among humans with immunopathological sequelae of *C. trachomatis* infection we evaluated sera from eight women with *C. trachomatis* associated ectopic pregnancy. All eight women had high titre of antibody to the chlamydial hsp60. Using synthetic peptides to map the major epitopic regions we determined that all eight women also had antibodies to human hsp60 peptide epitopes. These data suggested that autoimmune responses to self hsp60 may contribute to chlamydial immunopathology. To evaluate the condition under which autoimmune responses to hsp60 can be generated we next compared the immunogenicity of recombinant *C. trachomatis* and mouse hsp60 in CBA mice. We observed that mice are normally tolerant to mouse hsp60 and that immunization with chlamydial hsp60 alone does not induce strong autoimmune response to mouse hsp60. However under conditions of concurrent immunization with chlamydial and mouse hsp60, self tolerance breaks down. Tolerance to self hsp60 appears to be due to peripheral T cell anergy and is associated with antigen-specific IL-10 secretion. Co-immunization with chlamydia and mouse hsp60 resulted in reduction of antigen-specific IL-10 secretion and was associated with the induction of autoimmune responses to mouse hsp60. Chlamydia hsp60 deletion mutants were used to map the region within chlamydia hsp60 that provided help to break down tolerance when co-administered with mouse hsp60. Such sites were mapped to chlamydia hsp60 amino acid sequences 1 through 150 and 268 through 409. Thus T cell response to these regions of chlamydia hsp60 may be particularly important in *C. trachomatis* disease pathogenesis.

MATERIALS AND METHODS

1. Growth of Chlamydial Strains and Purification of EBs

The *C. trachomatis* serovars used were B (TW5/0T),C (TW3/0T),and L2 (434/BU) (courtesy of C-C Kuo, University of Washington, Seattle). This process involves the following four steps which have been standardized by our laboratory and others working with chlamydia.

(1) Hela 229 Cell line growth conditions. Hela 229 cells (American type Culture Collection, Baltimore, Maryland) were grown in Eagle's minimum essential medium (MEM) with 10% fetal calf serum (GIBCO) and 1mM glutamine (GIBCO). When cells becomed confluent, splited monolayer cell from one into two or three flasks: (i) Discard medium of an established cell culture flask (Nunc,175cm^2, GIBCO), then rinse the cells with GKNP 1x, about 5-10ml, 2ml of 0.1% trypsin solution and add just enough trypsin to cover monolayer (1-2mls for 175 cm^2 flask) and incubate at 37°C until the cell layer starts to detach. (ii) Pat the flask and quickly add 10 ml of MEM and pipet vigorously to disperse the cells and split the cell suspension to two or three flasks, then place about 30 ml of MEM in each flask and incubate at 37°C.

(2) Growing Chlamydia

Chlamydial stocks were grown in Hela 229 cells which were routinely maintained in the laboratory in Eagle's minimal Essential Medium with 10% fetal calf serum (FCS) and 1mM glutamate (complete MEM). Each chlamydial strain was inoculated onto 24-hour-old monolayer cultures of Hela 229 cells in 80 or 175 cm^2 flasks. The monolayers were pretreated with DEAE-dextran (30 μg/ml) (Pharmacia, Dorval, Quebec) for 20 minutes at room temperature. The inoculum was allowed to adsorb for 2 hours at room temperature. The monolayer was then rinsed once with Hanks' Balanced Salt Solution (HBSS) and incubated with complete MEM

containing 1 μg/ml cycloheximide. *C. trachomatis* serovars were incubated for 72 hours at 35°C before harvesting. To harvest the mature infected cultures, the growth medium was discarded and cells were rolled off the flask with approximately 30 glass beads in 10 ml of cold HBSS. The flask was rinsed with an additional 10 ml of cold HBSS. The cell suspension was sonicated on ice at an output of approximately 25 W for 35 seconds and then centrifuged at 500g for 15 minutes at 4°C. The pellet containing cell debris was discarded. The supernatant containing EBs and RBs was centrifuged at 30,000 g for 30 minutes at 4°C. The pellet containing chlamydiae was resuspended in sucrose-phosphate-glutamate buffer (SPG), pH 7.4, using a blunted spinal tap needle. This was used as inoculum for infecting more flasks or aliquoted and frozen at -70°C as stock.

(3) Purification of Chlamydia

To purify EBs or RBs from the flask cultures, the same harvesting procedure as previously described was used until the cell debris was discarded after the first centrifugation. Then the supernatant containing chlamydial EBs and RBs was layered on top of an 8 ml cushion of 35% Renografin (Renografin 76, Squibb, Canada) in HEPES buffer (0.01 M N-2-hydroxyethylpiperazine-N1-2-ethanesulfonic acid in 0.15 M NaCl) and centrifuged at 43,000 g at 4°C for 60 minutes in a SW27 rotor in the ultracentrifuge. The pellet containing EBs and RBs was resupspended in SPG and layered on top of a discontinuous gradient of 40-52% renografin in Hepes buffer and centrifuged at 45,000 g for 90 minutes at 4°C in a SW27 rotor in the ultracentrifuge. The EBs form a discrete band at the interface between the 44% and 52% renografin zones while the RBs form a band just below the 40-44% interface. The bands were harvested by suction with a pasteur pipette, washed twice in HBSS and resuspended in SPG

stored in -70°C.

2. Expression and Purification of Recombinant Heat Shock Proteins.

Mouse hsp60, chlamydial hsp60, or five deletion mutants of chlamydial hsp60 (PA1-265, C611, C86, C53, C6) were all cloned into a pGEx-2T vector and expressed as a fusion protein with 28 kd glutathione S-transferase (GST) as the fusion partner (Cerrone, 1991 and Yi, 1996). The following procedures were used to purify the hsp60 fusion protein (Smith, 1988). Overnight cultures of E. coli (DH5α strain)(started with a single clone) transformed with recombinant pGEx-2T plasmids were diluted 1:100 in 3000 ml of fresh medium (LB with ampicillin 100 μg/ml) and grown for 4 hours at 37°C (OD600 at 0.6) before adding IPTG (isoprophylthio-ß-D-galactoside) (Sigma, Chemical Co.) to 0.1 mM. After a further three to 4 hours of growth, cells were pelleted and resuspended in 1/50 to 1/100 culture volume of PBS (150 mM NaCl, 16mM Na2HPO4, 4mM NaH2PO4, PH7.3). Cells were lysed on ice by sonication and after adding Triton X-100 to 1%, and centrifuged at 10,000 xg for 10 minutes at 4°C. The supernatant was mixed at room temperature in a 50 ml polypropylene tube on a rotation platform with 5 ml 50% glutathione agarose beads (Sulphur linkage, Sigma). After absorption for 30 minutes, beads were collected by brief centrifugation at 500 xg and washed three times with 45 ml PBS. Fusion protein was eluted by competition with one bead volume of 50mM Tris.HCl (PH 8.0) containing 5mM reduced glutathione (Sigma) (fresh preparation required). Instead of elution, to cleave recombinant hsp60 from the GST binding beads, 100 units of thrombin (Sigma) was added, mixed with the beads in the 10 ml cleavage buffer containing 0.02 mM Tris, PH8.0, 0.15M

NaCl, 0.025 M $CaCl_2$ for 10 min at room temperature. The cleaved proteins were collected from supernatant by brief centrifugation. The samples were analyzed by optical density reading at 595nm, SDS-PAGE gel electrophoresis and immunodetection.

Regeneration of Gel. The glutathione agarose beads can be regenerated after four times washing with (1) at least 5 column volumes of 0.1 M borate buffer, PH 8.5 containing 0.5 M sodium chloride, (2) at least 5 column volumes of water, (3) at least 5 column volumes of 0.1 M acetate buffer, pH4 containing 0.5 M sodium chloride and (4) at least 5 column volumes of water. Finally the gel was equilibrated with equilibration buffer (PBS) before use.

3. Protein Concentration Determination

Microassay Procedure. Samples were diluted (1:100) in 800 μl PBS with a protein concentration between 1 and 20 μg/ml. Then 200 μl of Dye Reagent Concentrate (Bio-Rad protein quantization kit, Bio-Rad Laboratories, Richmond, CA) were added into the 800 μl sample and vortexed. Samples were incubated at room temperature for at least 5 minutes but less than 1 hour. The OD of samples would be measured at 595 nm. A standard curve was produced in the same way using bovine serum albumin. Protein concentrations in samples were calculated with reference to the standard curve.

4. Polyclonal Antibody Production

Rabbit antibody production. Female NZW rabbit (wight 2-2.5kg, from Roger Tessier, St.

Pierre-Jolys, Manitoba Canada) were used to raise antibodies against chlamydial antigens. Each rabbit was intramuscularly immunized with 5 x 10^8 inclusion forming units (IFU) purified organisms in equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI). Three weeks later, two intravenous injections of an aqueous EBs suspension (1 x 10^8 IFU/each) were given at 7 day intervals. Ten days after the final intravenous boost, rabbits were bled, Antisera are aliquoted and stored at -20°C.

Mouse antibody production. The CBA/j(H-2^k) mice (♀) were purchased from Jackson Laboratories (Bar Harbor, ME), and used at four to eight weeks of age. Groups of four mice were immunized subcutaneously with a total of 50 or 100 μg antigens such as hsp60, GST, or ovalbumin (ova) (Sigma Chemical Co.) per mouse emulsified 1:1 in incomplete Freund's adjuvant (Difco) in the base of the tail for antibody detection or foot pad injection for T cell proliferation. Mice were bled by cutting tail and sera were collected, stored at 4°C.

5. Peptide synthesis.

Overlapping 12-mer peptides of chlamydial hsp60 were synthesized using a commercially available kit (Cambridge Research Biochemicals, Cambridge, UK). Peptides were synthesized onto the solid polyethylene pins held by specially moulded polyethylene holders in the format and spacing of a 96-well microtiter plate using pentafluorophenyl (opfp) active esters of fluoroenylme-thyloxycarbonyl (F-MOC) protected L-amino acids with t-butyl derivatives as side-chain protecting groups except for arginine with methoxytrimethylphenyl sulphonyl (mtr) side chain protecting group (Geysen, 1987, Steward, 1984). The synthesis approach utilized a mild

base 20% freshly distilled piperidine in N_1N-dimethylformamide (DMF) purified with molecular sieve for repetitive N-deprotection starting with the first cleavage of F-MOC protecting group of beta-alanine residue precoupled to the polyethylene pins. Following the deprotection and DMF and methanol bath washing, 30 mM of the appropriate preactivated F-MOC-amino acid ester was dissolved in DMF containing 30 mM 1-hydoxybenzotriazole as catalyst and 100 μl aliquots of the solution is added into each corresponding well of polyethylene micro plates. The coupling reaction was carried out overnight at room temperature followed by DMF and methanol bath washing. The deprotection, washing, coupling and washing steps are repeated until 12-mer peptides were synthesized on all rods. After final deprotection and washing, the terminal amino groups were acetylated by reaction with DMF: acetic anhydride disopropyletylamine (50:5:1, v/v/v) for 90 minutes at room temperature and a single acid treatment with trifluoroacetic acid:phenol:1,2-ethanedithiol (95:25:2.5, v/w/v) for four hours at room temperature used as side chain deprotection followed by neutralization with 5% diisopropylethylamine in distilled dichloromethane. After wash with a final methanol bath for 18 hours and dried in vacuum over silica gel for 18 hours the pins with 12-mer peptides were ready to use. Successful synthesis was insured by the simultaneous synthesis of positive and negative control pins and comparison of the reactivity test mAbs to pins on which control tetrapeptides had been synthesized and supplied by the manufacturer. The amino acid sequence of C. trachomatis hsp60 as deduced from the DNA sequence (Cerrone et al,1991) was used to direct the synthesis of 533 overlapping 12-mer peptides covering the entire sequence and overlapping by all but a single residue (Yi et al, 1993).

6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The discontinuous buffer system (Laemmli, 1973) was used with all reagents being of electrophoresis purity grade (Bio-Rad). The unit was a Mini Protein II (Bio-Rad) with gel dimensions of 8 cm x 10 cm x 0.75 cm. On a given run, 2-5 μg protein was loaded per well after solublization. The gels were run in 0.025 M Tris/0.192 M glycine/0.1% SDS at 100 volts until the tracking dye reached the bottom of the gel. Estimation of molecular weights was done using the molecular weight standards. The gels were then either silver stained to visualize protein bands or electro-blotted to transfer the protein bands to a nitrocellulose membrane followed by staining with 0.1% protein dye amido black (Bio-Rad Laboratories, Richmond, CA) in solution (Methanol:HAc:dH_2O=9:2:9).

Silver staining of polyacrylamide gels. The procedure used to stain the gels for visualization of the protein bands was after the method of Morrissey (1981). Special care was taken to prevent dirt or grease from touching the gels which were handled only with disposable latex gloves. Following a PAGE run, the proteins were fixed by incubating the gel for 4-12 hours at room temperature with gentle shaking in at least 5 gel volumes of a solution of ethanol:glacial acetic acid:water (30:10:60). The fixing solution was discarded and the gel was incubated with at least 5 gel volumes of 30% ethanol for 30 min at room temperature with gentle shaking and repeat once. After the fixing solution was removed, 10 gel volumes of deionized water was added to the gel and the gel was incubated for 10 minutes at room temperature with gentle shaking and repeat twice. The gel swelled slightly during rehydration. After the last of water washing, 5 gel volumes of a 0.1% solution of $AgNO_3$ (freshly diluted from a 20 % stock, stored in a tightly

closed, brown glass bottle at room temperature) was added and incubated for 30 minutes at room temperature with gentle shaking. The $AgNO_3$ solution was discarded, and both sides of the gel were washed (20 seconds each) under a stream of deionized water.(Do not allow the surface of the gel to dry, otherwise staining artifacts will ensue). Then the gel was incubated with 5 gel volumes of a freshly made aqueous solution of 2.5% sodium carbonate, 0.02% formaldehyde at room temperature with gentle agitation. The gel was watched carefully. Stained bands of protein appeared within a few minutes. Continue incubation was needed until the desired contrast was obtained. The reaction was quenched by washing the gel in 1% acetic acid for a few minutes. Then the gel was washed several times with deionized water (10 minutes per wash). The gel was preserved by drying on filter paper.

7. Western Blotting

The procedure involves the transfer of proteins separated by SDS-PAGE onto a nitrocellulose membrane (NCM) allowing them to be reacted with immune sera or mAbs which is not possible when they are in gel matrix. A variation on the original method of Towbin et al (1979) was used in our laboratory.

Following the PAGE run, the gel would be immersed in transfer buffer (25 mM Tris/192 mM glycine/methanol, PH 8.3). The NCM (Bio-Rad) was used for electroblotting. The NCM was cut to fit the gel and presoaked in transfer buffer. A TRANS BLOT apparatus (Bio-Rad) was used for electroblotting. The NCM was placed on wetted 3 mm chromatography paper and the gel piece laid on top of it, making sure to exclude any air bubbles. Another piece of wet 3

mm paper was laid on top and this sandwich placed in between two pieces of pads and placed in a TRANS-BLOT holder such that the gel side was towards the cathode. The apparatus was filled to the top with transfer buffer. It was found that the best transfer of protein to the NCM was obtained with overnight blotting using 20 volts. The NCM with bound protein was immersed in a blocking buffer (2% BSA -PBS, PH7.4) for 90 minutes at 37°C with shaking. This was done to prevent non-specific binding of immunoglobulin to areas on the NCM where no protein had been attached. The antibody was diluted in 2% BSA-PBS-0.05% Tween 20 solution. Tween 20 is a detergent used to prevent nonspecific sticking of antibody to the NCM. The amount of antibody added to the NCM depended upon the titre of the immune sera or mAb, incubated at 37°C for one hour. The NCM was then washed three times over 30 minutes in wash buffer (PBS plus 0.02% Tween 20) and then the second antibody is added which was tagged with horse radish peroxidase (HRP) and was used at a dilution of 1:2000 in a 2% BSA-PBS-0.05% Tween buffer. The blot was developed with the substrate 4-chloro-1-naphthol (SIGMA Chemical) and H_2O_2. Full development of the coloration was allowed and then the NCM would be washed three times with dH_2O and blotted dry.

8. Standard ELISA

Microtiter plates (Corning, New York) were coated with 100 μl of hsp60 (2 μg/ml) in 0.1 M NaHCO3 buffer (PH 8.4). The microtiter plates were sealed and incubated overnight at 4°C. Wells were emptied and washed three times with PBS containing 0.05%-Tween 20 (PBS-Tween) and then incubated with 200 μl blocking buffer (PBS-Tween containing 2% bovine serum

albumin) for 2h at 37°C. The plates were washed once in PBS-Tween, and then 100 μl of sample antibodies diluted in blocking buffer was added to appropriate wells and then incubated for 90 minutes at 37°C. The plates were washed three times with PBS-Tween, and a 1:3000 dilution of horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin G or Goat anti-rabbit IgG (PIERCE) in 100 μl of blocking buffer was added to the wells and the plates incubated for 1h at 37°C. After washing three times, 100 μl of substrate (2,2-azino-bis-[3-ethyl-benzthiazoline-6 sulfonate], ABTS, in citrate buffer, PH 4.0) was added, and the plates incubated for 30 minutes at room temperature. Optical density was read at 405nm (Microplate reader, Bio-Rad Model 3550). Endpoint titrations were the highest dilutions of sera giving the optical density unit (0.3) which is at least four times higher than the control reading at 405nm.

9. Peptide-ELISA

The immobilized peptides were assayed by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacture, using polyclonal antisera or monoclonal antibodies (mAbs) raised against chlamydial antigens as first antibodies and goat anti-rabbit immunoglobulins (Cappel 3212-0213), or rabbit anti-mouse immunoglobulin G (Cappel) conjugated to horseradish peroxidase as second antibodies at a dilution of 1:1000. The colour was developed with ABTS (Sigma Chemical Co.) in the dark and read at 405nm, using a microplate reader (EL308; Bio-Tek Instruments, Inc.). The first antibodies were diluted in blocking buffer (1:250). The results were expressed in optical density (OD) values. The solid-phase peptides were reused after the bound antibodies were dissociated by ultrasonification for

30 minutes at 60°C in a solution containing 1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 0.1 M sodium phosphate (PH 7.2).

10. Lymphocyte Proliferation Assays

10 days after immunization with hsp60 in IFA in the foot pad of CBA mice, lymph nodes were collected aseptically and single cell suspensions were prepared and lymph node cells (LNCs) were washed and resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 15% FCS. LNCs ($5x10^5$/well) were cultured with and without antigen in flat-bottomed 96-well microculture plates (Costar 3596, Costar, Cambridge, MA). The plates were incubated at 37°C in a 5% CO_2, 95% air humidified incubator for 5 days. 1 μCi [^{3}H] thymidine (ICN Biomedicals, Inc., CA) was added to each well in 20 μl of RPMI 1640 18 hours before harvesting. The cultures were harvested using a phD cell harvester (Cambridge Technology Inc., MD, USA) and the incorporation of TdR into DNA was measured by liquid scintillation counter (Beckman Ls 5000 CE, Fullerton,CA). Results of representative experiments are presented as mean experimental DPM (Disintegrations Per Minute).

11. Spleen cell culture

Preparation of spleen cell suspensions. Mice were killed at various times following immunization. Mouse spleens were removed aseptically, and single cell suspensions were prepared. Debris was removed by passing the cell suspensions through nytex filters into

centrifuge tubes. Cells were spun down at 400 g for 10 min and washed once with serum free RPMI 1640 medium (no supplements). Cell pellets were resuspended in complete culture medium. The number and percentage of viable cells was determined by staining cell preparations with trypan blue and counting. Spleen cell suspensions were cultured at 1 x 10^7/ml (2 ml/well) alone or with different antigens (chlamydial hsp60, mouse hsp60, ovalbumin, 100 μg/ml), concanavalin A (Con A) at 37°C in complete medium. Duplicate cultures were established from the spleen cells of individual mice in each group. Culture supernatants were harvested at different time intervals (at 40 hours for IFN-gamma and at 72 hours for IL-10) as described (Chen et al, 1994) and stored at -70°C until cytokine production was analyzed.

12. Cytokine Determination

Murine IFN-gamma, IL-10, IL-2 and IL-4 were measured by a two mAb sandwich ELISA purchased from Pharmingen (San Diego, CA). e.g. IFN-gamma ELISA was carried out using xMG 1.2 as detection and R4-6A2 as capture antibody. IL-10 ELISA used JESS-2A5 and SXC-1 as capture and detection antibody respectively. 96 well plates (Corning, New York 14831) were coated with capture mAb at 2 μg/ml in coating buffer (0.1 M NaHCO3, PH 8.6). After overnight incubation at 4°C, the plates were blocked with 4% BSA PBS buffer for 2 hours at room temperature and washed extensively. Culture supernatant and cytokine standards (Pharmingen) were serially diluted and added to the plates. The plates were incubated at 4°C

overnight, and then washed four times. Biotinylated anti-cytokine detecting mAb (2 μg/ml) was added for 40 min at room temperature. The plates were washed four times and incubated with streptavidin-peroxidase at room temperature for 30 min. The plates were extensively washed and ABTS substrate was added and develop at room temp. The plates were read at 405 nm.

The quantikine TGF-ß1 immunoassay based on the "Quantikine™" kit (R&D SYSTEMS,Inc. Minmeapolis) was used by following the instruction of the manufacture. This assay employs the quantitative "sandwich" enzyme immunoassay technique. TGF-ß soluble receptor type II which binds TGF-ß1 has been coated onto the microtiter plate provided in the kit. Standards and samples were pipetted into the wells and any TGF-ß1 present was bound by the immobilized receptor. After washing away any unbound proteins, an enzyme-linked polyclonal antibody specific for TGF-ß1 was added to the wells to "sandwich" the TGF-ß1 immunobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of the TGF-ß1 bound in the initial step. The color development was stopped and the intensity of the color was measured by reading OD.

13. Preparation of T lymphocyte subpopulations and adoptive transfer of immune cells

Spleen cells from immunized or normal mice were enriched for T cells by panning with affinity purified sheep anti-mouse Ig coated plates to remove B lymphocytes. A 100 x 15 mm sterile tissue culture flask was coated with 5 ml sheep anti-mouse Ig (50 μg/ml) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) at 4°C overnight. After 1 hour blocking with 10

ml 10% FCS-PBS at room temperature and subsequent washing, $1x10^8$ spleen cells in a volume of 5 ml were applied to the flask. The flask was incubated for 60 min at 37°C. The nonadherent population, harvested with a pasteur pipette after gently rocking the flask, was predominantly T cell. These cells were further purified by passing through a nylon wool column (Nylon Fibre Column Wako Purechemical industries, LTD) twice as described (Julius, 1973). Briefly, the spleen cell suspension was loaded to the column which was previously equilibrated by running 25 to 50 ml of 37°C complete RPMI 1640 through the column and incubated in an upright position for more than 45 min at 37°C, 5% CO_2 humidified incubator and the cells were drained completely, then let cells stayed in the column for another 45 min in an upright position. The column was filled with 37°C complete RPMI1640 and the first 15 ml of the nonadherent, effluent cells (T cells) were collected. 1 x 10^8 purified immune T cells (at least 90% purity of T cells identified by antibody against Thy1.2 Ag) or unpurified spleen cells were adoptively transferred into recipient mice by intraperitoneal injection. At the same time the mice were injected in the base of the tail with 100 μg mouse hsp60 in IFA. Blood samples were collected at day 30 after immunization.

14. Bacterial Culture Media

(1) Luria-Bertani(LB) broth (one liter) contained 10 g bacto-tryptone (Difco), 5 g bacto-yeast extract and 10 g NaCl. The solution was adjusted to pH 7.0 and autoclaved.

(2) Luria-Bertani(LB) agar was made by adding 15 g Difco agar per liter to LB broth and was poured into 10 or 15-cm disposable dishes (approx.30 or 90 ml/dish) after autoclaving.

When needed, ampicillin (final concentration: 100 μg/ml) was added.

(3) SOC medium contained in one liter 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.2 g KCl, 10 ml of 1 M $MgCl_2$ and 20 ml of 1 M glucose and was adjusted to pH7.0. Medium containing no $MgCl_2$ and glucose was autoclaved. 1 M $MgCl_2$ and 1 M glucose solutions were prepared separately, filter-sterilized, and added into the autoclaved medium at final concentration of 10 mM $MgCl_2$ 20 mM glucose.

15. Cloning Vectors (Fig. 2)

pUC18 plasmids (Sambrook, 1989) obtained from GIBCO lack the rop gene which normally is located close to the origin of DNA replication and is involved in the control of copy number. As a result, these plasmids replicate to a much higher copy number than do other plasmids that carry a pMB1 (or ColE1) origin. The most important is that pUC18 vectors express the amino-terminal fragment of the lacZ gene product (ß-galactosidase) and display α-complementation in appropriate hosts. The recombinants containing inserts of interest can therefore be identified by histochemical screening.

pGEX-2T plasmid (Toye, 1990) features the glutathione S-transferase gene from *Schistosoma japonicum*, which forms an affinity tail on the protein products of genes inserted into the multiple cloning site. Expression is under the control of a tac promoter which enables inducible, high-level production of fusion proteins. The vectors contain the lac I^q gene, so they can be used in any *E. coli* strain. Intracellular fusion proteins are easily recovered from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B. Bound proteins are released from the matrix under mild, nondenaturing conditions (5 mM glutathione). This helps preserve

antigenicity and functional activity of the recombinant proteins. Recovery of the protein is completed by using a site specific protease to cleave GST from its fusion partner (thrombin for pGEX-2T). The protease recognition sequences are located between the GST gene and the multiple cloning site.

16. Oligo primer design and polymerase chain reaction (PCR)

All primers used in my studies are based on published sequence data (Cerrone, 1991 and Venner, 1990). Primers were synthesized on an oligonucleotide synthesizer (Oligo 1000 DNA synthesizer, BECKMAN INSTRUMENTS INC. Fullerton, CA).

M1: 5'GTT CCG CGT GGA TCC GCC AAA GAT GTA AAA TTT GGT GCG 3'

MR1.6: 5'TTT TCC CGG GGA TCC TTA GAA CAT GCC GCC TCC CAT ACC 3'

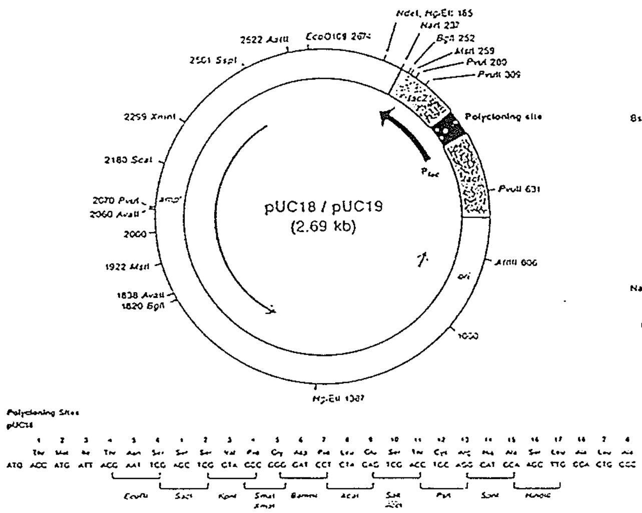
P250: 5'GT GGA TCC ATA GCA GAA GAC ATT GTA GGC 3'

P380: 5'GT GGA TCC CGC GTT GGA GCT GCA ACA GAG 3'

P545: 5'GT GGA TCC TTA ATA GTC CAT TCC TGC GCC 3'

P1: 5'CCG GGA ATT CAT ATG GTC GCT AAA AAC ATT AAA 3'

P150: 5'CG ATG AAT TCC AAT TGT TGC AAC TTG AGC AAT 3'


pUC18 / pUC19
(2.69 kb)
Polycloning site

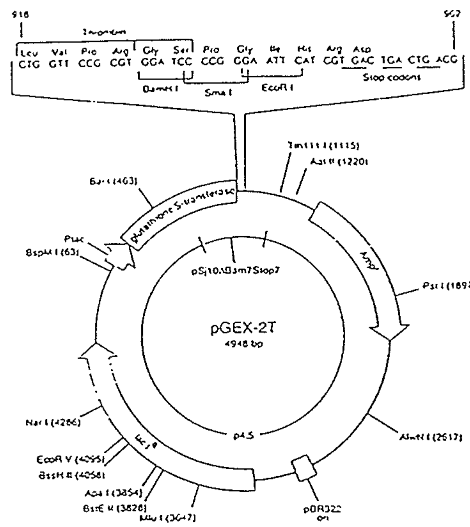

pGEX-2T
Stop codons

Figure 2. Maps of PUC 18 and the gene fusion vector pGEX-2T showing multiple cloning sites and genealogy.

The PCR cycling protocol was 25 cycles, each for 2 minute at 94°C, 2 min at 55°C and 2 min at 72°C. Each PCR reaction contained 50 pmol of each primer, 0.2mM dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH8.3), and 50 mM KCl and 0.5 unit of Taq polymerase (GIBCO/BRL) in a final volume of 100 μl.

17. Preparation of Plasmid

The alkaline lysis procedure was used for the isolation of small quantities of plasmid DNA from bacterial cells (minipreps), according to the method of Sambrook (1989). The large scale plasmid DNA preparation followed instruction of plasmid Maxi kit (QIAGEN, Chatsworth,CA)

18. RT-PCR cloning mouse hsp60 gene

Total mouse L cell mRNA purification, cDNA preparation and PCR amplification were performed following the manufacture's instructions (micro fast-track kit, cDNA cycle kit, In vitrogene San Diego, USA). Briefly, 5 x 10^6 mouse L cells were washed by 4°C PBS, pelleted down and lysed with 1 ml of Micro-FastTrack™ lysis buffer and passed through a sterile 21 gauge needle > 3 times and further incubated at 45°C for 15 minutes. 63 μl of the 5M NaCl stock solution were added to each 1 ml lysate and mixed thoroughly by passing through 21 gauge needle, Then one Oligo (dT) Cellulose tablet was added to the lysate and the tube was rocked gently at room temperature for 15 to 20 minutes. The oligo (dT) cellulose was pelleted down at room temperature at 2000 x g and washed by binding buffer for at least 3 times (OD_{260} of the

"flow-through" should be $\leq$ 0.05). The non-polyadenylated RNA was washed off by adding 200 μl of low salt wash buffer 3 times and mRNA was collected with 200 μl of elution buffer. The PolyA$^+$ RNA was precipitated by adding 10 μl of glycogen (2 mg/ml), 30 μl of 2 M sodium acetate and 600 μl of 100% ethanol and centrifuged at maximum speed (16,000 x g) for 15 min and the RNA pellet was resuspended in 11.5 μl of sterile water ($>$ 10 ng). cDNA was synthesized from RNA by priming this mRNA at 42°C for 1 hour in a final volume of 20 μl containing Oligo dT primer or random primers (1μl) at a final concentration of 0.5-1.0 μM, 1.0 μl RNase inhibitor, 4.0 μl 5x RT buffer, 1.0 μl 100 mM dNTPs, 1.0 μl 80 mM sodium pyrophosphate, 0.5 μl AMV reverse transcriptase. The solution was mixed by lightly tapping the tube, then spin it briefly. To remove the secondary structure, the vial containing only mRNA and primers was heated in a 65°C water bath for 10 minutes, then placeed at room temperature for 2 minutes before other reagents were added. The RNA-cDNA hybrids were denatured by 95°C for 2 minutes and spun and quickly placed on ice. This product was ready for any PCR reaction. Second-strand synthesis is not required or recommended. Mouse hsp60 specific oligonucleotides as primers (M1 and MR1.6) which locate at 5-' and 3'- primer end of mouse hsp60 gene (Venner et al, 1990) were designed with 5'- and 3'- BamH1 restriction endonuclease sites to allow cloning easily. The desired DNA insert verified by partial sequencing with known primer (M1 and MR1.6). The insert was isolated by gel electrophoresis. In order to reduce background, BamH1 cleaved vector DNA was treated with calf intestinal alkaline phosphatase (CIP). The concentration of insert DNA was relatively high (the molar ratio of insert to vector is 3 to 1) or equal (the ratio is 1 to 1) in order to facilitate ligation to vectors. The amount of DNA in a 20 μl ligation mixture was 0.1 μg. The products of the ligation were introduced into

competent E. coli (DH5α), and recombinant containing the gene for ampicillin resistance were selected directly on ampicillin (100 μg/ml) containing plates (LB agar). Through an intermediate puc 18 plasmid, the mouse hsp60 gene fragment (full size 1.6 kbp) was then subcloned into expression vector pGEx-2T with correct reading frame by electroporation in a Bio-Rad Gene Pulser with a mode of 2.5 kV/resistance high voltage, resistance of 200 Ω, charging voltage of 1.8 kV, desired field strength of 12.25 kV/cm and desired pulse length of 4-5 msec. The competent E. coli cells were prepared for electro-transformation by the method of Dower et al (1988). The complete mouse hsp60 express a glutathione S-transferase (GST) fusion protein induced by isopropyl-ß-D-thiogalactopyranoside (IPTG) induction. The positive clone was identified by western blot probed with mAb LK1 (Sigma Chemical Co.) which has unique specificity for mammalian hsp60 and the recombinant DNA insert was fully sequenced.

19. Construction of deletion mutants of chlamydial hsp60

Deletion mutant clones of chlamydial hsp60 (PA1-265, C611, C86, C53, C6) were constructed from plasmid pGEx-2T containing full length chlamydial hsp60 gene (Cerrone, 1991). PA1-265 resulted from chlamydial hsp60 gene fragment religation after deletion of fragment (amino acid position 266-545) cleavaged with EcoRI and expressed chlamydial hsp60 residues 1-265. Another fragment from EcoRI digestion was purified by agarose gel and religated with pGEx-2T (resulting in plasmid C86 gene expressing residues 268-409).

The plasmid C611, C53, C6 were obtained by Taq-polymerase-facilitated amplification of the corresponding DNA sequence using primers (P1 and P150 for C611, P250 and P545 for

C53, P380 and P545 for C6) containing restriction sites to allow cloning of the fragments into expression vector pGEx-2T. All constructs were checked by sequencing.

20. DNA Sequencing

Sequencing of cloned recombinant plasmids was done based on the protocols supplied with the dsDNA cycle sequencing kit purchased from BRL Life Technologies Inc. (Gaithersburg, MD). It is based on the chain termination DNA sequencing method (Sambrook, 1989) which includes labelling primer, cycle sequencing, gel electrophoresis (6.5% acrylamide:bis-acrylamide [29:1, w/w]). The labelled chains of various length were visualized after exposing to X-Omat AR film overnight at -70°C.

Comparison of the obtained sequence and known hsp60 gene and other analysis of hsp60 gene sequence data were done by using a PC/GENE software purchased from IntelliGenetics, Inc (Mountain View, California).

RESULTS

Part I. INTRODUCTION OF ANTIGENS USED IN THE STUDY.

The intention of the present study was to examine the host immune response to chlamydial hsp60 and mouse hsp60 in an animal model. A large amount of hsp60 was needed. Considering the limitations in purification of native hsp60 from either *C. trachomatis* or host cells, we decided to use an alternative approach to clone mouse hsp60 gene or chlamydial hsp60 gene fragments. Genes were expressed in *E. coli* via GST gene fusion system which combines innovations in vector design and purification to provide excellent performance in protein expression and recovery. The clone of recombinant chlamydial hsp60 fusion protein was a generous gift from R.S. Stephens (University of California, San Francisco). Since high expression systems often cause insoluble formation of recombinant protein (inclusion body), we initially made efforts to optimize technique conditions such as host strain, temperature, urea denaturation, detergent, times of sonification and so on for purification of a large amount of soluble hsp60.

1.1. Purity of recombinant hsp60

One of our major concerns was that *E. coli* proteins may co-purify with the GST fusion protein system since hsp60 has a physiological role as a chaperonin protein that binds nascent polypetides. A number of methods have been developed to stain polypeptides after separation by SDS-gel electrophoresis. A common reagent for staining proteins is Coomassie Brilliant Blue binding basic amino groups on proteins. Approximately 0.1 microgram (μg) of protein can be detected on a polyacrylamide gel. The most sensitive staining procedure for fixed proteins on

gels or blots involves silver staining , which can detect less than a nanogram (10^{-9} g) of protein. Thus, silver staining is the easiest and more practical way to check our products (hsp60) (Sambrook, 1989).

Fig.3 shows a silver stained SDS-GEL loaded with two purified recombinant chlamydial hsp60 samples lane 1 (1 μg loading), lane 2 (0.5 μg loading) and control protein samples of GST loading lanes 3,4 (2 μg/lane). There was a major band with molecular weight about 80 kD representing the fusion protein chlamydia hsp60 (GST-Chsp60) and no other protein can be found above them (lane 1,2,5,6,7). A degraded small band (ca. 52 kD) of chlamydia hsp60 was observed which can also be recognized by polyclonal antibody from rabbits immunized with chlamydial EB (Fig.3, lane 5,6,7). GST protein purified from *E.coli* containing only the vector itself (pGEX-2T) without an insert showed as a single band with molecular weight 27 kD without detectable contamination of other *E. coli* protein or chlamydial protein on the silver stained gel (lane 3,4). Therefore the GST hsp60 fusion system offer high purity recombinant proteins which were used in our study as antigens.

ELISA also can be used to check the purity of the recombinant hsp60 to determine whether contamination with GST, thrombin, or other hsp60 has occured. The ELISA plates were coated with GST, thrombin, mouse hsp60, or chlamydia hsp60 respectively. They were then incubated with normal mouse serum, mAb G57-19 (specific for chlamydia hsp60, see 1.3) and LK1 (specific for eukaryotic hsp60) and following standard ELISA protocol (Table 3). The polyclonal antibodies that were raised with purified chlamydial or mouse hsp60 did not bind to GST or thrombin (OD $<$ 0.00) which suggested that GST or thrombin contamination is negligible in our purified immunogens (chlamydia hsp60 or mouse hsp60). LK1 recognize mouse hsp60 with high

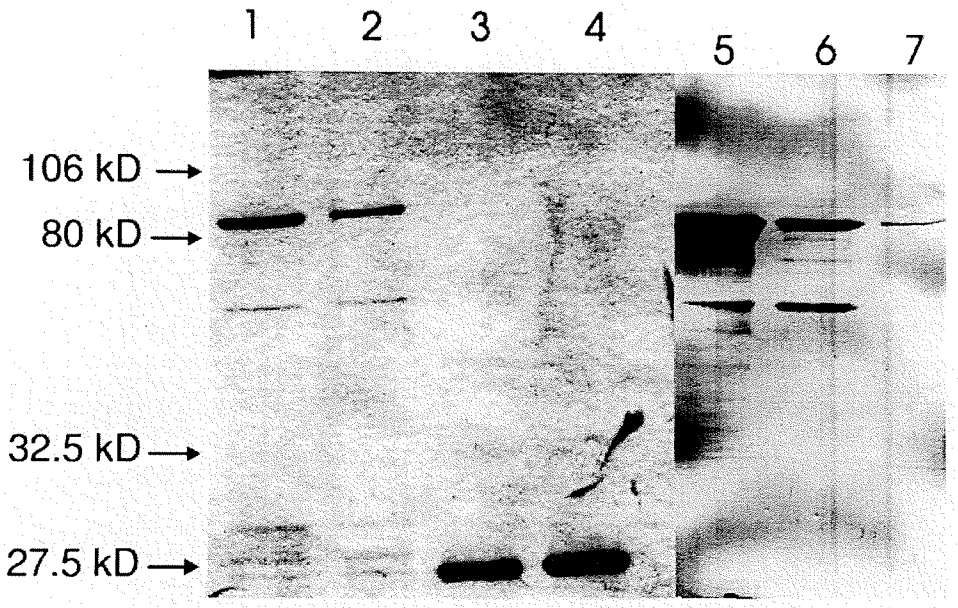

1
2
3
4
5
6
7
106 kD
80 kD
32.5 kD
27.5 kD

Figure 3. Purity of chlamydial hsp60 checked by silver staining and western blot. Purified fusion protein (lane 1, 2, 5, 6, 7) and GST (lane 3, 4,) were subjected to 12% SDS-polyacrymide gel separation and divided into two parts: part I (lane 1-4) was analyzed by silver staining, part II (lane 5, 6, 7) were transferred to nitrocellulose membrane for western blotting probed by rabbit immune serum against chlamydial EB at a dilution 1:200.

Table 3. ELISA check purity of hsp60

Ag coating / Antibodies (1:100)	GST	Thrombin	Mhsp60	Chsp60
Normal sera	0.000 (*)	0.000	0.002	0.000
mAbs				
G57-19	0.000	0.001	0.000	0.510
LK1	0.009	0.000	0.604	0.000
Immune sera against				
Chsp60	0.004	0.003	0.242	0.676
Mhsp60	0.000	0.000	0.315	0.125

* This is a mean OD(405nm) reading value from a standard ELISA protocol.

Chsp60 and Mhsp60 correspond to chlamydial hsp60 and mouse hsp60 respectively.

OD value (0.604) but no binding to chlamydia hsp60 whereas G57-19 showed high OD value (0.510) to chlamydia hsp60 and 0.000 to mouse hsp60. These data suggested that there was no detectable contamination between mouse hsp60 and chlamydia hsp60 samples. These results also showed that our ELISA system offer high specificity with very low background.

1.2.1. Cloning the mouse hsp60 gene

Since the nucleotide sequence of mouse hsp60 and chlamydia hsp60 are known from published data (Venner et al, 1990 and Cerrone et al, 1991), specific primer based PCR cloning was used to clone the mouse hsp60 gene and the chlamydial hsp60 partial gene fragments. Mouse hsp60 specific primers (M1, MR1.6) were used to amplify mouse hsp60 as a full length gene (1.6 kb) (Fig.4) from two mouse cDNA samples (cDNA2 reverse transcribed by poly T, cDNA3 made by 6 bp random primer) and also to screen recombinant *E.coli* clones (lane 1 to lane 12). Most *E.coli* clones did not contain the specific mouse hsp60 gene insert. One clone had correctly sized amplified product detected by PCR (lane 6, a band of 1.6 kb size).

Construction of deletion mutants of chlamydia hsp60 based on pGEX-2T containing chlamydia hsp60 gene. There are 3 EcoR1 sites located in the chlamydial hsp60 gene (Fig.5). The plasmid PA 1-265 resulted from religation of chlamydia hsp60 plasmid digested with EcoR1. The other EcoR1 fragment (PA 267-409, PA means protein amino acid position) was purified from agarose gel and ligated with EcoR1 treated pGEX-2T (resulting in clone C86). The rest of clones were obtained from specific primers amplified PCR products. C611 came from primer P1 and P150, C53 came from P250 and P545, C6 from P380 and P545. Amplified products were then cloned into pGEX-2T.

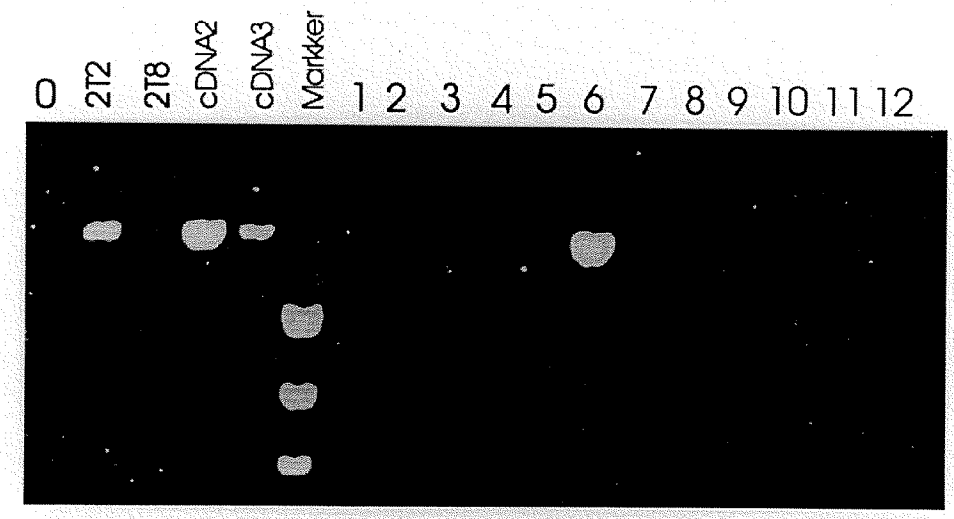

0
2T2
2T8
cDNA2
cDNA3
Markker
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4. Amplification and screening mouse hsp60 gene with PCR. DNA samples electrophoresis in a 0.8% agarose gel. Lane 0, only sample buffer loading. Lane 2T2, positive control sample of chlamydial hsp60 gene (1.6 kb). Lane 2T8, negative control in which pGEX-2T used as template. Lane cDNA2 and cDNA3 template are two mouse cDNA samples transcribed by either poly T primer (cDNA2) or 6bp random primer (cDNA3). Lane 1-12, different plasmids purified from E. coli clones which may contain mouse hsp60 gene were used as template in PCR.

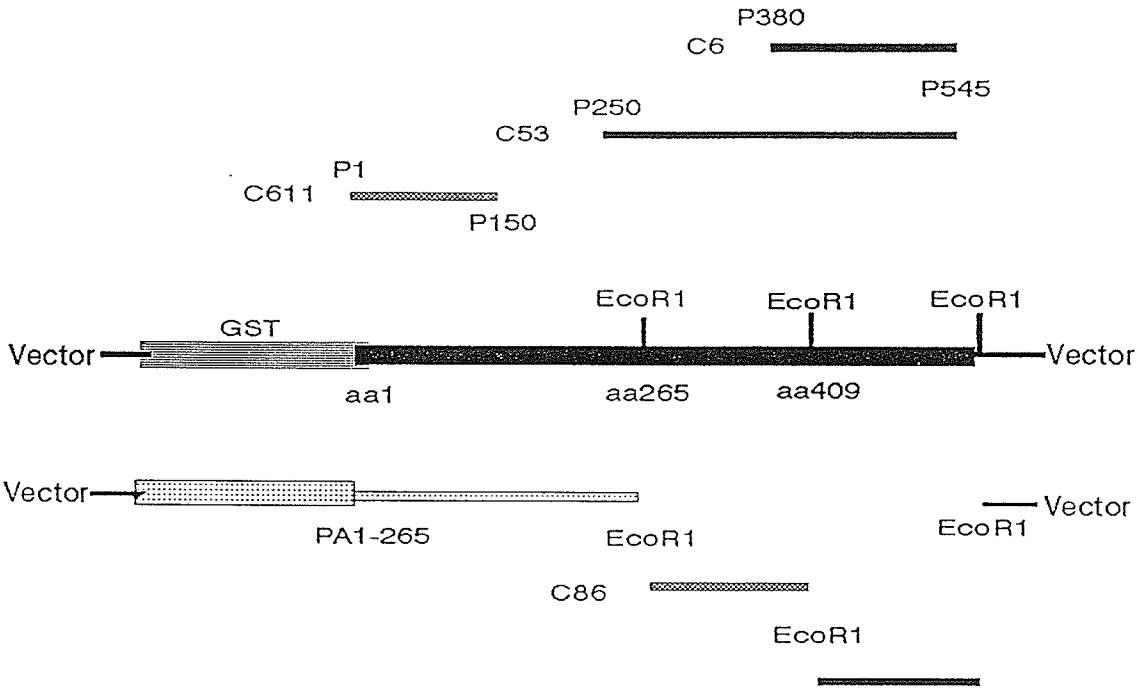

P380
C6
P545
P250
C53
P1
C611
P150
EcoR1
EcoR1
EcoR1
GST
Vector
Vector
aa1
aa265
aa409
Vector
Vector
PA1-265
EcoR1
EcoR1
C86
EcoR1

Figure 5. Strategy for construction 5 deletion mutants of chlamydial hsp60.

GST-chlamydial-hsp60 fusion protein (the centre line) was treated with EcoR1 to produce two small chlamydial hsp60 fragments including C86 and vector contain GST-PA1-265 with EcoR1 sites at 3' and 5' primer end. The chlamydial hsp60 gene fragments (C611, C53, C6) came from PCR amplification with specific primers (P1, P150, P250, P380, P545).

1.2.2. Expression hsp60 polypeptides

All deletion mutants of chlamydia hsp60 that were highly expressed in *E.coli* were seen on SDS-page gel stained by amido black (Fig.6). The major bands (about 30% of total *E.coli* proteins) are fusion proteins of chlamydial hsp60 deletion mutants with molecular weights between 57 kD to 41 kD (lane 1-5). These bands could be identified by western-blot with specific mAbs. For example, C6 clone expressing the C-terminal portion of chlamydia hsp60 (residues position 380-545, 43.5 kD) was recognized by mAb GP57-19 (lane 6-7).

1.2.3. Cleaved hsp60 from fusion proteins

All fusion proteins contained the N-terminus of the GST protein can be cleaved by the specific enzyme thrombin. Fig. 7 shows a western blot probed with mAb LK1 and demonstrates that mouse hsp60 (60 kD) is released in a time dependent manner from the GST-mouse-hsp60 fusion protein (84 kD) after binding to glutathione agarose. This allowed us to isolate enough cleaved mouse hsp60 and chlamydial hsp60 with the glutathione affinity column method.

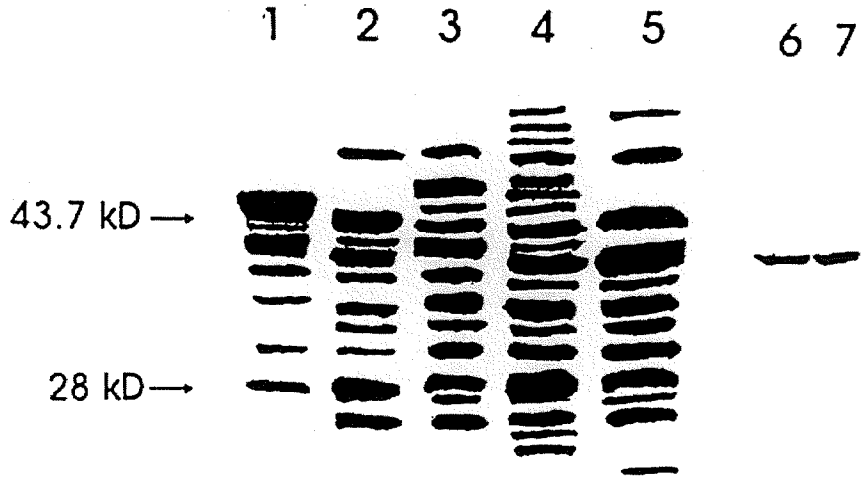

1
2
3
4
5
6
7
43.7 kD
28 kD

Figure 6. Total E. coli. proteins containing different chlamydial hsp60 fragments (lane 1, PA1-265 clone. lane 2, C611 clone. lane 3, C53 clone. lane 4, C86 clone. lane 5,6,7, C6 clone) were separated on SDS-page gel and transferred to NC membrane. Then lane 1-5 was stained by amido black and the lane 6,7 was analyzed by western blot with mAb G57-19.

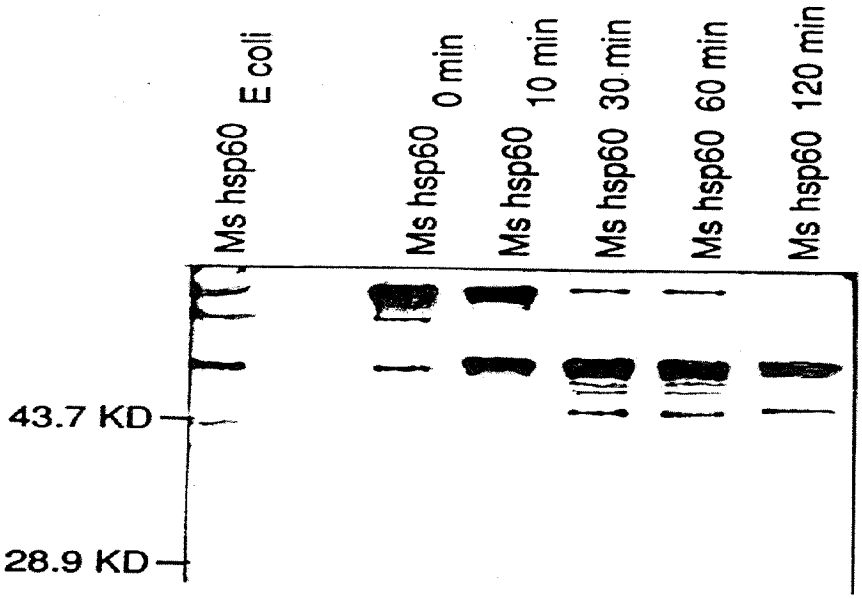

Ms hsp60
E coli
Ms hsp60
0 min
Ms hsp60
10 min
Ms hsp60
30 min
Ms hsp60
60 min
Ms hsp60
120 min
43.7 KD
28.9 KD

Figure 7. Western-Blot analysis of thrombin cleaved mouse hsp60. GST-mouse-hsp60 fusion protein was treated with thrombin for different time (10, 30, 60, 120 minutes) to remove GST and then loaded to SDS-PAGE gel. The NC membrane was probed by mAb LK1.

1.3. B cell linear epitopes mapping with mAbs

I synthesised 533 peptides encompassing the entire sequence of chlamydia hsp60 overlapping each other by a single amino acid residue. Yuan et al (1992) produced a panel of anti-chlamydial hsp60 monoclonal antibodies (mAbs) and defined their epitope location on the primary amino acid sequence of chlamydia hsp60 by immunoblotting against recombinant amino-terminal truncated hsp60 fusion polypeptides and overlapping synthetic peptides. For example, mAb A_{57}-E_4 reacted with sequence residues 117-122 and another mAb GP_{57}-19 recognized amino acid residue 517-522. In order to check our pin peptides and screen the expression library of chlamydia hsp60 deletion mutants (see 1.2.2), we mapped the epitopes recognized by these two mAbs. Table 4 shows the result. The A57-E4 bound to peptide (118-122) in which the core sequence is amino acid residues 118-122 and the epitope recognized by GP57-19 is peptide 516-522. These data which are consistent with Yuan's data suggested that our peptide synthesis was successful.

Table 4. Peptide mapping with mAbs

mAbs name	Position of Epitope (*)	Sequence of Epitope
GP57-19	516-522	-LLTTEAL-
A57-E4	118-122	-RGIDK-

* Position of epitopes represent the position of amino acid sequence of chlamydial hsp60

Part II. MAPPING CONTINUOUS B-CELL EPITOPES IN CHLAMYDIAL HSP60

2.1. Peptide epitope mapping with rabbit, human immune sera

Figure 8 shows the peptide epitope pattern in hsp60 recognized by the 13 rabbits immunized with EBs of serovar B, L2, and C respectively. A pattern of common epitopes among all rabbits was consistently observed, although individual heterogeneity was also apparent. No marked difference in epitope patterns was apparent among the three different serovars for immunized rabbits, consistent with the known conservation of hsp60 among all members of the genus *Chlamydia* (Morrison et al, 1990). Although epitopes are distributed throughout the entire sequence, the region from amino acid (aa) 283 to 409 was consistently immunorecessive. When both binding titers and reactive frequencies of all 13 rabbit serum samples were considered, five distinct immunogenic regions (aa 94 to 111, 147 to 163, 188 to 205, 258 to 294, and 410 to 491) were resolved within the linear sequence (Fig. 8B). Region 5, located toward the C terminus, is the most complex and is composed of multiple epitopic peaks. The other four regions have a single predominant or, at most, two epitopic peaks. Ten epitopes characterized by high response frequency (greater than or equal to seven rabbits) and high titers of binding ($\geq$0.2 optical density [OD] units) are located within the five regions.

Serum samples from eight women with *C. trachomatis*-associated ectopic pregnancies were next evaluated for the epitope specificity of the hsp60 antibodies in these sera by the pepscan assay and for comparison with the epitope map using rabbit antisera. All women had antibodies with high titers to the recombinant chalmydial hsp60 (Yi et al, 1993). Epitope scanning revealed

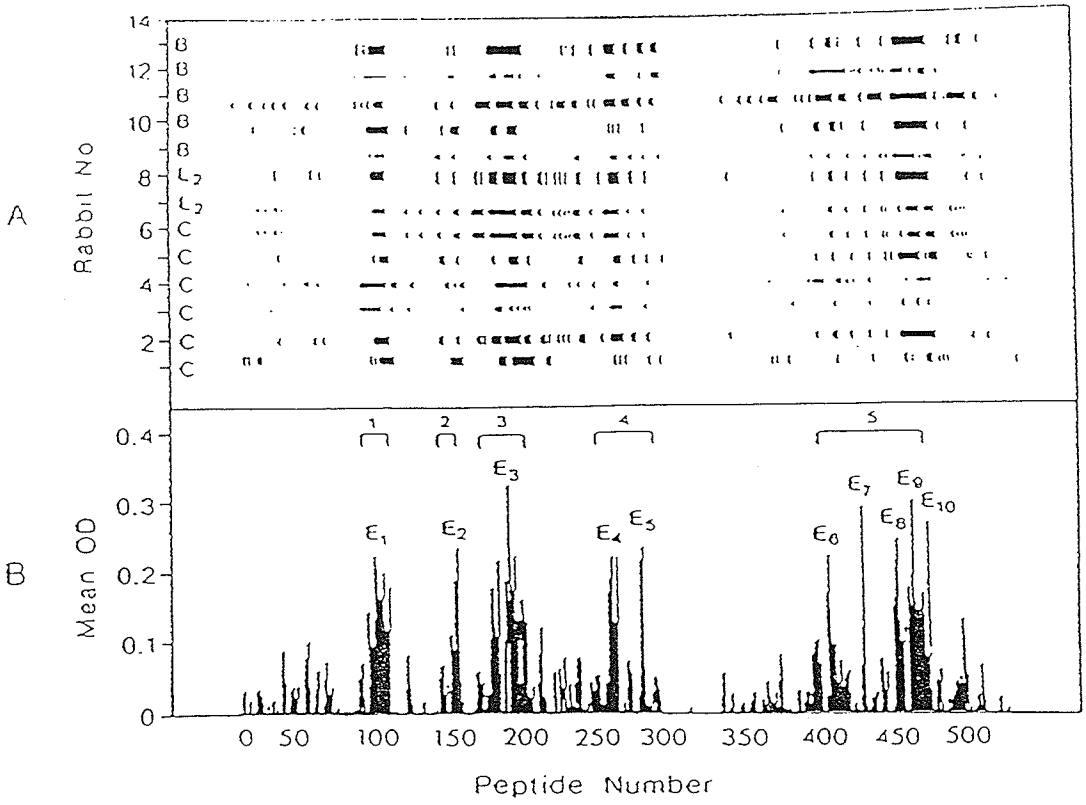

A
Rabbit No
B
Mean OD
Peptide Number
1
2
3
4
5
ϵ_1
ϵ_2
ϵ_3
ϵ_4
ϵ_5
ϵ_6
ϵ_7
ϵ_8
ϵ_9
ϵ_{10}

Figure 8. Antigenicity of 533 overlapping 12-mer peptides encompassing the entire hsp60 sequence of *C. trachomatis*. (A) Antisera were raised in 13 rabbits immunized with EBs of C. trachomatis serovars B (four rabbits), L2 (two rabbits), and C (six rabbits) as indicated. Bar codes signify reactive peptides. (B) Composite binding pattern averaged among the individual antiserum samples and expressed as mean OD units are shown. Sera were tested at a dilution of 1:250. The bracketed regions 1 to 5 indicate the five immunogenic regions.

a pattern of immunogenic regions similar to that observed with antisera of rabbits immunized with chlamydial EBs. In addition to the 10 epitopic peaks identified with the rabbit antisera, three new epitopes (H_1, H_2, and H_3 in Fig. 9) were detected with the human sera. The major new immunogenic region is aa 226 to 249, labelled epitope H_2. Two minor epitopes are located at aa 29 to 41, labelled H_1, and at aa 446 to 461, labelled H_3.

Two serum samples in which one came from a healthy woman without *C. trachomatis* infection and another from an unprimed rabbit were also tested by the pepscan assay. No peptides were bound with an OD of $\geq$ 0.1 with either sera (Figure 10.)

2.2. Search for cross-reactive epitopes

We next compared the chlamydial hsp60 amino acid sequences for the 13 major epitopes recognized by sera from humans with the homologous sequences in the human mitochondrial chaperonin 60 protein (Jindal et al, 1989) (Table 5). None of the epitopes showed 100% amino acid sequence identity between the two proteins. To determine if the homologous region in the human hsp60 sequence represented an autoantigen we synthesized 12-mer peptides for each epitope. Pin-bound peptides were then tested by the pepscan assay with the eight human serum samples (Fig. 11) and with mouse sera raised to the recombinant chlamydial hsp60 (Fig.12). In Fig.11 the immunologic cross-reactivity was determined by comparing relative antibody binding to peptides of chlamydial hsp60 with binding to homologous peptides of human hsp60. The data show that six epitopes (E_1, E_2, E_5, H_3, E_8, and E_9) are relatively specific to the chlamydial sequence, with the human antisera binding to the chlamydial peptide sequence at a significantly ($P<0.05$) higher OD than it does to the human peptide sequence. In particular, epitope E_8 is

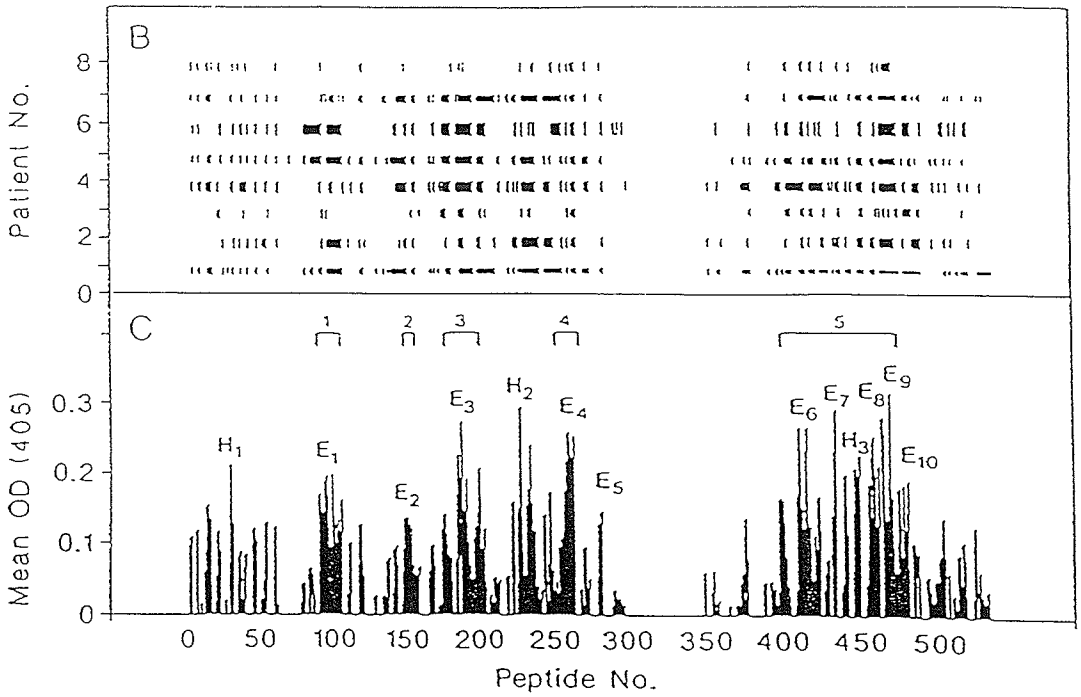

B
Patient No.
0
2
4
6
8
C
Mean OD (405)
0
0.1
0.2
0.3
1
2
3
4
5
H1
E1
E2
E3
H2
E4
E5
E6
E7
E8
H3
E9
E10
0
50
100
150
200
250
300
350
400
450
500
Peptide No.

Figure 9. Serum samples from eight women with *C. trachomatis*-associated ectopic pregnancies were assayed for peptide binding (B and C). Pepscan results for the eight human serum samples (tested at a dilution of 1:400) were displayed in the format described in the legend to Fig. 8. H1, H2, and H3 represent three new epitopes identified only with human sera. E1 through E10 represent the identical peptide epitopes as identified with rabbit antisera.

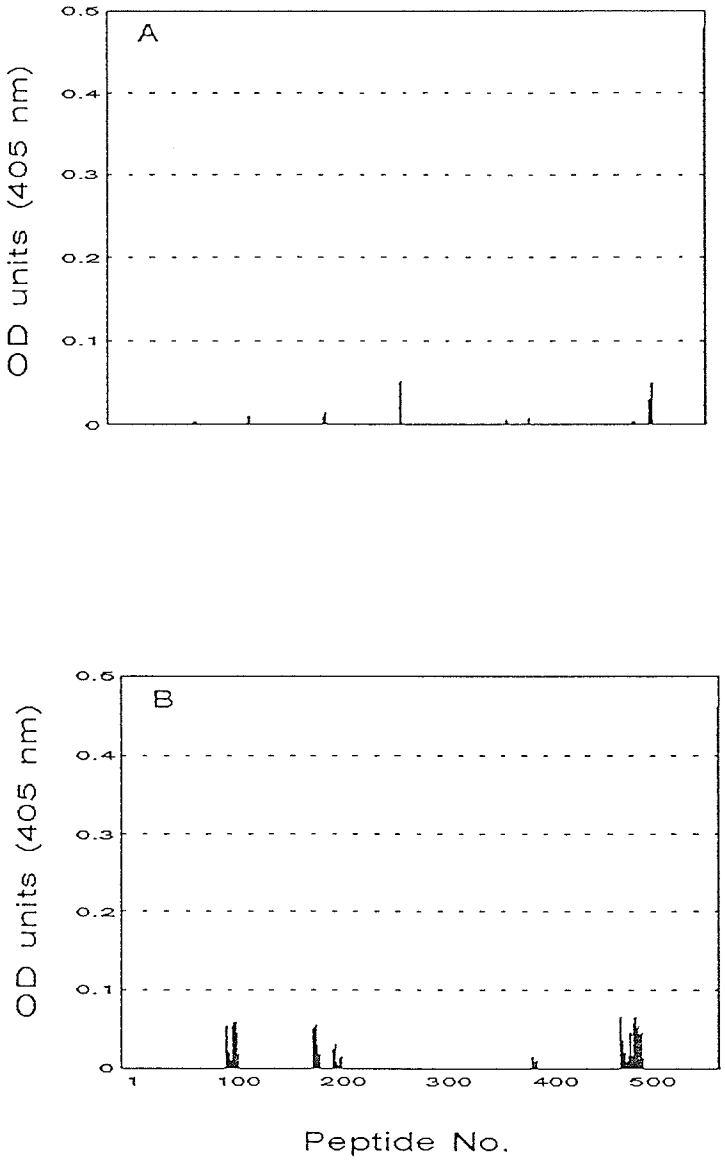

A
B
OD units (405 nm)
OD units (405 nm)
0.6
0.4
0.3
0.2
0.1
0
1
100
200
300
400
500
Peptide No.

Figure 10. Serum samples from one healthy women without *C. trachomatis* infection (panel A) and one unprimed rabbit (panel B) were used as a control and assayed for peptide binding (tested at a dilution of 1:250). No peptides were bound with an OD of $\geq$ 0.1 by either serum samples.

Table 5. Comparison of amino acid core sequence of *C. trachomatis* hsp60 peptide epitopes with that of human mitochondrial chaperonin 60 protein.

Epitope[a]	Peptide sequence[b]
H_1	^{29}VTLGPKGRHVVI
	^{27}**M*****T*I*
E_1	^{94}VLAEAIYTEGLR
	^{92}***RS*AK**FE
E_2	^{151}SANNDAEIGNLI
	^{149}***G*K****I*
E_3	^{188}DVVDGMNFNRGY
	^{186}EIIE**K*D***
H_2	^{228}SGIKDFLPVLQQ
	^{226}*S*QSIV*A*EI
E_4	^{260}ATLVGNRIRGGF
	^{258}S***L**LKV*L
E_5	^{282}GDRRKAMFEDIA
	^{280}**N**NQ*K*M*
E_6	^{411}ILPGGGTALIRC
	^{410}*VL***C**L**
E_7	^{435}NEDEQIGARIVL
	^{432}***QK**IE*IK
H_3	^{450}SAPLKQIAANAG
	^{447}KI*AMI**K***
E_8	^{459}NAGKEGAIIFQQ
	^{456}***V**SL*VEK
E_9	^{464}GAIIFQQVMSRS
	^{461}*SL*VEKI*QS*
E_{10}	^{470}QVMSRSANEGYD
	^{467}KI*QS*SEV***

[a] E_1 to E_{10} signify epitopes defined with both rabbit and human antisera, and H_1, H_2 represent epitopes seen only with human antisera.

[b] Top sequences are from C. trachomatis GroEL hsp60 (Cerrone et al, 1991) and bottom homologous sequences are from human mitochondrial chaperonin 60 protein (Jindal et al, 1989).

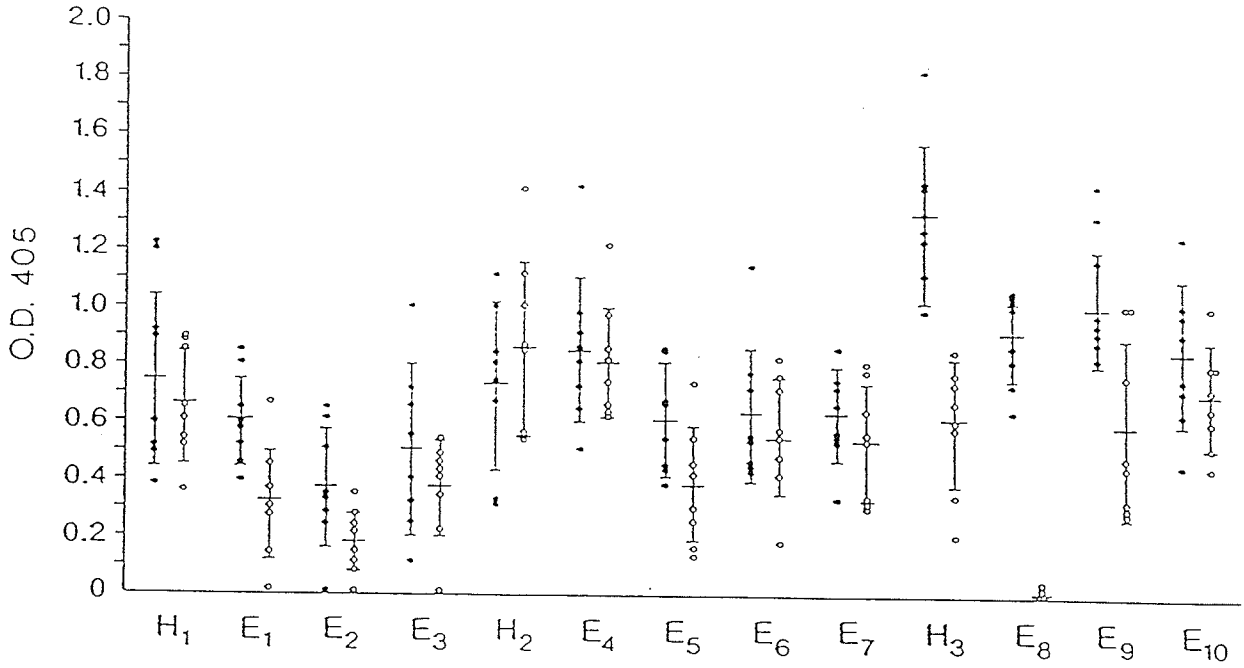

O.D. 405
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
H1
E1
E2
E3
H2
E4
E5
E6
E7
H3
E8
E9
E10

Figure 11. Thirteen major peptide epitopes from chlamydial hsp60 were used to detect homologous sequences in human mitochondrial hsp60. 12-mer peptides were synthesized in the pepscan format and tested with the eight serum samples from women with *C. trachomatis*-associated ectopic pregnancies (each sample was tested at a dilution of 1:200). Closed circles represent binding to the *C. trachomatis* sequences, and open circles represent binding to the homologous human sequences. Antibody binding was significantly ($P < 0.05$) greater to the *chlamydial* peptide than to the human peptide for E_1, E_2, E_5, H_3, E_8, and E_9. No human peptide bound antibody better than the *chlamydial* peptide, and H_1, E_3, H_2, E_4, E_6, E_7, and E_{10} were cross-reactive.

absolutely specific to the chlamydial sequence, The remaining seven epitopes (H_1, E_3, H_2, E_4, E_6, E_7, and E_{10}) were cross-reactive between the chlamydial and human hsp60 sequences.

The goal of this phase of the study was to determine whether the antibodies that are induced to chlamydial hsp60 are cross-reactive between human and chlamydial hsp60. The above data are consistent with the hypothesis that chlamydial disease sequelae may be due to autoimmunity to hsp60. To further address this hypothesis we next decided to map these autoantigen epitopes using highly immunized mouse sera raised against recombinant chlamydial hsp60. The data are shown in Fig.12. The white bar represent antibody binding to chlamydial hsp60 sequence and filled bar stands for mean OD to homologous peptide of human hsp60. Most epitopes (H1, E1, E3, E4, E5, E6, E7, E10) showed low affinity and partial cross reactivity to human hsp60 peptides. One epitope, E2, was strongly cross-reactive showing very high binding OD (1.313 and 1.046) to both the chlamydial hsp60 peptide and human hsp60 peptide. Epitopes (E8, E9) representing the C-terminus of chlamydial hsp60 were relatively specific to chlamydial hsp60 sequence without cross-reactive binding (Fig.11). Selected mouse sera were absorbed with GST-chlamydial-hsp60 fusion protein linked with agarose in order to remove antibodies against chlamydial hsp60. After absorption most epitope binding OD values were reduced by more than 50%, especially the E2 epitope. The OD value (0.188 for chlamydial hsp60 and 0.042 for human hsp60) (Fig.13) is 10 times lower than that before absorption (1.313 for chlamydial hsp60 and 1.046 for human hsp60). The sequence of E2 is highly homologous between chlamydia hsp60 and human hsp60 with only 3 amino acid residue substitutions in this 12-mer peptide (Table 5). These data suggest that anti-chlamydial-hsp60 antibody can cross-react with human hsp60 at selected antigenic regions although the number of

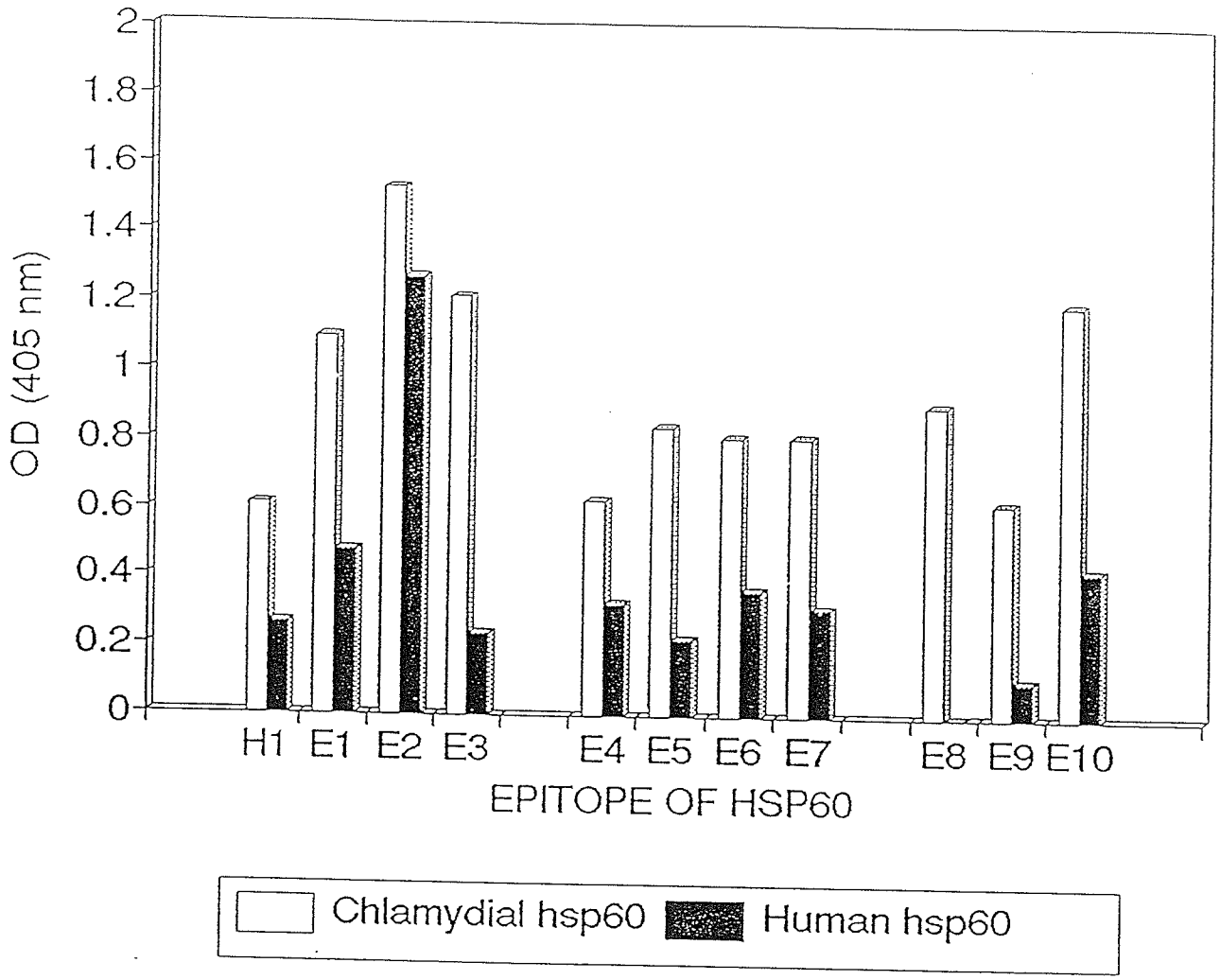

OD (405 nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H1 E1 E2 E3 E4 E5 E6 E7 E8 E9 E10
EPITOPE OF HSP60
Chlamydial hsp60
Human hsp60

Figure 12. Mapping cross-reactive epitopes before absorption. Both sets of 11 epitopes (listed in the Table 5) for chlamydial and human hsp60 have been synthesised and tested by the pepscan assay with mouse serum (1:500) raised with recombinant chlamydial hsp60. The white column represent antibody binding to the chlamydial hsp60 epitopes and filled column stand for antibody binding to homologous human peptides.

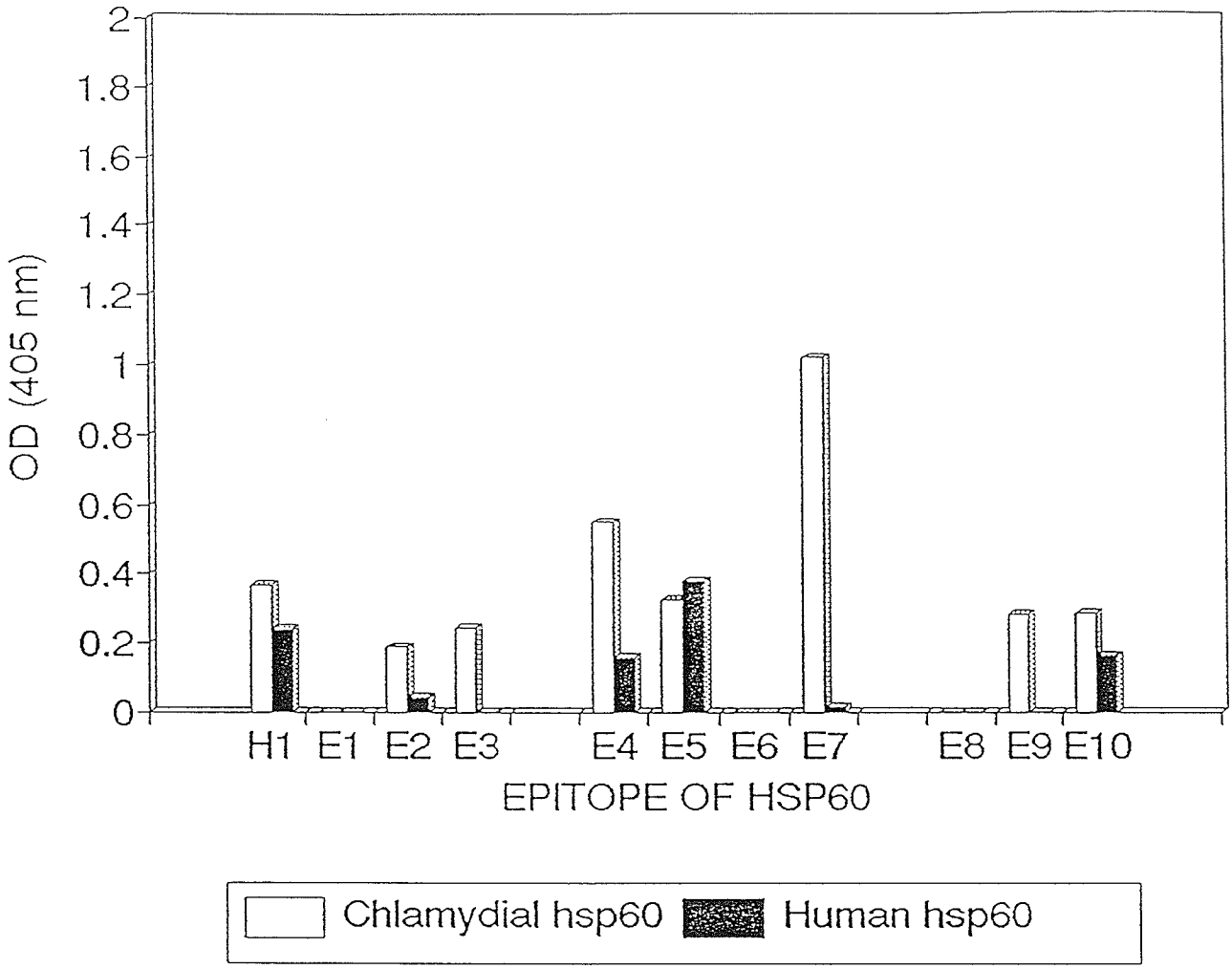

OD (405 nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H1 E1 E2 E3 E4 E5 E6 E7 E8 E9 E10
EPITOPE OF HSP60
Chlamydial hsp60
Human hsp60

Figure 13. Mapping cross-reactive epitopes after absorption. The same mouse serum used in the figure 12 was incubated with GST-chlamydial-hsp60 fusion protein linked with agarose at 4°C overnight before pepscan test. Open columns represent antibody binding OD to chlamydial-hsp60 epitopes and filled columns stand for antibody binding to human peptides.

cross-reactive epitopes may be limited (7 epitoptes in human sera and 1 in mouse sera).

2.3. Relationship between the crystallographically defined structural domains of hsp60 and antigenic domains of chlamydial hsp60

Based on the pepscan data there are five antigenic domains (or immunogenic regions) on chlamydia hsp60. They are domain 1 containing aa 94-111, domain 2 (aa 147-163), domain 3 (188-205), domain 4 (aa 258-294), domain 5 (aa 410-491). When we compared the location of these domains with the known structural and functional features of the crystallized *E. coli* GroEL/Hsp60 chaperonin (equatorial domain, intermediate domain, apical domain, see Introduction part 1.3.) (Fig.14), two interesting observations were made. (1) Most of the antigenic domains were located on the boundary regions between structural domains. e.g. antigenic domain 1 and 5 are located on the C-terminal of the first segment of equatorial domain (aa 6-133) and N-terminal of the second segment of equatorial domain (409-523). Antigenic domain 2 is located in the first segment of intermediate domain (aa 134-190). Antigenic domain 3 represents the N-terminal of the apical domain (aa 191-376), and (2) most antigenic domains (domain 1, 2, 4, 5) contained α-helices as their major secondary structural component. The exception is domain 3 which contains only ß-strands and extended strands. This information may be useful for the design of site-directed mutants of chlamydial hsp60 and in predication of immunogenic regions.

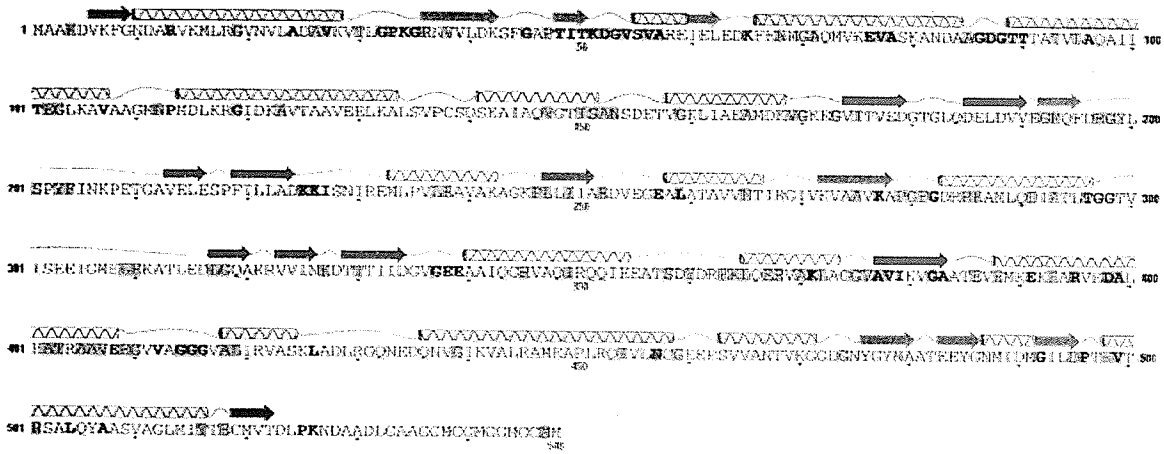


Figure 14. Amino-acid sequence and structural components of a subunit of GroEL/Hsp60. Secondary structural elements (defined by visual inspection), indicated by arrows (ß-strands) or sine wave in rectangles (α-helices), and extended strands. Colour-coding corresponds to the domain sequence segment; here, equatorial is green, intermediate blue, apical red.

The figure is from reference: Brag, K, et al. 1994.

Part III. IMMUNE TOLERANCE TO HSP60 AND AUTOIMMUNITY

3.1. *C. trachomatis* hsp60 and mouse hsp60 have different immunogenicity when used as individual immunogens.

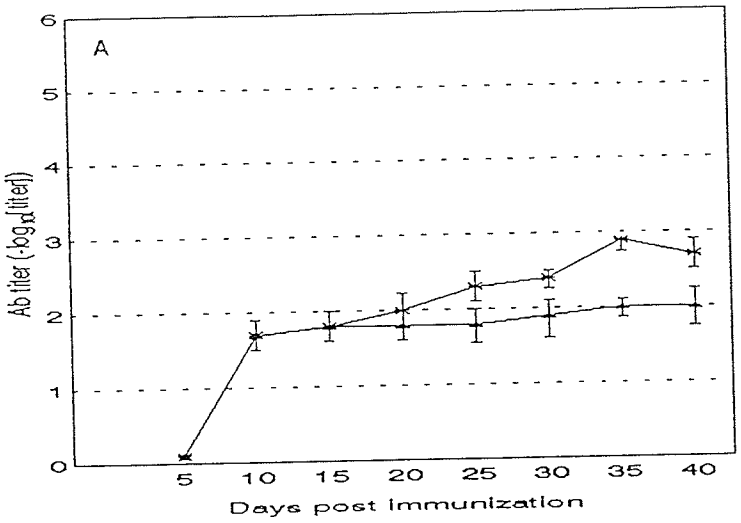
To understand this phenomenon more precisely we next immunized two groups of mice (4 CBA mice/group) with either chlamydial hsp60 or mouse hsp60 (100 μg/mouse). Sera were collected at 5 day intervals from day 5 to day 40. ELISA plates were coated with recombinant chlamydial hsp60 or mouse hsp60 and were used to measure the titre of hsp60 antibody. At day 5, CBA mice lacked detectable antibody to either *C. trachomatis* or mouse hsp60 (figure 15). The antibody titre gradually rose from day 10 onward and the highest titres were observed at day 35 for both mouse hsp60 and chlamydial hsp60 immunization. When CBA mice were immunized with chlamydial hsp60 alone, the antibody titre increased starting at day 10 reaching a maximum titre of between 1:10,000 and 100,000 between day 20 and day 30 (figure 15B). Antibodies generated by immunization with the chlamydial hsp60 reacted to higher titre with the chlamydial hsp60 antigen as compared with the mouse hsp60 suggesting that antibodies preferentially recognized chlamydial specific epitopes. When mice were immunized with mouse hsp60 they generated low antibody titre (less than 1:1000) to either chlamydial hsp60 or mouse hsp60 (Fig. 15A). These results suggest that while chlamydial hsp60 is strongly immunogenic in CBA mice, CBA mice are relatively tolerant to their own hsp60.

Similar patterns were also observed at the T cell level (figure 16). Day 10 after

immunization with recombinant mouse hsp60, lymph node cells (LNC) were cultured with mouse hsp60, chlamydial hsp60 or ovalbumin at varying concentrations from 1 μg to 100 μg/ml (Fig 16A). Neither mouse hsp60 nor chlamydia hsp60 stimulated T cell proliferation above background levels in immunologically naive mice although ConA (2.5 μg/ml) elicited proliferation in these cultures (77500 DPM, data not shown).

When CBA mice were immunized with chlamydia hsp60, LNCs strongly proliferated in response to chlamydial hsp60 in a concentration dependent manner (Fig. 16B). These levels of proliferation were 20 and 35 fold higher than in response to ovalbumin. Importantly when chlamydial hsp60 primed LNCs were cultured with mouse hsp60, intermediate levels of T cell proliferation occurred (17,000 DPM at 10 μg/ml, 20,000 DPM at 100 μg/ml) suggesting that greater cross-reactivity may be occurring at the T cell than the B cell level.

These data suggested that CBA mice are strongly tolerant to mouse hsp60 at the T cell level. Chlamydial hsp60, despite its high sequence similarity to mouse hsp60, exhibited marked immunogenicity. As was observed at the B cell level, chlamydia hsp60 elicited T cell responses which appear to be preferentially directed to foreign sequences in chlamydial hsp60 but which also exhibited some cross reactivity with mouse hsp60. Based on these data we conclude that chlamydial hsp60 alone does not induce high level autoimmune responses and that CBA mice are relatively tolerant to self hsp60 at both the B cell and T cell levels. Tolerance to self hsp60 appears to be similar at both the T cell and the B cell level, while immunization with chlamydial hsp60 induces greater cross-reactivity at the T cell than B cell level.


A
Ab titer (-log10[titer])
0
1
2
3
4
5
6
5
10
15
20
25
30
35
40
Days post immunization

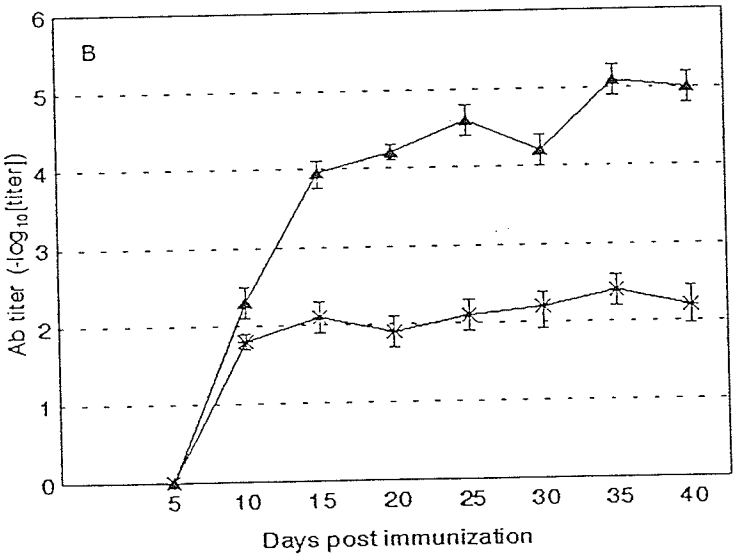
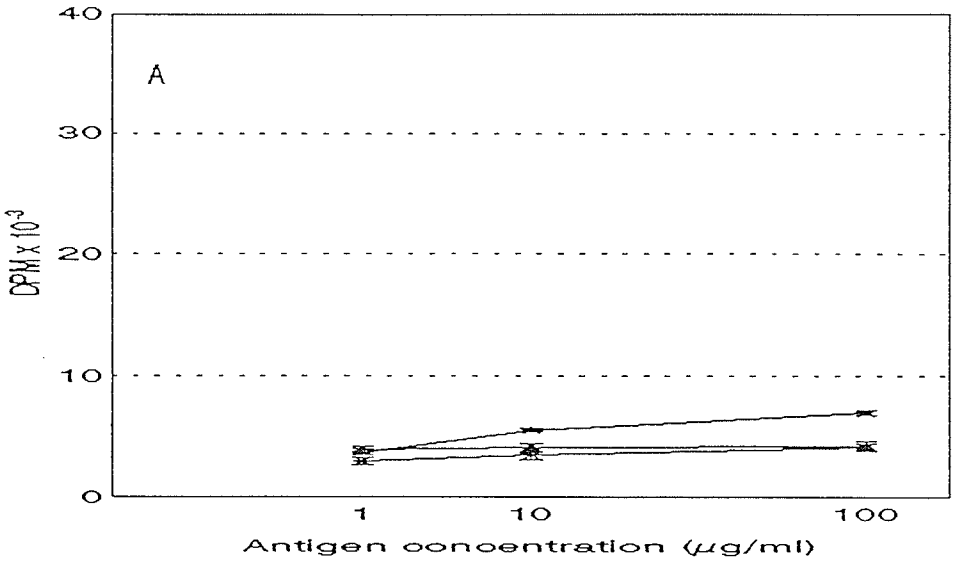

B
Ab titer (-log10[titer])
0
1
2
3
4
5
6
5
10
15
20
25
30
35
40
Days post immunization

Figure 15. Shown are the $\log_{10}$ antibody titre (mean value $\pm$ SD) to purified recombinant chlamydial hsp60 (▲) or to purified recombinant mouse hsp60 (*) at different days post immunization with either mouse hsp60 (panel A) or chlamydial hsp60 (panel B). Low level auto-antibodies were produced following immunization with mouse hsp60 or chlamydial hsp60 whereas high titres of chlamydial hsp60 specific antibodies were produced following immunization with chlamydial hsp60.


A
40
30
20
10
0
DPM x 10-3
1
10
100
Antigen concentration (μg/ml)

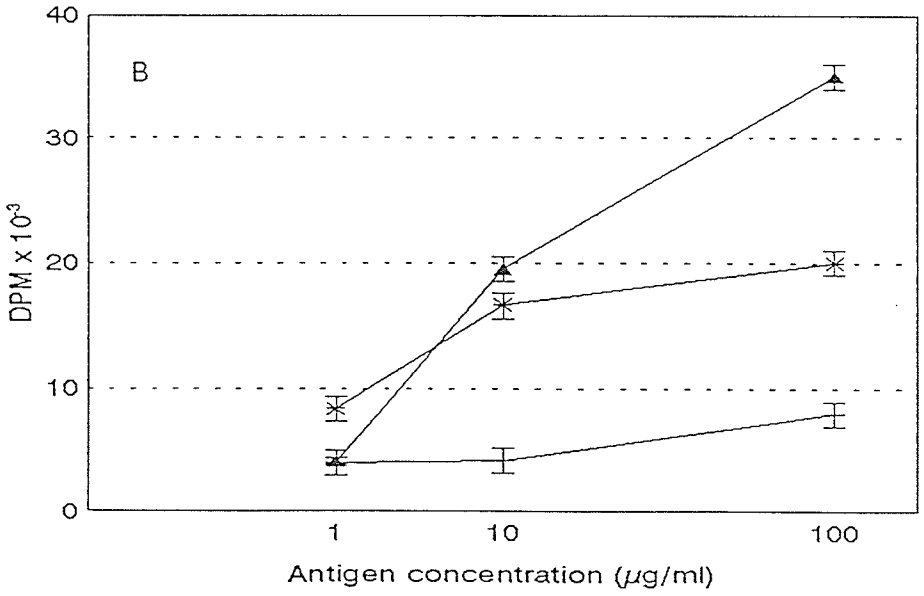

B
40
30
20
10
0
DPM x 10-3
1
10
100
Antigen concentration (μg/ml)

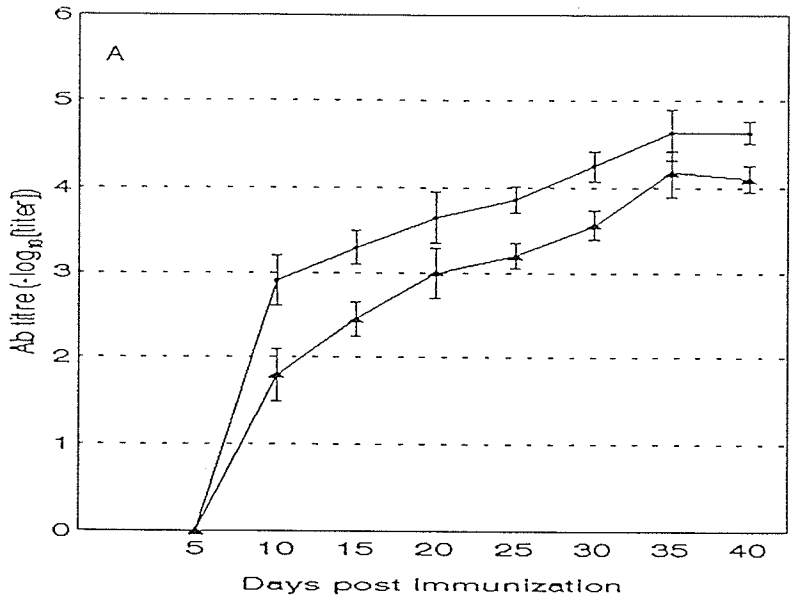
Figure 16. [^{3}H] thymidine incorporation by lymph node cells (LNC) collected from CBA mice immunized with mouse hsp60 (panel A) or chlamydial hsp60 (panel B) and stimulated for 5 days in vitro with mouse hsp60 (*), chlamydial hsp60 (▲) or ovalbumin (+). LNCs collected from mouse hsp60 immunized mice failed to respond to stimulation with mouse hsp60, chlamydial hsp60 and ova while responding to ConA (2.5 μg/ml) with 77.5 x 10^3 DPM. LNCs collected from chlamydial hsp60 immunized mice responded to both chlamydial hsp60 and mouse hsp60 but not to ovalbumin.

3.2. Autoimmunity is readily generated under conditions of concurrent immunization with chlamydial hsp60 and mouse hsp60.

Most autoimmune disease are characterized by high levels of high affinity autoantibody and/or increased numbers of autoreactive T cells. The results observed by immunizing with chlamydial hsp60 alone show that chlamydia hsp60 would be unlikely to induce a pathological autoimmune response by itself. However, during the stressed conditions of infection it may be that the immune system will be faced with both self and pathogen hsp60 in high concentration. A third immunization protocol was therefore designed to mimic this condition. CBA mice were immunized with equal amounts of chlamydial hsp60 and mouse hsp60 (50 μg each). Figure 17 shows the antibody and T cell responses to immunization with both mouse hsp60 and chlamydial hsp60 given concurrently. The data show that immunization with both antigens induced high titre antibody and T cell responses against both chlamydial hsp60 and mouse hsp60. The autoantibody titre reached 1:10,000 at day 35 after immunization (Fig. 17A) and autoreactive T cell response to mouse hsp60 (28,000 DPM at 10 μg/ml) is even higher than T cell proliferation to chlamydia hsp60 (19,000 DPM at 10 μg/ml) (Fig. 17B). To determine if mouse hsp60 contains cryptic determinants which are not generated under physiological conditions by antigen presenting cells and thereby fails to induce immune response, mice were immunized with different doses of mouse hsp60 (0, 3.1, 6.2, 12.5, 25, 50 μg per mouse) plus 50 or 100 μg chlamydia hsp60 respectively. Figure 18 shows the comparison of antibody titre for mouse hsp60 at day 40 post immunization. The autoantibody titre increased when a larger amount of mouse hsp60 were

given. The data suggests that a quantitative change in mouse hsp60 peptide processing may be involved in breaking tolerance to mouse hsp60 suggesting that high levels of cryptic mouse hsp60 epitopes may be generated and presented to mouse immune system when mice are immunized with a high dose of mouse hsp60.

To determine if the shared sequence between chlamydia hsp60 and mouse hsp60 were necessary for the induction of self hsp60 autoimmunity or whether any concurrent antigen could elicit this response, we repeated the experiment replacing chlamydia hsp60 with ovalbumin (ova,50 μg) together with mouse hsp60 in the immunization protocol. Figure 19 shows that the poor immunogenicity of mouse hsp60 is unchanged when compared with mouse hsp60 alone (figure 15) and mouse hsp60 plus ova. The antibody titre to mouse hsp60 remains low (less than 1:1000) (Fig. 19A) and T cell response to mouse hsp60 remained at background levels ($\leq$1000 DPM) (Fig. 19B) although a strong immune response to ova are observed at either B cell or T cell level.


A
Ab titre (-log10 titer)
0
1
2
3
4
5
6
5
10
15
20
25
30
35
40
Days post Immunization

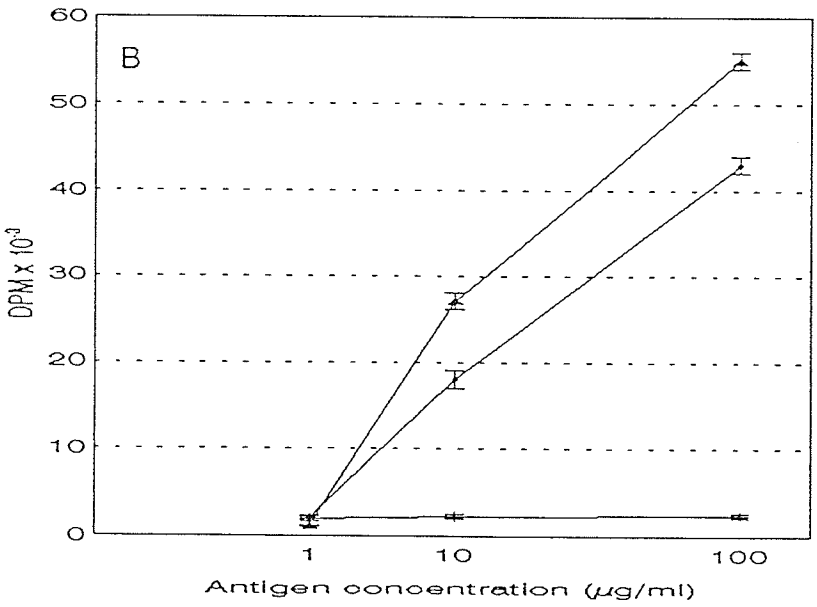

B
DPM x 10-3
0
10
20
30
40
50
60
1
10
100
Antigen concentration (μg/ml)

Figure 17. Groups of CBA mice were immunized with purified recombinant mouse hsp60 (50 μg) and chlamydial hsp60 (50 μg) subcutaneously in incomplete Freund's adjuvant. Sera were collected at five day intervals post immunization and tested at varying dilutions in an ELISA (panel A) for binding to mouse hsp60 (▲) or chlamydial hsp60 (•). Lymph node cells were collected 10 days after immunization and tested in a lymphocyte proliferation assay (panel B) for [^{3}H TdR] thymidine incorporation in response to varying concentrations of mouse hsp60 (▲), chlamydial hsp60 (•) or ovalbumin (+). Immunization with both mouse hsp60 and chlamydial hsp60 elicited strong B and T cell response to both antigens.

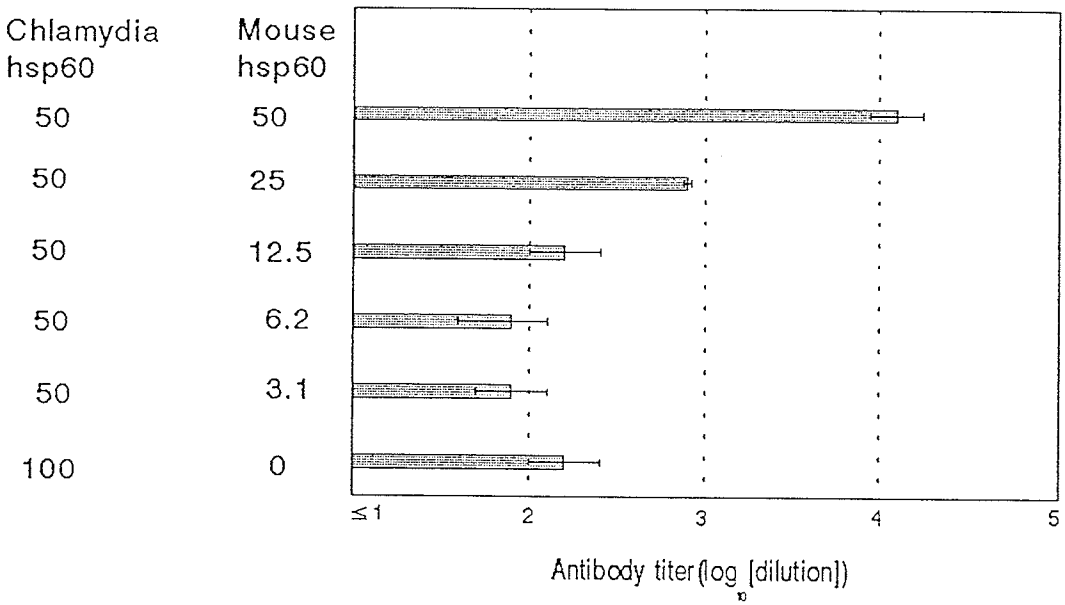
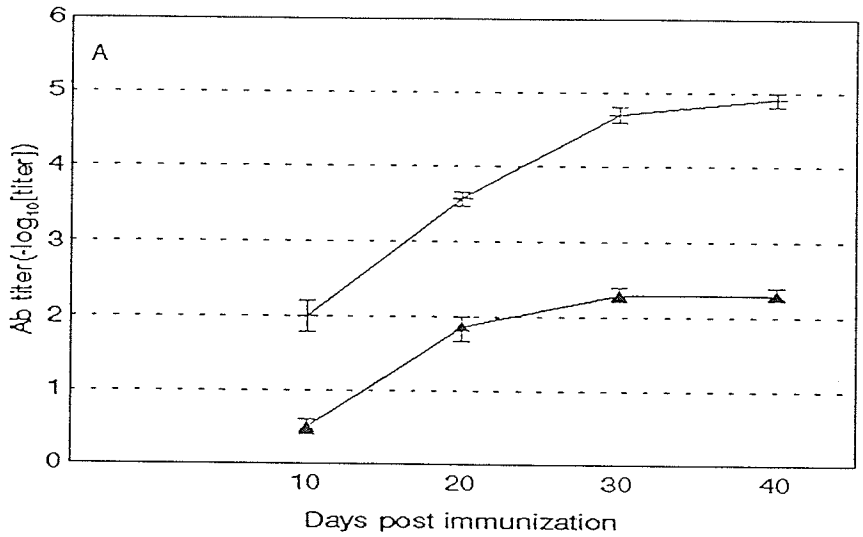

Chlamydia hsp60
Mouse hsp60
50
50
50
25
50
12.5
50
6.2
50
3.1
100
0
≤1
2
3
4
5
Antibody titer (log10 [dilution])

Figure 18. Groups of mice were immunized with 100 μg chlamydial hsp60 alone or 50 μg chlamydial hsp60 plus different dosage of mouse hsp60 (3.1, 6.2, 12.5, 25, 50 μg per mouse respectively). Serum samples were tested for antibody binding titre to mouse hsp60 40 days after immunization. A large dose of mouse hsp60 are required to elicit high level of autoantibody.


A
Ab titer(-log10[titer])
0
1
2
3
4
5
6
10
20
30
40
Days post immunization

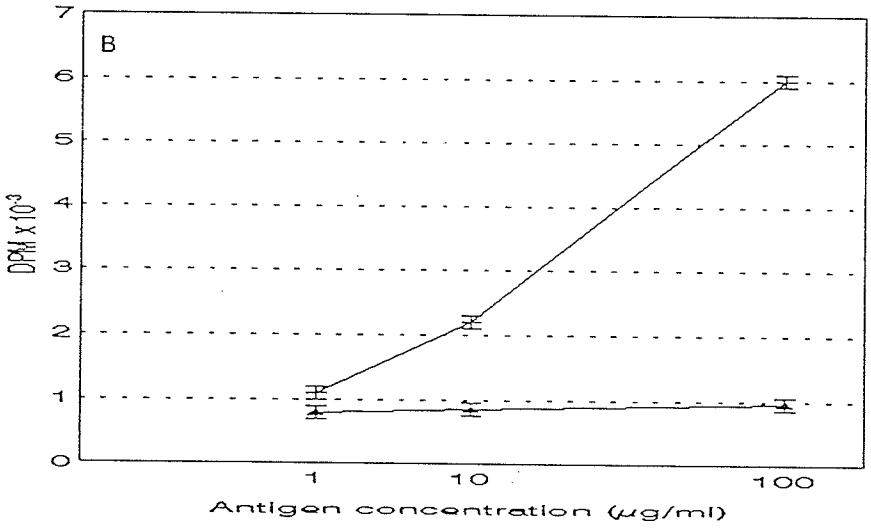

B
DPM x10-3
0
1
2
3
4
5
6
7
1
10
100
Antigen concentration (µg/ml)

Figure 19. CBA mice were immunized with purified recombinant mouse hsp60 (50 μg) and ovalbumin (50 μg) subcutaneously in incomplete Freund's adjuvant. Sera were collected at 10 day intervals post immunization and tested at varying dilutions in an ELISA (panel A) for binding to mouse hsp60 (▲) or ovalbumin (┼). Lymph node cells were collected 10 days after immunization and tested in a lymphocyte proliferation assay (panel B) in response to varying concentrations of mouse hsp60 (▲) or ovalbumin (┼). Immunization with both mouse hsp60 and ovalbumin elicited strong T and B cell responses to ova but weak B cell and absent T cell response to mouse hsp60.

Part IV. MECHANISMS OF THE IMMUNE TOLERANCE MODEL

4.1.1. T cell tolerance to mouse hsp60 is controlled through peripheral anergy

To determine if peripheral anergy rather than clonal deletion accounts for tolerance to self hsp60, we repeated the lymphocyte proliferation assays using LNC from mouse hsp60 immunized animals after adding recombinant IL-2. If peripheral anergy accounts for T cell tolerance to mouse hsp60 we hypothesized that added IL-2 should overcome the anergic state as this has been been frequently observed in other experimental system (Schwartz 1990, and Umlaue et al, 1993). Cultures stimulated with mouse hsp60 (20 μg/ml) alone failed to respond whereas the cultures responded strongly if supplemented with IL-2 (either at 10 or 100 units/ml) (figure 20). IL-4,IL-10 and IFN-gamma (100 units/ml) provided poor or negligible co-stimulation for mouse hsp60 induced T cell proliferation (figure 21). Control LNCs without mouse hsp60 stimulation exhibited low level proliferation to IL-2 only at the highest IL-2 concentration (100 u/ml) tested (Figure 20). These results clearly show that T cells reactive to self hsp60 are found in the periphery apparently escaping thymic deletion and thus are available for activation under appropriate conditions.

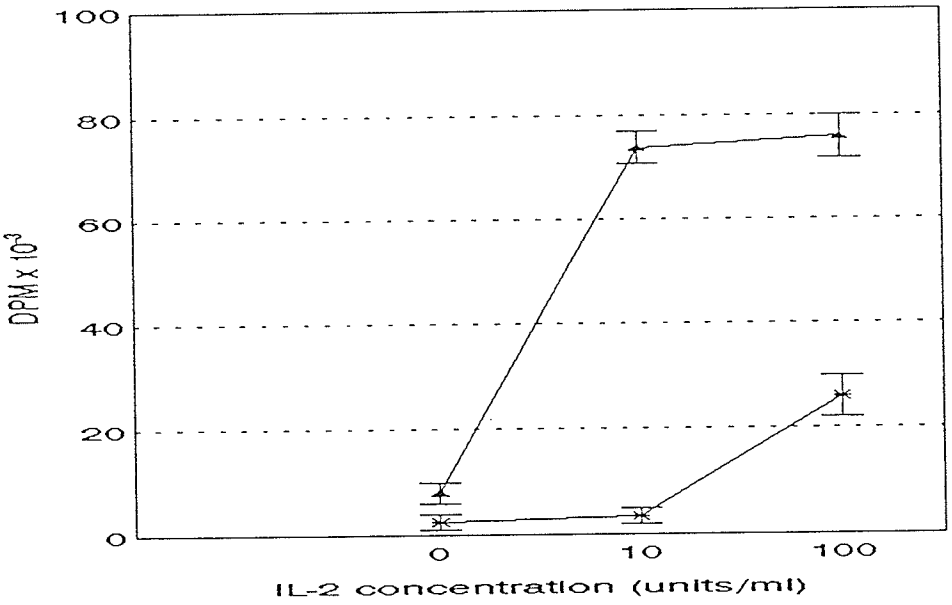

100
80
60
40
20
0
DPM x 10³
0
10
100
IL-2 concentration (units/ml)

Figure 20

Lymph node cells (LNCs) were collected from CBA mice immunized with mouse hsp60 10 days earlier and cultured in vitro with varying concentration of IL-2 alone (0, 10, 100 units/ml), or mouse hsp60 (20 μg/ml) plus IL-2 (▲). LNCs proliferated in response to mouse hsp60 when supplemented with IL-2.

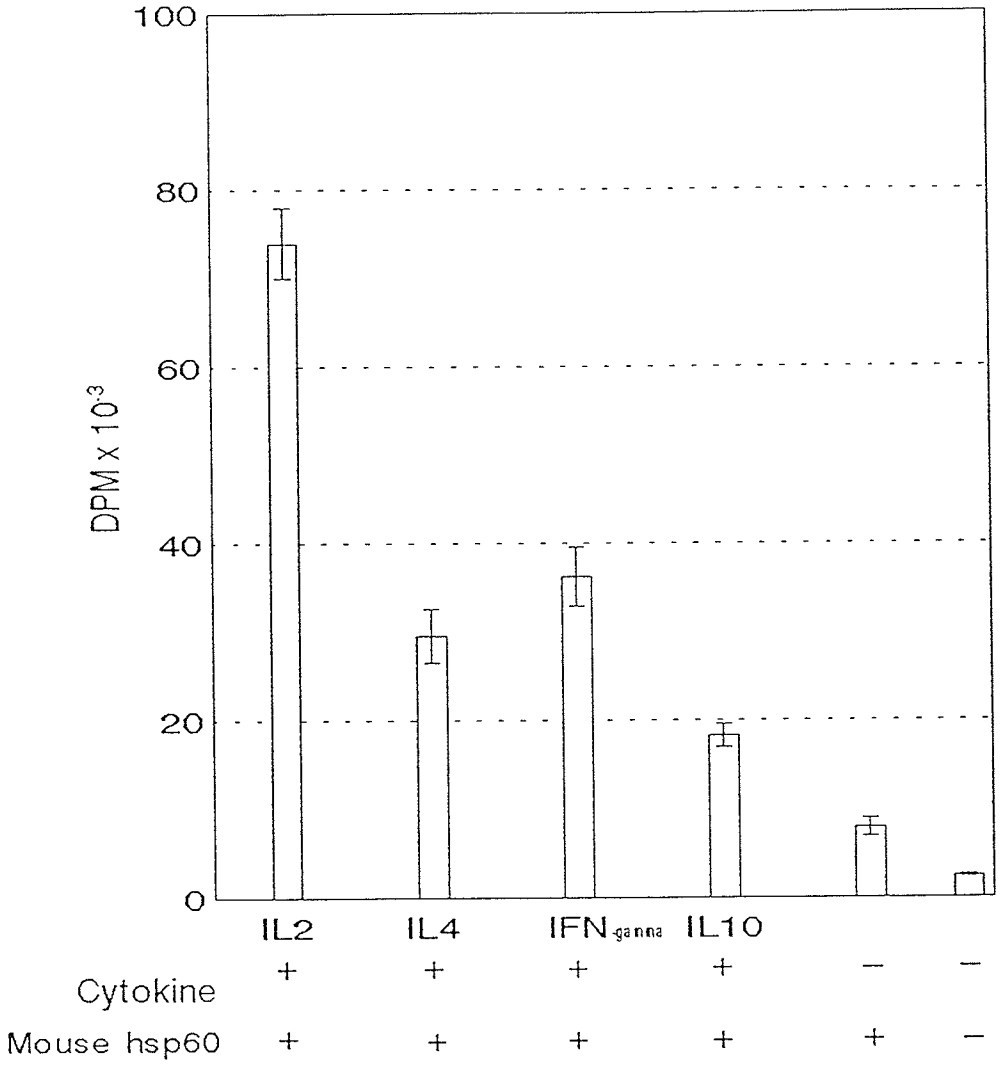

100
80
60
40
20
0
DPM x 10-3
IL2
IL4
IFN-ganma
IL10
Cytokine
Mouse hsp60
+ + + + − −
+ + + + + −

Figure 21

Comparison of anergic T cell proliferation to cytokines. LNCs were collected from mice immunized with mouse hsp60 and cultured in vitro with or without mouse hsp60 (20 μg/ml) and mouse hsp60 plus different cytokines (100 units/ml, IL-2, IL4, IFN-gamma, IL-10). Only the culture supplemented with IL-2 strongly respond to mouse hsp60.

4.1.2. Cytokines induced by hsp60.

To investigate whether a unique cytokine pattern characterized hsp60 anergic T cells we next determined cytokine production by spleen cells collected from mouse or chlamydial hsp60 immunized mice after in vitro stimulation with mouse or chlamydial hsp60 or non relevant control antigens (ovalbumin, cytochrome c). The cytokine production (IFN-gamma, IL-2, IL-4, IL10, TGF-ß) was examined by ELISA assay. Table 6 shows the results. When immune spleen cells were stimulated with ovalbumin, cytochrome c, or without antigen, the cytokine production was undectable or low level except for TGF-ß production. Substantial amounts of TGF-ß were detected in all tested samples. We also failed to detect significant levels IL-2 or IL-4 production in hsp60 stimulated samples and therefore the difference among samples was slight. We think that the "fresh" lymphocytes we examined directly ex vivo may have failed to produce IL-4 upon short-term culture and that the secreted IL-2 may have been quickly taken up by T cells through an autocrine or papracrine mechanism. The consensus from several groups is that T cells with the potential to become IL-4 secretors exist in vivo in the form of precursors which require 4-12 days of culture and one or more cycles of rest and restimulation with mitogen or antigen before they become detectable as lymphokine-secreting cells (Swain, 1988; Powers, 1988; Hayakawa, 1989; Fox, 1989; Powell, 1990; Weinberg, 1990; Rocken, 1991).

The remaining two cytokines, IFN-gamma and IL-10, had differential secretion patterns which depended on the antigen used for immunization and during in vitro stimulation (Table 7). Chlamydia hsp60 induced a high level of IFN-gamma production and low levels of IL-10 production. Mouse hsp60 induced low level IFN-gamma and high levels of IL-10.

Table 6 Cytokine production profile

Immunization	Stimulation in vitro	Cytokine production (pg/ml)				
		IFN-r	IL-2	IL-4	IL-10	TGF-ß
Mhsp60	No Antigen	< 39	ND	ND	< 20	ND
	Ovalbumin	< 39	130±11	55±4	210±31	970±19
	Cyt c	< 39	ND	ND	< 20	ND
	Chsp60	934±33	450±27	62±7	1390±124	1200±162
	Mhsp60	194±25	330±16	39±5	2186±103	1450±270
Chsp60	No Antigen	< 39	ND	ND	< 20	ND
	Ovalbumin	< 39	145±23	83±19	193±8	1280±121
	Cyt c	< 39	ND	ND	< 20	ND
	Chsp60	8032±134	340±12	74±16	507±35	1350±215
	Mhsp60	433±31	272±31	59±9	1227±80	1400±310
(M+C)hsp60	No Antigen	< 39	ND	ND	< 20	ND
	Ovalbumin	< 39	170±36	92±17	< 20	650±53
	Cyt c	< 39	ND	ND	< 20	ND
	Chsp60	4723±187	650±72	88±11	424±8	1100±126
	Mhsp60	417±31	330±43	95±15	366±42	630±81

Note: Groups of mice were immunized with mouse hsp60 (Mhsp60), chlamydia hsp60 (Chsp60) or both Mhsp60 and Chsp60 [(M+C) hsp60] 10 days earily and spleen cells were stimulated in vitro with the relevant antigen and supernatants were collected at 24, 48, or 72 hours. Cytokine concentrations were determined by ELISA as described in Material and Methods. Cyt c represents cytochrome c, ND means not done.

Table 7 Comparison of cytokine production and immune response

Group	Immunization	In vitro stimulation	IFN-γ (pg/ml)	IL-10 (pg/ml)	IFN-γ/IL-10 ratio	Antibody titre	T cell proliferation
1	Mhsp60	Mhsp60	194±25	2186±103	0.09	2.4±0.30	2.0±0.4
2	Mhsp60	Chsp60	934±33	1390±124	0.67	1.9±0.21	4.2±0.6
3	Chsp60	Chsp60	8032±134	507±35	15.8	4.2±0.15	43.6±1.2
4	Chsp60	Mhsp60	433±31	1227±80	0.35	2.2±0.18	8.4±0.1
5	M+C	Chsp60	4723±187	424±8	11.4	4.2±0.28	19±2.1
6	M+C	Mhsp60	417±31	366±42	1.14	3.6±0.20	20±5.1

Note: Six group mice (4 mice/group) were immunized with mouse hsp60 (Mhsp60), chlamydia hsp60 (Chsp60), mouse hsp60 plus chlamydia hsp60 (M+C) respectively. Immune spleen cells and LNCs were isolated at day 10 after immunization and were stimulated with Mhsp60 or Chsp60 in vitro. Cytokine production were measured from spleen cell supernatants and T cell proliferation data (DPM x 1000) come from LNC cultures. Serum samples were collected 30 days later post immunization and tested in ELISA plates coated with Mhsp60 (group 1,4,6) and Chsp60 (group 2,3,5) for antibody titres represented as $-\log_{10}$ (dilution).

Importantly IL-10 production elicited by mouse hsp60 (either in vivo or in vitro) was markedly reduced in mice co-immunized with both chlamydia hsp60 and mouse hsp60. The IFN-gamma/IL-10 ratio was best correlated with the autoimmune response induced by hsp60. When the ratio was greater than one, a high level autoimmune response to mouse hsp60 was observed at both the B and T cell level. When the ratio was less than one, autoimmune responses to self hsp60 was not detectable.

We next investigated the effect of IL-10 on IL-2 reversal of T cell responses to mouse hsp60. We reasoned that if IL-10 production in vivo maintains T cell anergy to mouse hsp60 in vivo, IL-10 should prevent IL-2 from reversing T cell tolerance in vitro. Figure 22 shows the result. LNCs primed with mouse hsp60 that were cultured with mouse hsp60 (20 μg/ml) and IL-2 (50 units/ml) proliferate strongly (11,800 dpm) again showing that IL-2 easily reverses T cell anergy to mouse hsp60. IL-10 suppressed IL-2 reversion of T cell anergy in a dose dependent manner between 5 and 50 ng/ml. Thus IL-10 appears to be a key cytokine maintaining tolerance to mouse hsp60 .

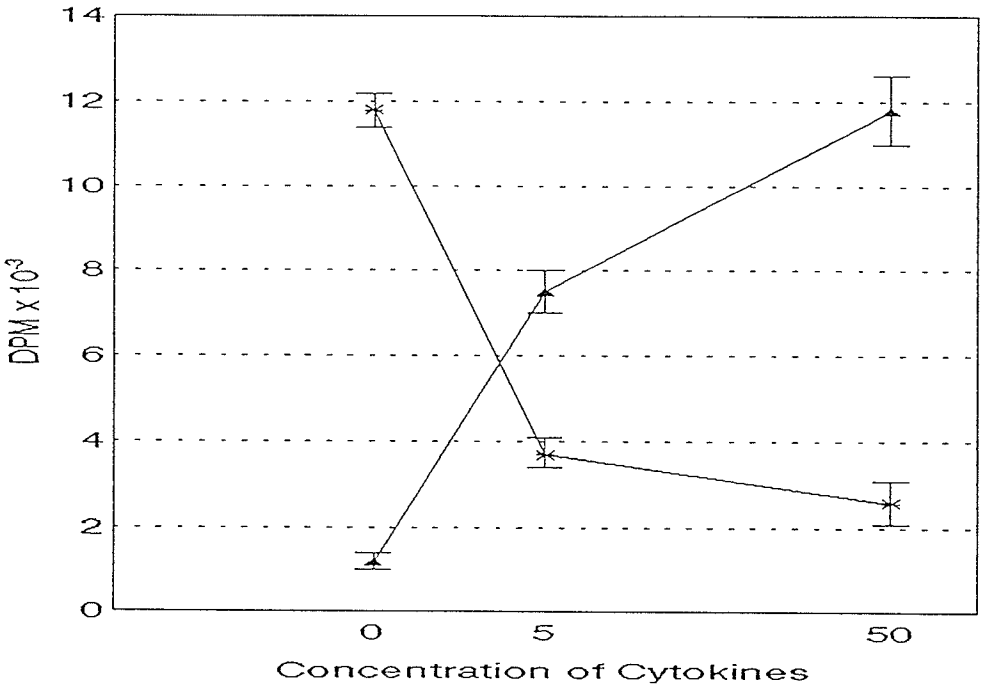

14
12
10
8
6
4
2
0
DPM x 10^3
0
5
50
Concentration of Cytokines

Figure 22

Lymph node cells (LNCs) were collected from mice immunized with mouse hsp60 and cultured in vitro with mouse hsp60 (20 μg/ml) (▲ without IL-2 added) or mouse hsp60 plus varying concentration of IL-2 (line -▲-, at 5, 50 units/ml). IL-10 (line -*-) with varying concentrations (5, 50 ng/ml) were added to the IL-2 cultures containing mouse hsp60 (20 μg/ml) and IL-2 (50 units/ml). IL-10 inhibited the proliferation induced by IL-2 cultures.

4.2.1. T helper function for mouse hsp60 can be bypassed by a foreign carrier protein

To determine if the absence of a B cell response to mouse hsp60 was due to the absence of T cell help, antibody response to mouse hsp60 was compared among groups of CBA mice immunized with mouse hsp60 alone or with mouse hsp60 as a fusion protein containing the N-terminal 280 amino acids from *Schistosome japonicum* glutathione-S-transferase (GST). Antibody titres at day 30 after immunization were compared (figure 23). Immunization with mouse hsp60 fusion protein or mouse hsp60 plus chlamydia hsp60 induced high antibody responses to mouse hsp60 whereas immunization with mouse hsp60 alone or mouse hsp60 plus soluble GST did not elicit high titers of antibodies to mouse hsp60. These results suggest that self-reactive B cells for mouse hsp60 require T cell help from a foreign epitope sequence in order to induce high titres of antibodies to mouse hsp60.

4.2.2. T cells primed by immunization with chlamydial hsp60 adoptively transfer T cell help for autoimmune response to mouse hsp60

Since chlamydia hsp60 also contains foreign epitope sequences we hypothesised that immune T cells induced by chlamydial hsp60 may also help mouse hsp60 break tolerance. To test this hypothesis, immune T cells primed in vivo with chlamydia hsp60 were adoptively transferred into naive CBA mice followed by immunization with mouse hsp60 and autoantibody titers to mouse hsp60 were assayed 30 days after immunization (Table 8). The control groups of mice that received immune serum (Group 5) or spleen cells primed with mouse hsp60 (Group

4) had low $\log_{10}$ titre (2.0 and 2.5 respectively) of antibody to mouse hsp60 at day 30 after immunization with mouse hsp60. Mice that were adoptively transferred with immune spleen cells or T cells previously primed in vivo with chlamydial hsp60 (Group 1, 2) had high titres of mouse hsp60 antibody (4.1 and 3.7 respectively). The data suggest that chlamydia hsp60 specific T cells can provide helper T cells to mouse specific B cells. In aggregate with these data as shown in figure 20A, the induction of high titre of mouse hsp60 antibodies requires that mouse hsp60 be co-administered with chlamydia hsp60 and does not occur after immunization with chlamydial hsp60 alone.

4.2.3. Mapping T helper sites from chlamydial hsp60

Since chlamydial hsp60 can induce T cells involved in autoantibody production against mouse hsp60, we reasoned that chlamydial hsp60 should contain one or more T cell sites capable of providing help for autoreactive B cells. We constructed five deletion mutants of chlamydia hsp60 in order to map T helper autoantigenic sites. Each deletion fragment was used to co-immunize with mouse hsp60 and antibody titres to mouse hsp60 were measured 30 days later (Table 9). Among the deletion fragments only C6 (amino acids 380 to 545) failed to induce autoreactive B and T cell response to mouse hsp60 after co-administration with mouse hsp60. Since the four other fragments were able to induce autoimmune responses to mouse hsp60, these data suggest that multiple T helper cell sites are located in the N terminal two-thirds of the protein.

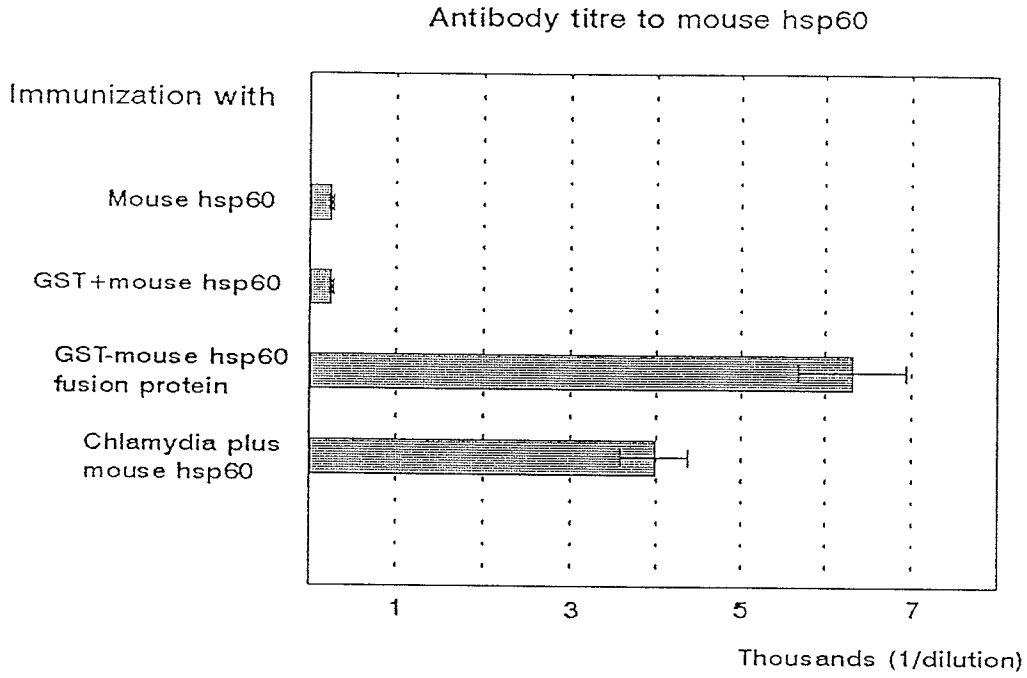

Antibody titre to mouse hsp60
Immunization with
Mouse hsp60
GST+mouse hsp60
GST-mouse hsp60 fusion protein
Chlamydia plus mouse hsp60
1
3
5
7
Thousands (1/dilution)

Figure 23.

Four groups of CBA mice (four mice/group) were immunized with 100 μg purified recombinant mouse hsp60, mouse hsp60 fusion protein in which the N-terminal of mouse hsp60 was fused to glutathione S-transferase (GST), mouse hsp60 plus GST, or mouse hsp60 plus chlamydial hsp60 subcutaneously in incomplete Freund's adjuvant. Sera were collected at day 30 post immunization and tested at varying dilution in ELISA for binding to mouse hsp60. Immunization with GST-mouse hsp60 fusion protein elicited strong B cell response to mouse hsp60.

Table 8. Autoimmune response to mouse hsp60 is dependent on chlamydial hsp60 specific T cells

Group	Immunization of donor mice	Adoptive transfer	Immunization of recipient mice	Antibody titre ($-\log_{10}$) for mouse hsp60
1	Chlamydia hsp60	spleen cells	Mouse hsp60	4.1±0.19
2	Chlamydia hsp60	T cells	Mouse hsp60	3.7±0.17
3	Chlamydia hsp60	spleen cells	Ovalbumin	1.4±0.10
4	Mouse hsp60	spleen cells	Mouse hsp60	2.5±0.15
5	Chlamydia hsp60	sera	Mouse hsp60	2.0±0.12

Sera,spleen cells, or purified T cells (see material and methods) were collected from donor mice primed with either chlamydia hsp60 (group 1,2,3,5) or mouse hsp60 (group 4) and adoptively transferred to recipient mice followed by immunization with mouse hsp60 (group 1,2,4,5) or ovalbumin (group 3). Antibody binding to mouse hsp60 was tested by ELISA at day 30 post immunization and is shown as $-\log_{10}$(dilution).

Table 9. Mapping helper sites of chlamydia hsp60

Immunization with Mhsp60 plus Chsp60 and deletion fragments		Antibody titre for Chsp60	Antibody titre for Mhsp60	T cell stimulated by Chsp60	T cell stimulated by Mhsp60
1 — 545	Hsp60	4.9±0.17	4.0±0.25	20.3±2.2	20.8±2.5
— 265	PA1-265	4.3±0.20	3.9±0.25	61.4±1.7	64.6±2.2
— 150	C611	2.3±0.25	3.8±0.21	9.0±0.4	6.7±0.2
250 —	C53	4.8±0.10	4.5±0.20	22.2±1.2	6.5±0.3
268 — 409	C86	2.1±0.13	4.0±0.15	27.2±1.4	8.6±0.5
360 —	C6	3.4±0.20	2.1±0.22	3.6±0.3	1.0±0.2

Notes: Antibody titres shown in the table as -log (dilution) were measured 30 days after immunization.
CBA mice were immunized with equal amount (50 ug/mouse) of mouse hsp60 (Mhsp60) and chlamydia hsp60(Chsp60) or deletion mutants. LNCs were stimulated with either Chsp60 (10 ug/ml) or Mhsp60 (10 ug/ml) in vitro. T cell proliferation represent as disintegration per minute (dpm x 1000). The background in the proliferation assays were between 700 to 3000 dpm.

DISCUSSION

It is well known that *C. trachomatis* infections can cause infertility and adverse pregnancy outcome. In an in vitro fallopian tube organ culture, addition of *C. trachomatis* induced little damage to the integrity of the fallopian tube epithelial cells (Hutchinson, 1979). Similarly in vivo, primary infection of *C. trachomatis* into the fallopian tubes of nonhuman primates resulted in only a self-limited infection with no long-lasting damage (Patton, 1990). Repeated infections, however, led to permanent fallopian tube scarring (Patton, 1990). Analogous to the better elucidated mechanism of immune-mediated damage in trachoma, a chlamydial eye infection (Taylor, 1984), it appears that an immune response to *C. trachomatis* infection also play a prominent role in causing fallopian tube pathology. A remarkable gradient in seroprevalence to the chlamydial hsp60 is observed among women with different manifestations of chlamydial infection. Sixteen percent to twenty-five percent of fertile microimmunofluorescent antibody-positive women, in contrast 36%-44% of women with *C. trachomatis* cervicitis, 48%-60% of women with *C. trachomatis* pelvic inflammatory disease (PID), and 81%-90% of women with *C. trachomatis*-associated fallopian tube obstruction manifesting as ectopic pregnancy or tubal infertility have chlamydial hsp60 antibody (Brunham, 1994). Furthermore, among women with laparoscopially visualized chlamydial PID, those with the highest antibody titres to chlamydial hsp60 have significantly more severe inflammatory manifestations (Stamm, 1994).

The strong correlation observed between the prevalence of antibody responses to chlamydial hsp60 and the severity of chlamydial disease raises a central issue about immunopathological responses to the chlamydial hsp60. How does the immune system mount such strong response to such highly conserved protein (chlamydial hsp60) without risking autoimmunity? The goal of

this thesis was to look for those answers. The work presented in this thesis establish several facts regarding the immunology of hsp60 (both chlamydial and mouse hsp60). (1) Autoantibodies to human hsp60 peptides are present in the sera of women with *C. trachomatis* associated ectopic pregnancy. (2) Chlamydial hsp60 itself fails to induce high levels of autoimmune responses. (3) Strong autoimmune response to host hsp60 occurs under specific immunization conditions; for instance, when mice were coimmunized with chlamydial hsp60 and mouse hsp60. (4) The shared amino acid sequence between chlamydial hsp60 and mouse hsp60 as well as high concentrations of self hsp60 are essential to induce autoimmunity. (5) Tolerance to mouse hsp60 is due to T cell anergy and active cellular suppression mediated by antigen-specific IL-10 secretion. (6) At least two T helper autoantigenic sites are located at regions (aa1-150, aa268-409) on the chlamydial hsp60 and are responsible for high levels of autoantibody induced by the co-immunization protocol.

I. Chlamydial hsp60 and mouse hsp60 have different immunogenicity.

Heat shock protein 60 (hsp60) are among the most abundant and most conserved polypeptides in the biosphere (Kaufmann, 1991). The omnipresence of hsp60 in the microbial world means that the chance that the vertebrate immune system will frequently come into contact with them is extraordinarily high. Interestingly many microbial hsp60 including chlamydial hsp60 are also dominant targets of immune responses. Kaufmann (1987) showed that about 20% of Mycobacteria-reactive T cells from mice immune to *M. tuberculosis* responded to hsp60.

Importantly, the existence of hsp60 in mammalian cells requires that immune tolerance be established in order to avoid self-attack. Because hsp60 are such highly conserved proteins, these unique features pose central problems for the immune system. The high degree of homology between microbial and mammalian hsp cognates requires that the immune system needs to decide whether to accept hsp60 as a dominant microbial antigen, or as a harmful self-antigen. It appears that the immune system tries to do justice to either possibility. Often, this is achieved surprisingly well; sometimes, however, failures seem to be unavoidable and such failures may be associated with the induction of autoimmune diseases. Reactivity against the mycobacterial 65-kDa hsp has been described in various forms of arthritis (Res, 1988; Gaston, 1989, 1990) and synovial fluid-derived Yersinia-reactive T cells responding to human 65-kDa heat-shock protein and heat-stressed antigen-presenting cells have been reported (Hermann, 1991). Thus a commonly held postulate regarding the immunopathogenesis of hsp60 related diseases is that of autoimmunity triggered by antigenic mimicry.

We found that *C. trachomatis* hsp60 and mouse hsp60 have different immunogenicity when used as individual immunogens. When CBA mice were immunized with chlamydia hsp60 alone, the antibody titre reached 1:100,000 between day30 and day40 (Fig. 15B). Lymph node cells (LNC) strongly proliferated in response to chlamydia hsp60 which was 20 and 35 fold higher than background proliferation (Fig. 16B). The cross-reactivity to mouse hsp60 elicited by immunization with chlamydial hsp60 was quite low at either the antibody (Fig. 15B,less than 1:500 for mouse hsp60) or T cell level (Fig. 16B, 3 to 4 fold higher than background). Thus chlamydial hsp60 alone does not induce high level autoimmune responses although chlamydial

hsp60 is a dominant antigen with strong immunogenicity. When mice were immunized with mouse hsp60 they generated low antibody titres (Fig. 15A) and only background levels of T cell proliferation to either mouse or chlamydial hsp60 (Fig. 16A). CBA mice had relative tolerance to self hsp60.

Although CBA mice were relatively tolerant to self hsp60 at the B cell level, the production of autoantibodies to self hsp60 was not entirely prevented (Fig.15). An example of partial "breakage" of B cell tolerance has also been observed in mice that express hen egg lysozyme (HEL) as a transgene-encoded self antigen (Goodnow, 1988). In this model system incomplete tolerance in the B cell repertoire was shown to be due to the failure to induce tolerance among low affinity B cells (Goodnow, 1990). In physiological terms, the failure to induce tolerance in low-affinity anti-self B cells to many self antigens (Goodnow, 1990; Benjamin, 1984) appears to pose little risk of autoimmune disease in most cases, presumably because the titres and affinity of such antibodies are normally below those needed to initiate tissue destruction or to interfere with biological functions.

II. The induction and maintenance of anergy to mouse hsp60.

2.1. General mechanism of immune tolerance

The establishment and maintenance of tolerance is based on multiple events that occur in the thymus and the periphery and that result in either deletion of self-reactive T cells or the

induction of nonresponsiveness. The repertoire of mature T cells found in the blood and peripheral lymphoid organs is selected by MHC molecules and self-antigens expressed in the thymus. Immature thymocytes that express a TCR with the "appropriate" affinity for MHC-self-peptide complexes preferentially emigrate from the thymus into the periphery (positive selection), whereas autoreactive thymocytes that recognize self-antigen-MHC complexes with a high affinity are deleted (negative selection). Self-tolerance due to central mechanisms is established in large part through negative selection.

For autoreactive T cells that escape thymic selection peripheral mechanisms such as anergy induction, downregulation of TCR, or active suppression also exist to maintain tolerance (Kappler, 1987; Kisielow, 1988; Sha, 1988; Nikolic-zugic, 1990; Rocha, 1991; Lo, 1991; Schonrich, 1991; Takahama, 1992; Simpson, 1993). Under normal conditions, anergy to self proteins is probably most effectively maintained at the T cell level as compared to the B cell level. It has been shown that at very low concentrations of self-antigen (below 10^{-10} M) self-reactive T cells become tolerant; B cell tolerance however requires 10 to 100 times higher antigen concentration for induction. In addition tolerized B cells remain able to make high-affinity antibody only when given appropriate T cell help (Adelstein, 1991). The distinct levels of anergy are characterized by the phenotypic appearance of tolerant T cells and their resistance to activation in vitro; they range from cells without phenotypic change, a relatively mild form of tolerance, to cells with complete downregulation of T cell receptor and accessory molecules, the most stringent level of anergy (Arnold, 1993).

2.2. T cell tolerance to mouse hsp60 is controlled through peripheral anergy.

Immunization with chlamydial hsp60, a protein that shares 48 % amino acid sequence identity with mouse hsp60, generates mainly B and T cell responses to chlamydial specific epitopes. Tolerance to mouse hsp60 appeared to be due to T cell anergy since it could be overcome by exogenous IL-2 as this has been identified as a marker of peripheral T cell anergy in other antigen systems (Fig. 20, Fig. 21). Mature peripheral T cells can be silenced by incubating T cells with a high concentration of peptide in the absence of antigen-presenting cells (APC) preventing the cells from responding to a subsequent challenge with antigen-pulsed APC. They are said to have become "anergic" (Lamb, 1983). Furthermore, interleukin-2 (IL-2), but not interferon-gamma or IL-1, inhibited tolerance induction and the addition of IL-2 reversed established tolerance (Essery, 1988). Similar in vitro experiments with T-cell clones showed that anergic T cells were unable to produce their own growth factor IL-2, following restimulation (Schwartz, 1990). In general, more in vivo works including our data support the two-signal model of Bretscher & Cohn (1970). They proposed that the distinction between immunity and tolerance depends on whether the immunocompetent cell receives a "second" or costimulator signal in addition to the antigenic epitope presented by the MHC.

It is possible that mouse hsp60 naturally exist in vivo at very low concentration and only can be presented by resting B cells or other APCs that lack costimulator molecules such as B7 (Freeman, 1989; Hathcock, 1993). Foreign hsp60 such as chlamydial hsp60, however, do not usually confront the immune system as a soluble protein antigen (mouse hsp60). It is likely that foreign antigens such as microbial hsp60 appear in a multi-valence display, as a part of

infectious organisms rather than as a soluble protein, and enter the body at sites (e.g., skin, mucosal membrane and lungs) where potent APCs such as dendritic cells and macrophages with costimulator molecules pick them up. In addition, many microbial substances (e.g., lipopolysaccharide) are excellent inducers of costimulatory activity in APC, ensuring that T cells activation will occur when these APC present antigen. There is also evidence that the induction of anergy in vivo to foreign antigens can occur if they follow presentation conditions similar to self proteins. For instance, T cells chronically exposed to high circulating levels of *Mycobacterium lepra* antigens in patients with lepromatous leprosy appear to be unresponsive to antigen (GayLord, 1987). This may also explain the anergic state induced in vivo following intravenous injection of anti-CD3 antibody (Hirsch, 1988), high doses of soluble antigen (Dixon, 1955), or superantigens (Webb, 1990; Kawabe, 1991).

The critical change characteristic of anergic T cells is their inability to produce the autocrine growth factor IL-2. They are unable to proliferate in response to antigen and normal APCs because of this fundamental defect. Northern blot analysis showed that anergic T cells stimulated by anti-CD3 plus anti-CD28 antibodies do not produce detectable IL-2 mRNA (Johnson, 1994), IFN-gamma and IL-3 mRNA production are detectable though greatly reduced (Jenkins, 1991)

The molecular changes in anergic T cells responsible for the defect in IL-2 production are not yet clear. Mueller and colleagues (Jenkins, 1991; Mueller, 1989) reported that signal transduction in an anergic murine T cell clone was unaffected at the level of inositol phosphate generation and concluded that the TCR on anergic cells appeared to be coupled to normal intracellular signalling pathways. Gajewski et al (Gajewski, 1994), however, reported multiple TCR-associated signalling defects in anergic T cell clones, including elevated basal levels of

intracellular calcium and altered tyrosine phosphorylation patterns. Although the TCR can be transiently down-modulated following receptor occupancy, TCR/CD3 expression returns to normal in anergic cells (Quill, 1987; Jenkins, 1990; Blackman, 1990; Kang, 1992). Anergic cells also express normal levels of high affinity IL-2 receptor, CD4, LFA-1, ICAM-1, and CD28 (Jonhnson, 1994) on their surfaces. In one report of cloned human T cells, however, *Staphylococcus* enterotoxin-induced anergy was associated with a down-modulation of the TCR and CD28 (O'Hehir, 1990). The anergic T cell clones which constitutively express the high affinity IL-2 receptor are still able to proliferate in response to exogenous IL-2 (Geenen, 1993) and are rescued from their anergic state by exogenous IL-2 (Desilva, 1991; Beverly, 1992). Recently Li et al (1996) reported that the analysis of CD3- and CD28-induced signal transduction revealed reduced ERK (extracelluar signal-regulated protein kinases) and JNK enzyme (c-Jun NH_2-terminal kinases) activities in murine anergic T cells. Both of these kinases are thought to play key roles in the signal transmission from the outside of the T cell to AP-1-binding DNA sequence inside the nucleus. The amounts of ERK and JNK proteins were unchanged, and the kinases could be fully activated in the presence of phorbol myristate acetate. Dephosphorylation of the calcineurin substrate NFATp (prexisting nuclear factor of activated T cells) also remained inducible. These results suggest that a specific block in the activation of ERK and JNK contributes to the defective IL-2 production that characterizes clonal T cell anergy.

2.3. IL-10 is a key cytokine for active suppression.

It has been my interest to investigate the possibility that cytokines may play an important

role in tolerance to mouse hsp60, by investigating which cytokines are associated with the induction or reversion of T cell anergy to self hsp60.

In vitro proliferative responses to measure the presence of T cell immune response to a particular antigen have been extensively used. When the central role of cytokines in the immune response was elucidated, measurement of proliferation was said to be too complicated a system that did not correlate with resistance in many model of infectious diseases. Cytokine levels, however, were shown in many instances to correlate well with resistance or disease susceptibility (Belosevic, 1988, Liew, 1989). T cell proliferation is, however, a measurement of a culmination of multiple processes and networks of cytokine production, utilization and interactions. Proliferative assays may still be useful in elucidating some of the relevant cytokines that may be essential for one of the fundamental facets of the immune system (APC processing and presentation of antigen and TCR recognition of antigen that leads to proliferation of antigen-specific T cells).

Direct measurement of cytokines in vitro were performed by analyses of secreted cytokines in culture supernatants collected from "fresh" spleen cells directly ex vivo in response to antigen specific stimulation (Yang, 1993a). We detected significant levels of antigen-specific IFN-gamma and IL10 production in our system. However, we failed to detect significant amounts of IL-2 and IL4 production. Autocrine IL-2 consumption occurring simultaneously with production in the bulk culture system may be a reason. This or other reasons may also be responsible for the absence of detectable IL-4 production by freshly derived T cells. Other reasons could include (1) inhibitory effects of Th1-derived cytokines (Powell, 1990), (2) IL-4 secreting cells require additional differentiative steps before detectable IL-4 is produced (Swain, 1988; Powers, 1988;

Powell, 1990) or (3) IL-4 inhibitory activity of the adjuvant used in the immunization (Kishimoto, 1982, Yang, 1993b).

Active cellular suppression of immune responses has been studied extensively over the years and has remained illdefined due to difficulties in cloning suppressor cells and defining their mechanism of action. More recently, it appears that one of the primary mechanisms of active cellular suppression is via the secretion of suppressive cytokines such as transforming growth factor (TGF-ß), IL-4, and IL-10 after antigen-specific triggering of T cells (Weiner, 1994). TGF-ß-secreting myelin basic protein (MBP)-specific $CD4^+$ T cell clones from the mesenteric lymph nodes of SJL mice (Chen, 1994) were structurally identical to Th1 disease-inducing clones in T-cell receptor usage, major histocompatibility complex (MHC) restriction, and epitope recognition but suppressed rather than induced experimental autoimmune encephalomyelitis (EAE). Because of this finding we examined the production of TGF-ß induced by mouse hsp60 here. However, TGF-ß was produced in similar amounts by T cells from all mice irrespective of different stimulation protocols either in vivo or in vitro, which argues against a central role for this cytokine in the process we are studying. Rather our data show that tolerance to mouse hsp60 is directly correlated with the net secretion of IL-10 and inversely correlated with the ratio of IFN-gamma/IL-10 net secretion (Table 7).

IL-10 was originally identified as a product of $CD4^+$ Th2 cells that inhibited proliferation of CD^+ Th1 cells via down-regulation of IFN-gamma and IL-2 production (Fiorentino, 1989; Fernandez-Botran, 1988). IL-10 is well known to de-activate macrophages and downregulate Th1 immune responses (Moore, 1993). In several model systems, IL-10 has been demonstrated to be a critical cytokine in the regulation of T cell differentiation in terms of cytokine phenotype

and function (Mosmann, 1989, 1994). The production of IL-10 often correlates with strong Th2 responses and inhibition of cell-mediated Th1 responses (Li, 1994; Mosmann, 1989; 1994). In addition, IL-10 prevents the infiltration of mononuclear cells into inflammatory sites (Powrie, 1993b).

More recently IL-10 has been implicated in T cell tolerance. Enk et al (1994) reported that in vivo application of IL-10 before allergen treatment induced antigen-specific tolerance in mice. They suggested IL-10 might act via inhibition of proinflammatory cytokines because injection of IL-10 into mice significantly impeded the induction of proinflammatory cytokines IL-1ß, tumour necrosis factor α and IL-1α. It was also demonstrated that IL-10 inhibits the induction of proliferation of Th1 cell clones by freshly cultured Langerhans cells (LC). This effect was independent of Ag-processing and shown to be mediated via inhibition of a costimulatory signal on LC (Enk, 1993). Current evidence favours the idea that IL-10 inhibits production or function of costimulator molecules such as B7/BB1 (Linsley, 1991; Freedman, 1991), as the absence of costimulation in the presence of antigen-presentation leads to T cell anergy (Schwartz, 1989). Complete activation of T cells requires two signalling events, one through the antigen-specific receptor and the other through the receptor for costimualtory molecules. In the absence of the latter signal, the T cell makes only a partial response and, more importantly, enters an unresponsive state know as clonal anergy in which the T cell is incapable of producing interleukin-2 upon restimulation (Schwartz, 1990). This model can explain our observations. Mouse hsp60 induced high IL-10 production which directly correlated with tolerance to mouse hsp60. Autoimmunity to mouse hsp60 was correlated with reduced levels of IL-10 production and high levels of IFN-gamma (table 7). In vitro IL-2 reversed mouse hsp60 specific T cell

anergy presumably because exogenous IL-2 bypasses the requirement for the second signal costimulation that is controlled by IL-10 (Fig. 20, Fig. 22). In other system it has also been reported that the inhibitory effect of IL-10 was at least significantly, if not completely, overcome by exogenous IL-2 (Fiorentino, 1989; Ding, 1992). In aggregate these results suggest that IL-10 maintains antigen specific T cell tolerance via inhibition of IL-2 production by impeding costimulatory signal expression at the APC level. The net effect of IL-10 appears to depend on the precise balance between IL-10 and IL-2. The result shown in figure 22 illustrates that exogenous IL-10 (at 5 ng/ml) can inhibit IL-2 (50 units/ml) reversion of T cell anergy to mouse hsp60. IL-10 is known to down regulate expression of class II MHC or costimulatory B7 on monocyte/macrophage and IL-2R on T cells (de Waal-Malefyt, 1991). All of the molecules (MHC class II, B7, IL-2R) are essential for T cell proliferation, thereby IL-10 act antagonistically against IL-2 function by interfering with antigen presentation, IL-2 production and IL-2 autocrine binding to IL-2R. Therefore, we conclude that two mechanisms by which tolerance to mouse hsp60 is mediated are via the generation of active cellular suppression and by clonal anergy.

III. The induction of autoimmunity.

3.1. The role of Th1/Th2 regulatory cells.

Our data (table 7, 8, 9; fig. 20, 21, 22) suggest that mouse specific B cells are in an immunoignorance state and that autoimmunity is controlled by the balance of Th1 and Th2 cells.

Th1 cells, producing IL-2 and IFN-gamma, mainly mediate cellular immune reactions to chlamydial hsp60. High levels of autoimmunity inversely correlated with Th2 type cytokine IL-10 production. We hypothesize that the chlamydial specific T regulatory cells interact with mouse hsp60 specific B cells and anergic T cells through cross-priming (molecule mimicry, Fig. 16B) and paracrine secretion of IL-2 or IFN-gamma. This Th1 type cytokine can inhibit IL-10 production and furthermore may cause antigen-presenting cells to alter the pattern of protein processing that generates differential protein cleavage patterns, leading to the expression of cryptic epitopes and presentation to autoreactive T cells and thereby stimulating autoimmune disease. We also propose that this effect can be counteracted by Th2-associated cytokines that modulate APC function (Elson, 1995).

3.2. Cryptic epitopes of mouse hsp60

The importance of coimmunization in the induction of autoimmune responses to a self antigen highlights the importance of high concentrations of self antigen and homologous foreign antigen being present at the immunizing site. High concentrations of the self antigen (mouse hsp60) plus homologous microbial antigen (chlamydial hsp60) appeared to be required to break tolerance (Fig. 18).

Through what potential mechanisms can an ubiquitously expressed antigen such as hsp60 produce an organ-specific disease? One explanation could involve a localized induction of the heat shock response. Histochemical studies of the brains from patients with multiple sclerosis (MS) or with gliomas reported expression of hsp60 in only some but not all astrocytes,

oligodendrocytes and reactive macrophages (Georgopoulos, 1993). Human hsp60 is strongly expressed by mononuclear inflammatory cells in colon and ileum tissue sections from patients with Crohn's disease and ulcerative colitis but not in biopsy specimens from patients with acute self-limited colitis and controls. As well hsp60 is expressed by giant cells and mononuclear cells present in the granulomas of patients with Crohn's disease (Peetermans, 1995).

Autologous APC have been reported to stimulate hsp60-specific T cells following heat shock (Hermann, 1991) or interferon-gamma treatment (Koga, 1989), indicating that endogenous hsp60 can be processed and presented on MHC. These findings raise the interesting possibility that under certain circumstances, possibly infection, some cell populations may constitutively exhibit enhanced levels of protein mis-sorting leading to the appearance of hsp60 in unexpected locations.

Evidence for increased hsp 60 expression in autoimmune lesions has also been presented (Jones, 1990; Karlsson-parra, 1990; Rajagopalan, 1990; Selmaj, 1991). Thus, heightened hsp60 levels have been found in the synovial lining and other cells present in rheumatoid arthritis lesions, oligodendrocytes of multiple sclerosis, and pancreatic cells of IDDM patients. An inflamed infection site likely presents stressed conditions to both pathogen and host cells and there is evidence to suggest that inflammation results in up-regulation of hsps. As well T cell clones that are specific for self hsp60 can be isolated from local inflammatory sites in mice and humans (Polla, 1989; Anderton, 1993).

T cell tolerance depends on the presentation of self-proteins to T cells and therefore can only be established to those self-determinants which, under steady-state conditions, are generated in sufficient amounts to be recognized by T cells undergoing deletion in thymus or anergy in the

periphery. Thus, there is large number of self-determinants that are cryptic because they are not generated at all or are generated at subthreshold levels. T cells specific for these cryptic epitopes are present in the normal repertoire and might become activated and autoaggressive if the epitopes are presented at higher concentrations. This concept, which had been originally proposed by Sercarz and colleagues, represents today one of the major hypothesis for the pathogenesis of autoimmune diseases (Sercarz, 1993)

There appears to be two functional sets of self-determinants within the mouse hsp60 molecule. Those that are easily processed and presented, readily tolerizing developing T cells and comprising the dominant self antigen, and those that are more difficult to present and apparently do not tolerize, thus comprising the cryptic self antigen. Up-regulation of the synthesis of host hsp60 may occur under inflammation or immune-mediated circumstances and thus present the immune system with high concentrations of self hsp60 epitopes by increasing antigen delivery to the processing compartment, modulation of antigen processing, or by increasing expression of adhesion and costimulatory molecules on antigen presenting cells (Lanzavecchia, 1995).

It is remarkable therefore that immunization with chlamydial hsp60 itself does not generate strong self reactive antibody to mouse hsp60 (fig. 15). This is probably due to the fact that under these condition little mouse hsp60 is present in vivo and most activated B cells are specific to determinants unique to chlamydial hsp60. Our data indicate that the autoantibody titre for mouse hsp60 depends on the concentration of mouse hsp60 co-immunized with chlamydial hsp60(fig.18).

3.3. Molecular mimicry and the carrier effect

Tolerance to mouse hsp60 is not a single immunologic event. As previously reported by Lin et al (1991) autoimmune response are relatively easily induced when two homologous antigens, one of which is a self antigen, are administrated concurrently. We observed that co-immunization with mouse hsp60 and chlamydia hsp60 induced strong B and T cell responses to mouse hsp60 (Fig. 17). The induction of autoimmune responses depended on the shared amino acid sequences between mouse and chlamydia hsp60 since it was not observed when nonhomologous but highly immunogenic proteins such as ovalbumin (Fig. 19) or glutathione-S-transferase (Fig. 23) were co-administered with mouse hsp60. The importance of shared sequences implicates cross-reaction between the antigen recognition structures on T and/or B cells in the autoimmune response rather than bystander activation due to nonspecific cytokine release. Clearly these observation implicate molecular mimicry as the basis for autoimmune induction by hsp60, at least under the circumstances examined in these experiments.

Many other murine models of human autoimmune disease implicate Th cells as a central component in driving autoantibody responses (Steinberg, 1980; Santoro, 1988; Wofsy, 1987). These T cells presumably have not been deleted from the peripheral repertoire and may await induction by the appropriated self or cross-reactive foreign antigen (molecular mimics).

Transgenic mice carrying a gene construct encoding hen egg lysozyme (HEL) under transcriptional control of the mouse albumin promoter (Goodnow, 1990) express lysozyme as a self antigen, resulting in tolerance to lysozyme within the T cell compartment (Goodnow, 1988). The functional absence of lysozyme-specific helper T cells can be circumvented by

challenging mice with a conjugate of lysozyme coupled to foreign (sheep) red blood cells (SRBC), creating a situation where lysozyme-specific B cells can collaborate with SRBC-specific helper T cells (Goodnow, 1990).

Since antigen-specific B cells need to collaborate with antigen-specific T cells in order to mount efficient antibody responses to foreign antigens (Claman, 1966; Vitetta, 1989), the failure to produce high-affinity autoantibodies to mouse hsp60 could merely reflect T cell clonal anergy rather than any change in the B cells themselves.

It is easy to understand the mechanism by which helper T cells recognizing foreign epitopes on GST may stimulate autoreactive B cells (specific for mouse hsp60) if the foreign epitopes are linked to a self protein as a GST-mouse hsp60 fusion protein, because mouse hsp60 reactive B cells recognize and bind mouse hsp60 through their sIg, then process it and present the linked GST epitopes on the surface as a MHC class II complex which are recognized by activated T cell (specific for GST). We think that the co-immunization with mouse and chlamydial hsp60 also allows the chlamydial hsp60 specific Th cells to interact with mouse hsp60 specific T/B cells through a similar cross-priming process. For example, when chlamydial hsp60 primed LNCs were cultured with mouse hsp60, intermediate levels of T cell proliferation occurred (Fig. 16B).

Therefore, high levels of autoantibody can be induced by providing T cell help through the incorporation of a foreign T cell site in mouse hsp60 by creating a self plus foreign fusion protein (Fig. 23). Adoptive transfer of immune T cells from mice immunized with chlamydia hsp60 also demonstrated that chlamydial hsp60 contains T cell sites which activate cross-reactive T helper cells to help autoreactive (mouse hsp60 specific) B cells (Table 8).

3.4. Epitope mapping.

Since chlamydial hsp60 can induce T cells involved in autoantibody production against mouse hsp60 (Fig. 23, Table 8), I reasoned that chlamydial hsp60 should contain one or more T cell sites capable of providing help for autoreactive B cells. All previously mapping studies on hsp60 focused on defining the cross-reactive epitopes that may be involved in autoimmunity.

Numerous studies have identified T-cell responses to hsp60, for example adjuvant arthritis can be induced in the rat with an epitope of mycobacterial hsp60 (aa180-188), and the disease can be transferred with T cells specific for this epitope (Van Eden, 1988). T cell recognition of epitopes conserved between mycobacterial and mammalian hsp60 has also been reported previously. Murine gamma/δ T cell hybridomas generated against PPD and specific for residues 180-196 of mycobacterial hsp65 responded to the homologous region of human hsp60. (Born, 1990). Following bulk stimulation of PBL from healthy donors with killed *M. tuberculosis,* MHC class II-restricted CTL were generated that lysed autologous target cells pulsed with synthetic peptides of human hsp60 (Munk, 1989). In another study, T cell lines from ascitic fluid of a tuberculosis patient which were specific for residues 195-219 and 390-412 of mycobacterial hsp65 also responded to whole human hsp60 (Lamb, 1989). A T cell response to hsp60 and, in particular, to aa337-460 has also been demonstrated in NOD mice developing diabetes mellitus (DM). These T cells also respond to the membrane of insulin secretory granules and hsp60 may be targeted to these granules (Elias, 1991). Cross-reactive T cell recognition of hsp65 and rat hsp60 was limited to a single epitope (aa: 256-265), recognized after hsp65 immunization

which protects rats against adjuvant arthritis (Anderton, 1994).

Epitope mapping studies show that sera from patients with *C. trachomatis* induced pelvic inflammatory disease or ectopic pregnancy have antibodies that preferential react to the carboxyl-terminal half of the chlamydial hsp60 (residue position:274-545) (Cerrone, 1991). Arno (1995) further mapped antibodies to amino acids 201 to 300 of *chlamydial* hsp60 in women with tubal infertility. We previously mapped several B cell peptide epitopes to this region and identified that they cross-reacted with homologous peptide epitopes from human hsp60 (Yi, 1993). To test whether chlamydial salpingitis induced sensitization to conserved epitopes, patients'PBLs were tested for their ability to respond to five synthetic peptides corresponding to regions of identity or near identity between the *C. trachomatis* and human hsp (Witkin, 1994). Since hsp functions as a molecular chaperon and readily binds other proteins, the use of synthetic peptides in these investigations assured that no false-positive responses occurred as a result of stimulation by a contaminating native or recombinant peptide. PBLs from 4 of 10 (40%) women with recurrent salpingitis, but none of 9 women with a first episode of salpingitis and none of 32 women with no evidence of salpingitis, responded to a peptide corresponding to amino acids 275-283 in the chlamydial hsp.

I constructed five deletion mutants of chlamydial hsp60 in order to map in vivo T help sites capable of providing help for autoreactive B cells (Table 9). Two helper T cell sites in chlamydial hsp60 (amino acid residue: 268-409, 1-150) were identified that can induce autoimmune responses if they were co-immunized with mouse hsp60. But it is not clear whether these fragments induce a specific T regulatory cells releasing unique cytokines or by cross-priming self-reactive anergic T cells and B cells. An exciting result has been reported recently

(Brocke, 1996). The T cell clone L10C1 recognizes the myelin basic protein (MBP) epitope p87-99 and causes severe experimental encephalomyelitis (EAE). Administration of the altered peptide ligand (APL, 96P→A), in which a phenylalanine at residue 96 was replaced by alanine, prevented EAE or reversed paralysis. This peptide bound poorly to MHC class II molecules and induced a lower proliferative response in L10C1 than original peptide P87-99. The reversal of EAE with APL depend on the availability of the Th2 cytokine IL-4. Neutralizing monoclonal antibody directed against IL-4 block the therapeutic effect of APL.

Therefore future studies will be certainly needed to map more precisely the immunodominant T cell epitopes within these two regions. The epitope may be useful as a marker for chlamydia associated immunopathology or designing altered peptide ligand for treatment of autoimmunity induced by chlamydia hsp60.

IV. Conclusions and Speculations

The data available thus far show that the immune system does indeed attempt to produce a compromise in the face of the problem noted at the beginning of the discussion. That is, the immune system has decided to accept chlamydial hsp60 as a dominant microbial antigen and to keep cross-reactivity at low levels for the shared epitopes in order to reduce the risk of autoimmunity. The immune system relies on regulatory mechanisms inducing periphery anergy and active suppression, which can be overcome in vivo if the responding lymphocytes encounter both mouse hsp60 and chlamydial hsp60 in sufficient amounts. The danger of immunopathogenic effects following chlamydial infection may prevail under such circumstances.

The role that IL-10 production by hsp60 stimulated T cells could play in *C. trachomatis* infection is uncertain. In IL-10-deficient mice, generated by gene targeting, lymphocyte development and antibody response are normal, but most animals are growth retarded and anaemic and suffer from chronic autoimmune enterocolitis that appears to be triggered by intestinal bacteria. Alterations in the intestine include extensive mucosal hyperplasia mononuclear inflammatory reactions, and aberrant expression of major histocompatibility complex class II molecules on epithelia. When IL-10 deficient mice are kept under specific pathogen-free conditions they develop only a focal inflammation limited to the proximal colon. These results indicate that bowel inflammation in the IL-10 knock out mutants originates from uncontrolled immune responses stimulated by enteric bacterial antigens and that IL-10 is an essential immunoregulator in the intestinal tract (Kühn, 1993). It is very interesting to wonder whether GroEL from *E. coli is* involved in the chronic enterocolitis in this IL-10 deficient mouse strain or what would happen if mutant mice were immunized with mouse hsp60.

The effects of IFN-gamma on chlamydial growth are clearly concentration-dependent and can lead to either complete inhibition of chlamydial growth (2.0 ng/ml) or to persistence (0.2 ng/ml) (Beatty, 1993). However, the reported concentration of IFN-gamma in cervical secretions and serum of women with chlamydial cervicitis and pelvic inflammatory disease is much higher than that required for persistent chlamydial infection (Arno, 1990; Grifo, 1989). Therefore, secretion of IFN-gamma at local sites of infection could reach concentrations that would completely inhibit chlamydial growth and resolve infection. Accumulating evidence does show that IFN-gamma is the key cytokine involved in host defence against *C. trachomatis* infection (Yang, 1996; Williams, 1993; Beatty, 1993). Unfortunately, this is not the case in vivo. Data

from animal infection models and human epidemiological studies demonstrate that the pathologic changes such as chronic inflammation, tissue damage, and scarring of mucosal epithelium are quite common after repeated infections (Morrison, 1992). *C. trachomatis* like other intracellular infection may upregulate host cell expression of self hsp60. Our data suggest that the self-hsp60 could activate anergic T cells to secrete high level IL-10. IL-10 could then down-regulate T cell production of IFN-gamma, thereby providing a prolonged antigenic stimulation that promotes chronic chlamydial inflammation and immune-mediated disease. We hypothesize that chronic, repeated or persistent infection which appears essential to the immunopathological response that characterize chlamydia inflammatory disease may be controlled by the balance of the ratio of IFN-gamma/IL-10 among responding antigen specific lymphocytes.

Could immune responses to stress proteins have a role in protection against infection? One view is that they do not, since infection by any one pathogen generally does not protect an individual against infection by another. An alternative view is the model of immune surveillance in which self-reactive T cells provide a first line of defense against infection by recognizing and helping to eliminate stressed autologous cells, such as cells infected with chlamydia. During everyday life the host will frequently encounter microbes, most of which are nonpathogenic, but which may survive for a limited time period. Although such microbes will be eliminated before they cause clinical disease, hsp synthesis may be induced. Therefore, the host's immune system may be frequently boosted by microbial hsp derived from various microorganisms. In this way, constant T cell stimulation may occur, and T cells specific for epitopes of hsp shared among various microbes could provide an early defence mechanism against pathogenic microbes (Kaufmann, 1990a).

The present study supports a model of molecular mimicry for the induction of autoimmune responses. Hsp60 makes an appealing model of foreign/autoantigen immunologic discrimination since the amino acid sequences from more than 50 species are known to show a high degree of evolutionary conservation. As well, the structural characteristics of GroEL have been described by crystallography, the immunobiology of foreign hsp60 has been well studied, and the mouse hsp60 molecule has 97% homology at the amino acid level with the human hsp60 (Elias, 1991). The animal model system for induction of autoimmunity to hsp60 that we have described will be useful to evaluate for accelerated pathology following primary chlamydial infection.

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