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Antibody Gene Markers and their
Relationship to the Chlamydial major outer membrane protein
(MOMP) Immune Response.

by

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IV. OVERALL SUMMARY OF DISCUSSION AND SPECULATION:

Abbreviations:

EB	Elementary body (infectious form of chlamydial species)
LPS	Lipopolysaccahride
mAb	monoclonal antibody
MOMP	Major outer membrane protein of *Chlamydia trachomatis*
MoPn	Mouse pneumonitis strain of *C. trachomatis*
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PID	Pelvic inflammatory disease
RACE	Rapid amplification of cDNA ends
SDS	Sodium dodecyl sulphate (detergent)
TdT	Terminal deoxynucleotidyl transferase
C VD-1	Serovar C MOMP variable domain 1 peptide epitope; DVAGLQND
V-gene	Immunoglobulin variable region gene (antigen binding domain)
V-marker	An immunoglobulin allele that encodes epitope specific antibody

"No man is born smarter than another we just have different jobs."
(paraphrased from a Tom Clancy novel)

This thesis is dedicated to my family: We have the collective belief that you can do anything if you really want to do it, and it is true. We have worked hard as tradesmen (Berry; de Moissac), carpenters (Berry; Morrison), farmers (Berry; Cassan; Flynn; Yelland), miners (Berry; Cassan; Hood), bus drivers (Cassan), mailmen (Hunt), teachers (Cassan, Clerihew), scientists (Berry; Faraday; Hunt), and doctors (Livingstone). Our most important roles have always been as Moms and Dads due to our love of children.

To: Tracy, Jordan-Lee, and Erin.

Love: Dad

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I.   *Abstract:*

The chlamydial major outer membrane (MOMP) protein has attracted much interest in the design of first generation chlamydial subunit vaccines. As yet, few constructs elicit high titre neutralizing antibody to the native MOMP proteins, and no chlamydial candidate vaccine has been able to create broad immunity. In the absence of precise structural information regarding MOMP we studied the molecular basis of antibodies raised to the native MOMP in order to guide vaccine design. Both polyclonal and monoclonal immunoglobulin responses to a neutralizing epitope on the MOMP of *Chlamydia trachomatis* (serovar C) were comprehensively analyzed in Balb/c mice. This thesis has defined the molecular basis for the classic serologic phenomenon termed "original antigenic sin" and has generated precise new data regarding host antibody gene usage in response to an antigenic site on a major pathogen protein that is of general significance to the field of immunobiology and relevant to vaccine design. Sequence analysis of MOMP variable domain 1 (VD-1) specific mAbs revealed a patterned usage of particular V-genes with structural commonalities, which suggests that antigen-mediated clonal expansion exerts a powerful selective force upon B cell clones that are commonly found within the host's available antibody repertoire. Novel reverse-transcriptase PCR studies support the contention that the host responds to MOMP with a specific increase in expression levels of the same V-genes alleles used to recurringly encode VD-1 mAbs. For example, a V_{k21}-J_{k2} gene, used in 50% of VD-1 mAbs, was up-regulated in all mice immunized with serovar C EBs, in some mice recieving VD-1 synthetic peptides, but not in mice recieving irrelevant bacterial antigens or peptides.

Predominance of the same V_{k21}-allele has been seen previously with other pathogenic and
 "self" epitopes which raises the possibility that V_{k21} is overabundant through
developmental mechanisms, and selected for frequent use against protein antigens on
common pathogens. This relationship between a variable pathogen epitope and variable
host paratopes, determinants of molecules that juxtapose the two, supports the co-
evolutionary paradigm between antigenic determinants of pathogens and immune
recognition molecules found within the host genome.

II. INTRODUCTION

Chlamydiae are obligate intracellular eubacterial parasites composed of four recognized species *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci*, and *Chlamydia pecorum*. The chlamydiae collectively cause a wide range of human and animal diseases. Diseases caused by *C. trachomatis* are a major public health concern and are generally initiated by ocular or genital infection. *C. trachomatis* is responsible for trachoma, lymphogranuloma venereum, saplingitis, cervicitis, and can lead to blindness and infertility. Although *C. trachomatis* is primarily a human pathogen, the related mouse pneumonitis (MoPn) strain of *C. trachomatis* causes lethal respiratory infections in mice. *C. pneumoniae* has been shown to be a major cause of acute respiratory disease in humans and more recently has been associated with lesions in developing artherosclerosis. *C. psittaci* is mainly an animal pathogen infecting birds but also causes economically important reproductive disease in sheep, cows, and goats. *C. psittaci* can cause serious pneumonia in humans by zoonotic transfer or through accidental laboratory acquisition. The chlamydiae are in general mucosal pathogens and strategies designed to reduce chlamydial transmission and acquisition must ultimately transpire at mucosal sites. Despite the availability of excellent anti-microbial therapy (Peeling and Brunham, 1996) *Chlamydia trachomatis* remains a predominant human pathogen. The reasons for the prevalence of human chlamydial disease worldwide are complex, and include socio-economic factors, and the inability of antibiotics to prevent re-infection. Vaccines for the prevention of chlamydial disease are clearly desirable.

Since the early triumph of Jenner in his historic studies of human immunity (Brunham and Coombs, 1998) scientists have been working towards the development of vaccines for the prevention of most important infectious diseases. The advent of high level and multi-drug resistance in recent years has re-fueled efforts for vaccines that *a priori* prevent bacterial infection rather than treat an infection after it has presented. The value of vaccination for improving the human condition is indisputable (Krause et al., 1997).

Active vaccination is based upon two fundamental principles of the adaptive immune system. These principles are specificity and memory. The capability of antigen-specific lymphocytes to undergo clonal expansion allows the host to deal with pathogens whose life cycles are many times faster than the host itself, at the single cell level. Indeed, the exquisite specificity of the host response is due to antigen selection and clonal expansion of specific T and B lymphocytes (Paul, 1995; figure 1). Appropriate differentiation and maturation of these cells ensures that a memory pool is created to be drawn upon in future encounters. While the factors that drive memory pool formation are not entirely understood, it is clear that a rapidly inducible, higher magnitude (higher antibody concentration), and longer lasting secondary response results following secondary exposure to most protein antigens. These defenses have evolved to ensure that a single host is best equipped to deal with the myriad of infections or toxic insults they face over their lifetime.
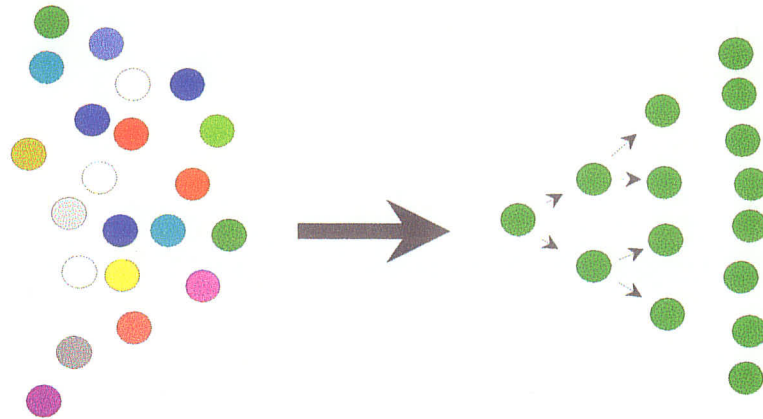
Figure 1: Clonal expansion. B cells with surface bound immunoglobulin directed to dominant epitopes of an antigen are selectively expanded out of the naive pre-immune B cell population.

Clearly, there has been powerful natural selection on the immune system for providing host defense. Vaccines educate the immune system (without causing disease) to specifically recognize and "remember" the antigens of a pathogen such that immunity is conferred at the time of encounter via the presence of circulating antibodies, or is rapidly engendered at the time of the encounter via the recall of immune defenses.

Many scientists believe that all of the "easy" vaccines have already been made. "Easy" vaccines can be defined as those that confer immunity to reinfection by immunization with either the killed or attenuated native organisms or their products. Creating a vaccine for many other organisms has proved much more difficult. *Chlamydia trachomatis* falls into the classification of "difficult" for vaccine design. Reasons for this include the short-lived and sometimes inapropriate immune responses produced in humans after vaccination with parenterally administered noninfectious whole chlamydial organisms (Grayston, 1971; Grayston and Wong, 1975). The presence of pathological antigens is further confounded by the inability to genetically transform *C. trachomatis* in order to produce an attenuated strain. Furthermore, the obligate intracellular nature of chlamydial organisms prevents easy cloning in pure culture and makes their laboratory study complicated as well as expensive. Indeed, the intracellular habitat of chlamydial organisms likely evolved in order to limit direct contact of the pathogen with the humoral immune system and is recognized as a virulence factor.

Many pathogenic microorganisms have intricate virulence mechanisms that enable them to subvert or avoid host immune defenses (Marrack and Kappler, 1994). Improved

knowledge of the host immune system as well as better basic understanding of virulence

factors posessed by chlamydial pathogens can benefit chlamydial vaccine development.

For example, the chlamydial RecA gene-homolog was identified via complementation,

and has a potential role in chlamydial virulence for genetic processes requring

recombination. Recombinatorial mechanisms are commonly used by bacterial pathogens

for creating antigenic variation in surface exposed structures (Borst and Greaves, 1987;

David and Maizels, 1989; Spanopoulou et al., 1996) and this is important to vaccine

design. The recent sequence determination of the entire chlamydial genome holds great

promise for identification and characterization of other chlamydial virulence factors

involved in antigenic variation and intracelular metabolism by comparison with known

virulence genes in other microorganisms. Ultimately, this knowledge may allow for

targetted gene removal to aid in attenuation, identification of previously unknown

vaccine candidate antigens, or perhaps lead to rationally designed media formulations for

host cell free growth of chlamydiae.

A rational approach for the design of subunit vaccines consists of three main steps

(Lanzavecchia, 1993). I will address each in turn in terms of relevance to the status of

chlamydial vaccines; First, protective effector mechanisms must be identified. Second, an

antigen that consistently induces the desired immune response must be chosen. Third, the

vaccine must be delivered in an appropriate fashion in order to induce the correct immune

response. The correlates of protective cell mediated immunity to *C. trachomatis* have been

established in mouse models (Yang and Brunham, 1997). A T_H-1 like polarized response

with the induction of cell mediated immunity and secretory IgA antibody (sIgA) are

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correlated with immunity. In the murine immune system a cell mediated response to a limited chlamydial infection leads to rapid resolution and clearance of the organism. How these data translate to human chlamydial disease is not entirely clear and is under intense investigation in many laboratories. However, in order to prevent the pathological damage caused by repeated and/or chronic chlamydial infection, sterile immunity is the ultimate goal of a chlamydial vaccine. Therefore, a chlamydial vaccine that induces protective antibodies is also desired because antibody is the only host defense capable of completely preventing infection (Ada and Ramsey, 1996). However, the correlates of antibody mediated protection in humans are not well understood. A direct exploration of the role of antibodies in preventing chlamydial infection may necessitate the cloning of mucosal antibodies from convalescent human patients. The role of antibodies in chlamydial infection is discussed further below.

The second principle of vaccine development concerns choosing an antigen that consistently elicits the desired immune response. Unfortunately, most recombinant subunit vaccines do not trigger identical immune responses compared to those elicited by the native antigen (Eisenstein and Engleberg, 1986). This is likely due to differences in the ability of a recombinant product to portray the target antigen in terms of three dimensional structure, local conformation of carrier moieties, as well as the differential ability of subunit vaccines versus live pathogen in terms of competing antigens, proinflammatory signalling, and in localization and persistence in the correct microenvironment in order to stimulate appropriate immune defences. These differences may contribute to the generally lower immunogenicity of subunit vaccines.

The chlamydial MOMP is the major protective antigen of *C. trachomatis*. Recently, studies in our laboratory have shown that the MOMP is unequivocally a protective antigen for *C. trachomatis* (Zhang et al., 1997). Indeed, DNA - based subunit vaccines have shown protection *in vivo* against the *C. trachomatis* MoPn (mouse pneumonitis) strain in a live mouse lung infection model. Importantly, these vaccines induce a T_H-1 like immune response similar to the response engendered against the native organism. However, the antibody response to the DNA vaccine was inconsistent, of low titre, and did not include IgA antibodies.

Protective MOMP B cell epitopes map to the surface exposed domains of the MOMP. The omp1 gene that encodes the MOMP has many allelic forms as the result of immune selection pressure over time (Stothard et al., 1998). MOMP variation has produced antigenically variant chlamydial serovars. Neutralizing antibodies are directed to either either group, subgroup, or type specific epitopes of the MOMP with approximately 20% of the total anti-MOMP antibody response being directed to the four sequence variable domains (VDs) (Guangming Zhong, University of Manitoba, Ph.D. Thesis). The immunodominance of the VDs is consistent with the serovar specific protection that has been demonstrated in immunized humans (Jawetz et al., 1961) and animal models (Brunham et al., 1984). Unfortunately, MOMP antigenic variation and type specific immune responses create an additional level of complexity in vaccination for broad immunity. Additional studies are needed to identify vaccines capable of inducing broad protection against infection with *C. trachomatis*. However, in the absence of an animal model for human *C. trachomatis* strains, our observations have been limited to in vitro

analysis. For this thesis MOMP peptides and synthetic peptide vaccines have been used as in vitro tools for the dissection of the chlamydial antibody response. Experiments on the effect of sequential vaccine delivery on antibody cross-reactivity was also examined in this thesis as they act as a reasonable estimate of B cell diversity (Fish and Manser, 1989). Improved understanding of the parameters that lead to enhanced cross-reactivity among the antigenic variants of the MOMP is considered significant in order to exploit for vaccination against *C. trachomatis*.

Synthetic peptides have shown limited value as chlamydial vaccines. Studies by our lab and others using synthetic (Su and Caldwell, 1992;Zhong et al., 1993) or recombinant MOMP polypeptides (Toye et al., 1990; Hayes et al., 1991) have helped to provide information on MOMP immunogenicity and antigenic structures. To date, no chlamydial peptide vaccine has shown significant efficacy in animal models. On the other hand, MOMP synthetic peptide vaccines have not yet been designed nor tested for protection against *C. trachomatis* MoPn strain, the only readily accessible *C. trachomatis* strain for which an in vivo protection model exists with an associated mortality due to infection. Intuitively, synthetic peptide vaccines may not be adequate for priming protective immunity. Reasons for this thinking include the fact that peptides are structurally very simple, delivered extracellularly, and contain a very limited number of T helper cell epitopes. Recently we observed that conformationally constrained MOMP peptides, with affinity selected structures, exhibit improved immunogenicity over linear constructs when both are carried on filamentous phage (Zhong et al., 1994b). Such polypeptides may have use in the future perhaps as a booster immunization in combination

with DNA vaccines (Letvin et al., 1997). Studies on the immunogenicity of constrained VD-1 polypeptides in the absence of the phage carrier are included in this thesis. We predicted that constrained VD1 peptides would biologically portray the native MOMP peptide epitopes and engender a host antibody response more similar to that elicited by the native protein.

The third step in rational design of subunit vaccines concerns the appropriate method of vaccine delivery. This is particularly important for a chlamydial vaccine where entrance and infection by the pathogen occur at mucosal surfaces. Little is known on the chlamydial protective effector mechanisms found on the mucosa and much remains to be explored. However, mucosal IgA may mediate protection since secretory IgA antibody to *C. trachomatis* in the cervical mucus has been inversely correlated with quantitative shedding of the organism in humans (Brunham et al. 1983). While the role of human cellular immunity to *C. trachomatis* on the mucosal surface is largely unknown (Cotter and Byrne, 1996) it is likely to involve a triggering of dendritic cells which have been found to be critical in stimulation of IgA production (Fayette et al., 1997). Mucosal delivery of DNA vaccines creates nearly sterile immunity in mouse lung infection animal models, however the results are inconsistent (due to variations in delivery of the nucleic acids to the cells beneath the mucosal layer; Dr. Dongji Zhang, University of Manitoba, personal communication). Combination vaccines will need to be studied at the mucosal surface. Especially in light of the absence of immune protection despite systemic immune responses following combination DNA/ protein vaccination for HIV-1 (Putkonen et al., 1998).

The host antibody gene repertoire is potentially vast. Recent immunological studies have provided more information on host antibody gene diversity to epitopes on foreign proteins. Despite the complexity of antigens, such as whole bacterium, the host antibody response is focussed to a remarkably small number of surface exposed epitopes. Initially, many B cells may recognize various epitopes on an incoming pathogen and all are turned on against it (Weiss, 1993). This is supported by the finding that many variable region genes are turned on early in an immune response (Kelsoe et al., 1989; Kelsoe, 1991). But over time the complex and cooperative mechanisms within the immune system gradually select for dominant B cell specificities which best "fit" the evoking antigen . Indeed, affinity selection of antibodies during immune responses depends upon two mechanisms: The first molecular, involving the specific introduction of somatic mutations in rearranged immunoglobulin genes ; the other cellular, involving the clonal activation and expansion of B cells expressing a surface bound immunoglobulin with higher affinity for antigen (Andersson et al., 1998). The somatic mutational mechanism is central to the developing response except when the primary response is already of high affinity (Foote and Milstein, 1991; Roost et al., 1995). The selective amplification of a few B cells expressing receptors of higher affinity is not entirely understood. However survival in the germinal centre reaction is dependent upon improved binding of its receptor to the antigen, either through sucessful scavenging of minute amounts of antigen, improved BCR and CD21/19 complex mediated signal transduction (to prevent apoptosis) (Carter and Fearon, 1992), or improved recruitment of T cell help (Batista and Neuberger, 1998). A subset of cells proliferate more rapidly and terminally differentiate into plasmablasts, and these cells

generate the bulk of the antibody response. The dominant antigens elicit stronger immune responses. In some cases, antibody responses to dominant epitopes have been found to be encoded by a relatively limited set of recurrently used antibody genes (Kavaler et al., 1990). Given the well-known differences in B cell repertoire expression associated with polymorphic differences in mouse and human V_H (Brodeur and Riblet, 1984; Yancopoulos et al., 1988; Kofler et al., 1989; Adderson et al., 1993; Milner et al., 1995; Sasso et al., 1995) , Vl (Laskin et al., 1977; Ulrich et al., 1997; Juul, et al., 1998), mouse DH (Riley et al., 1988; Atkinson et al., 1994) and JH (Solin and Kaartinen, 1992) gene segments, there is unquestionably an evolutionarily determined influence on repertoire expression (Klinman and Linton, 1988; Kofler et al., 1992)

Understanding V-gene usage in antibody responses is important to vaccine design for several reasons: 1) Not all combinations are equally protective (Briles et al., 1982). 2) Some protective antibody responses are limited to only a small number of variable region gene combinations (Anderson et al., 1992; Carroll et al.,). 3) Some protective antibody responses are germline encoded and require little affinity maturation (Kalinke et al., 1997). Thus, the ideal vaccine should portray the native antigen in these regards in order to maximize protection. While antibodies to unique artificial antigens can be created (eventually) via somatic mutational processes, beginning from even very limited germline repertoires (Cascalho et al., 1996), in reality, some pathogens will not give the host immune system the luxury of multiple boosts in order to do so. Indeed, in mice lacking terminal deoxynucleotidyl transferase (and therefore N region somatic diversification) produced antibodies with the same specificity and affinity for VSV epitopes as was found

in the normal littermates (Gilfillan et al., 1995). The benefits of a rapid, and high affinity germline antibody response has recently been demonstrated in the murine anti-VSV response (Kalinke et al., 1997). Finally, the observation that many epitope-specific antibody responses are patterned in their recurrent usage of particular V-gene elements (references in table 2) supports the notion that germline selection for protective V-gene elements has occured in the primary immune repertoire (Claflin and Berry, 1988), and that antigen-driven events select optimized somatic variations of these precursors (Fish et al., 1991).

Commonalities in V-gene usage are reflected in structural commonalities observed in antigen antibody recognition (Geysen et al., 1987). Throughout a host's lifetime different germline V-gene combinations are preferentially found in the available B cell repertoire (Kraj et al., 1997). The temporal emergence of other V-genes contributes to repertoire "shift", which is the usage of alternative V-genes to encode antibodies to the same epitope, at different stages of immunity (Berek and Milstein, 1987). For the majority of non-lethal pathogens, the host defenses provide time for the fine-tuning of the antibody response. Somatic mechanisms coincidentally exist for the creation of antibodies with improved affinity for antigen. For example, somatic changes in the antigen binding domains, such as junctional diversity, created upon initial imprecise joining of the germline V-D- J elements (Sanz, 1991), or somatic hypermutation (insertions, deletions, and substitutions) (Ohlin and Borrebaeck, 1998) and possibly receptor editing (substitution with a new upstream V-gene) (Nadel et al., 1998) are all subjected to stringent antigen selection whereby beneficial variant changes are selected and observed

as repertoire "drift" (Berek and Milstein, 1987). Affinity improvements, created through any mechanism (known or unknown), are compared in an clone-autonomous fashion relative to the clonal precursor within the germinal center. Even if the original V-gene encodes an antibody with high affinity, the mutation process ensures global diversity (Vora and Manser, 1995). Thus affinity is an important criterion for entry into the memory B cell compartment. The major goal of this thesis is to determine if a molecular genetic relationship exists between host antibody genes used to encode monoclonal antibodies against the variable domain - 1 (VD-1) epitope on the major outer membrane protein of *C. trachomatis* serovar C, and antibody responses to peptides that portray the same epitope. Therefore, this project entails a comprehensive study of B cell diversity to an immunodominant and neutralizing MOMP epitope in order to generate a molecular profile of the immunoglobulin genes it recruits. The variable region genes (V-genes) (the part of the antibody gene that encodes the antigen binding domain) of VD-1 specific mAbs were sequenced in order to determine if structural commonalities exist. Such commonalities have potential application as molecular markers for an *in vivo* measure of biological mimicry based upon the ability of candidate MOMP vaccines to upregulate their specific expression. Logically, if the native epitope recurrently recruits a limited set of antibody gene structures, and a vaccine portrays the epitope, in a similar fashion, we would expect to find expression of the same or a subset of the same recurrent V-genes in response to the vaccine. This notion is supported by several studies, including one in which the ability of haptenic-B cell epitopes were observed to recurrently elicit mAbs encoded by the same antibody genes, independent of the carrier molecule (Fish and

mAbs encoded by the same antibody genes, independent of the carrier molecule (Fish and Manser, 1987). This has allowed us to explore how the knowledge of epitope specific antibody repertoires might be used specifically to guide the design of chlamydial subunit vaccines, and for measuring antibody responses to pathogens in general.

I. LITERATURE REVIEW

1.1 THE PROKARYOTE

1.1.1.   Taxonomy and Life cycle

The order Chlamydiales consists of one family, the Chlamydiaceae. There is a single genus *Chlamydia* within this family (Moulder et al., 1984) which has four recognized species. These are *C. trachomatis, C. pneumoniae, C. psittaci,* and *C. pecorum* (Grayston et al., 1989; Fukushi and Hirai, 1992). All members of the genus *Chlamydia* are highly specialized gram-negative eubacterial organisms, with diverse disease spectrums and DNA compositons (table 1). Despite these differences all chlamydial species are obligate intracellular pathogens of eukaryotic cells, and none have been grown free of the host cell. These organisms divide by binary fission within a unique inclusion vesicle, are sensitive to antibiotics, have their own ribosomes, and have dual trilaminar membranes (Moulder, 1991). Many chlamydia-like and related orgnisms have recently been identified in invertebrates and from aquatic environments demonstrating the ecological success of this organism (Ward, 1998).

The chlamydia are morphologically unique. The extracellular EB is approximately 200-300 nm in diameter and has a rigid outer membrane structure (Fields and Barnes, 1990). The outer membrane, in all chlamydial species, is dominated by the major outer

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Table 1 (facing : Chlamydial organisms and disease spectrums.

Characteristics of the Species Chlamydia

Characteristics	C. trachomatis	C. pneumoniae	C. psittaci	C. pecorum
Hosts	Humans, mice, pigs	Humans, horses	Birds, humans*, other mammals	cows, sheep
Morphology	round	tear-drop	round	round
Iodine Stain	Yes	No	Yes	Yes
Number of Serovars	>15	1 - ?	?	3
Infections	Genital- STD, LGV, PID, ectopic pregnancy, HIV co-factor; Ocular- trachoma, conjunctivitis; > 70% asymptomatic*	Respiratory- pneumonial disease; Athersclerosis- Ct Ag; Lung Cancer? often asymptomatic	pneumonia; abortion, arthritis, systemic disease	Respiratory, CNS, gut, often asymptomatic
Homology	92% 1-7 1-33 1-10	94-96 1-8 10	14-95 1-20	88-100

membrane protein (MOMP) which accounts for 60% of the total protein composition (Hatch et al., 1981). *Chlamydiae* also contain a rough-type lipopolysaccharide in the outer membrane which contains antigenic determinants both unique to chlamydial-species and common to other gram negative organisms (Caldwell and Hitchcock, 1984). Unlike all eubacterial organisms, however, Chlamydial species lack detectable peptidoglycan (Caldwell et al., 1981), despite the fact that they contain genes that encode homologs for peptidoglycan biosynthesis (Stephens, 1998) . The recent whole-genome sequence determination of *C. trachomatis* serovar B has resulted in a flood of new genetic information, some of which I have included comments about below.

The chlamydial genome is relatively small. At roughly 1 million basepairs, the chlamydial genome contains only 875 protein coding genes (Stephens, 1998). It is thought that the dimunitive genome reflects the progressive deletion of genes made redundant in the nutrient rich intracelluar environment of the eukaryotic cell. Despite this small size, however, the chlamydial genomes contains all the genes necessary for the synthesis of phospholipids, LPS, branced chain amino acid transporters, and many genes involved in nucleotide metabolism (Grant McClarty, Department of Medical Microbiology, University of Manitoba, 1998). Recently, genes were identified that encode a putative type III secretion system in chlamydia (Hsai et al., 1996), which is a known virulence factor for *Yersina* species (Cornelis, 1997). Type III secretion systems enable the delivery of prokaryotic molecules across eukaryotic membranes (Rosqvist et al., 1994; Persson et al.,1995). The putative presence of a type III secretion system in chlamydial species supports the potential for cross-talk between pathogen and host. There

is no evidence of a restriction modification system as is found in *E. coli*, which may facillitate the, one day, sucess of genetic transformation of chlamydia.

The chlamydiae have a unique dimorphic lifecycle. Chlamydial organisms phase between an extracellular infectious form called the elementary body (EB), and an intracellular metabolically active form the reticulate body (RB). The elementary body is a rigid structure about 0.3 um in diameter, whose DNA nucleoid is highly condensed onto histone-like proteins (Pederson et al., 1996).

Chlamydial EBs attach to host cells through a variety of low avidity ligands (Ward, 1997). Chlamydial adhesion may be the result of a large number of low avidity interactions involving several chlamydial surface components. A tri-molecular mechanism of attachment has recently been postulated, in which host ligand and chlamydial attachment proteins are bridged by heparin-sulfate like molecules (Zhang and Stephens, 1992)

Chlamydial entry is dependent on host cell interactions. Entry involves both clathrin- and microfilament-dependent mechanisms and coincident tyrosine kinase activation (Birkelund et al., 1994). EBs differentiate into RBs and begin to divide. Shortly after entry into the host cell the EB differentiates into the RB and begins to divide by binary fission (about 8 hours). The RB is roughly 1 um in diameter. The chlamydial RBs remain within an inclusion membrane of unknown origin. Chlamydial organisms have evolved complex mechanisms of acquiring host metabolites while remaining within this unique vacuole (McClarty, 1994). 24-48 hours after initial entry, the RBs change back to the EB form and at 48-72 hours are released again from the host cell.

1.1.2.   Antigenic Composition of *Chlamydia*

The following section comprises a brief review of serologically defined chlamydial antigens. Several comprehensive reviews on chlamydial antigens have recently been published (Brunham and Peeling, 1994a; Raulston 1995). I will focus upon surface exposed B cell vaccine candidate antigens as little is known concerning chlamydial antigens recognized by T cells (Brunham and Peeling, 1994a).

There is a great deal of variation in the measurable human serum antibody responses to specific chlamydial antigens (Brunham and Peeling, 1994a). Similarly heterogeneous antibody responses are also observed in human sera during infection with the facultative intracellular pathogen *Mycobacterium tuberculosis* (Lyashchenko et al., 1998). The intracellular habitat of both mycobacterial and chlamydial organisms ostensibly causes similar variations in the decay of antigen depots established upon initial infection thus causing variation. The MOMP and Omp-2 are the dominant antigens, both eliciting a serum antibody responses in 70% of infected humans. However, the Omp-2 antigen is not surface exposed and does not generate in vitro neutralizing antibodies (Maclean et al., 1987). Other candidate antigens that generate neutralizing antibodies include the heat shock protein 70 (MaClean et al., 1987), and the MIP homologue (Lundmose et al.,1992). Little is known about the human antibody response to these antigen and they only elicit measurable antibody in the serum in 45% and 15% of infected patients respectively (Brunham and Peeling, 1994b). Clearly, more studies are needed to elucidate the dominant chlamydial antigens recognized by human mucosal antibody as the specificity patterns of human antibodies for bacterial antigens may be different from those in

autologous mucosal secretions (Berneman et al., 1998). Heterologous antibody responses are also seen in murine antibody responses to chlamydial antigens and are linked to major histocompatibility (MHC) genes and non-MHC genes (Zhong and Brunham, 1992).

The MOMP dominates the outer membrane of all chlamydial species. MOMP general structure is highly conserved. MOMP is a 40-kd, surface exposed, integral membrane protein and is the immunodominant antigen. The outer membrane of *C. trachomatis* is proposed to be organized in a hexagonal lattice (Chang, 1982) and may constitute a bacterial S-layer (Brunham and Peeling, 1994a). The MOMP has been estimated to account for 60% of the total protein mass in the outer membrane (Bavoil et al., 1984; Hatch et al., 1984). Recently, MOMP has been shown to posess porin-like properties in vitro (Wyllie et al., 1998), and MOMP may have additional roles in host cell attachment (Su et al., 1990). Consistent with the finding that MOMP is the major protective antigen for *C. trachomatis* (Zhang at al., 1997) the MOMP protein contains the main antibody neutralization epitopes for the organism (Brunham and Peeling, 1994a).

The omp-1 gene, that encodes the MOMP, is found as a single copy in the genome. The omp-1 open reading frame encodes a 22 amino acid residue leader sequence, and is expressed constitutively during the chlamydial developmental (Stephens et al., 1988). MOMP structure appears to be modular and composed of canonical sequences (Brunham and Peeling, 1994a). Indeed, comparative analysis of the entire omp-1 sequence among several serovars revealed that sequence variation is clustered into four sequence variable domains (Baehr et al., 1988). The variable domain sequences are known for the 15

classical *C. trachomatis* serovars (Yuan et al., 1989) and have been grouped according to serological relatedness into the B, C and intermediate serogroups (Stephens et al., 1987; see below). Members of the same serogroup are highly related and differ by amino acid substitutions. These appear to be point mutational changes representative of antigenic drift (Brunham and Peeling, 1994a; Stothard et al.,1998).

Other surface antigens may have potential protective value for chlamydial vaccines including the unique macromolecular surface projections (Matsumoto, 1982), and chlamydial LPS, which has recently been observed to elicit neutralizing antibodies for *C. pneumoniae* (Peterson et al., 1998). Interestingly, multiple putative outer membrane protein genes have been identified via genome sequencing (Stephens, 1998). These genes may encode novel vaccine candidate antigens and will no doubt be the subject of intense investigation.

1.2   THE PATHOGEN

1.2.1.   Human Chlamydial Disease

Members of the *C. trachomatis* species are mainly human pathogens. They cause a wide spectrum of human diseases including trachoma, lymphogranuloma venereum, salpingitis and can lead to blindness and infertility. There are more than 500 million human chlamydial infections per year. About 7 million people go blind due to trachoma every year mainly in third world countries. There are an estimated 90 million cases of genital chlamydial infections world-wide per year with 4 million in the USA alone (Peeling and Brunham, 1996). *C. pneumoniae* infection is implicated in the pathogenesis of atherosclerosis and pneumonia, which is a causative agent of respiratory illness in

humans (Peeling and Brunham, 1996). Genital chlamydial infection has also been implicated as a risk factor for the acquisition of HIV-1. Chlamydial organisms are also important agricultural pathogens of livestock, including sheep, cows, horses, and birds (Ward, 1997). A vaccine that would reduce chlamydial transmission and infection would clearly be beneficial.

1.2.2.   *Virulence and Pathogenesis*

Chlamydial organisms infect a wide range of host cells. Serovars A, B, Ba, and C cause trachoma, serovars D-K are the major causes of genital infection, while L1-3 cause lymphogranuloma venereum (Kuo, 1988). The LGV biovar can be distinguished from the other serovars by its ability to infect lymphatic as well as epithelial cells. While the intracellular niches of these biovars are different, the differences are not reflected in their omp1 structures and other, as of yet, unknown differences are responsible for determining host cell tropism (Stothard et al., 1998).

Chlamydial organisms have several important virulence factors. The intracellular lifecycle of chlamydial organisms ensures that they are hidden from humoral immune responses during their replicative phase. Cell mediated immunity may be important for the host to identify and remove chlamydial infected cells. Chlamydial organism prevent phagosomal-lysosomal fusion by two mechanisms. The first is inherent to the EB structure, and ensures early survival of the EB early-on after entry; The second requires early gene expression and is an active mechanism (T. Hackstadt, NIH Labs, Montana, 1998) Recently, it has been shown that chlamydial organisms can prevent the *in vitro* apoptosis of the host cells early-on in the lifecycle (Fan et al., 1998). This is perhaps in

order to "hijack" the cell for its own uses and may establish a reservoir for persistent cryptic infection. Others have found that *C. psittaci* causes apoptosis in the later stages of replication *in vitro* (~20 hours) , which may be related to the release-stage (Ojcius, et al., 1998). Clearly much remains to be discovered about the cell biology of chlamydial infections, in particular the relevance of these findings to *in vivo* chlamydial infection.

1.2.3.   Major Outer Membrane Protein (MOMP): Antigenicity and Variation

The different strains of *C. trachomatis* are natural antigenic variants. Differential serological reactivities to *C. trachomatis* isolates formed the original basis for sero-typing by micro-immunofluorescence antibody assay (Wang and Grayston, 1974). Serotyping lead to the classification of 15 *C. trachomatis* serovars and organized these into 3 serogroups based on cross-reactivity. These are the B serogroup (B, Ba, D, G, L2, L1,) C serogroup (A,C, H, I, J, K, and L3); and the intermediate serogroup (E, F) . None of the 15 classical serovars of *C. trachomatis* are sequence identical in all four MOMP variable domains which suggested that MOMP variable domain variation is at least partially responsible for serovar specificity seen in the MIF (figure 2). Numerous serovar-, subserovar-, and serogroup specific epitopes have been identified (Wang and Grayston, 1982) and many of these are localized in the MOMP

Figure 2 (facing): Primary sequence structure of the *C. trachomatis* major outer

membrane protein (MOMP). Shown are the variable domains (VD) from serovars used in

this study. Members of the same serogroup are variable in VD-1 by point mutations

causing substitutions. Inter-serogroup variation is larger and appears to result from a

larger scale, possibly recombinatorial changes. The VD-4 sequence is largely species

conserved. Synthetic peptides corresponding to the serovar C parental VD-1 sequence,

DVAGLQND, and the VD-4 conserved sequence TTLNPTIA, were used in this study to

analyse antibody responses. * indicates a deletion; - indicates sequence homology.

The ribbon diagrams are derived from the crystal structure of another porin from

Escherichia coli omp F. The omp F protein also forms outer membrane trimers. The VD1

(green) and 4 (pink) loops of *C. trachomatis* are superimposed upon this structure based

upon comparative structural modelling studies performed using PC-Gene. The size of the

outer membrane complex can easily accomodate antibody Fab binding to the putative

MOMP surface exposed loops that contain the VD epitopes.
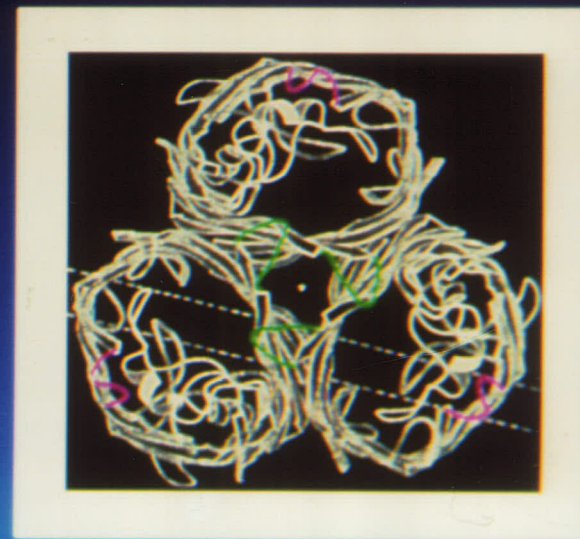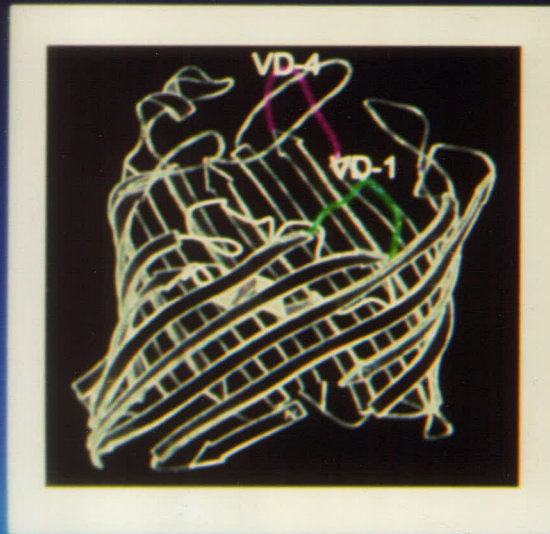
(Zhang et al., 1989). MOMP antibodies tend to map to the variable domains, as observed with recombinant soluble polypeptides (Toye et al., 1990; Stephens et al., 1988), recombinant bacteriophage (Baehr et al., 1988; Zhong et al., 1997) and/or synthetic peptides in a pepscan format (Conlan et al., 1988; Stephens et al., 1988; Toye et al., 1990; Zhong and Brunham, 1990a, 1990b, 1991; Villeneuve et al., 1994; Batteiger, 1996) with immune sera and monoclonal antibodies. While most of the epitopes are inferred to be surface accessible via binding studies on native organisms (Zhong and Brunham, 1990a) there has only been one reported study, which utilized anti-peptide antibodies, to directly confirm surface exposure of a MOMP epitope using immunoelectron microscopy (Conlan et al., 1989). The molecular basis of the omp1 gene diversity among the serovars is a clustered nucleotide substitution, within serogroups, and insertions or deletions, between serogroups (Stephens et al., 1987).

MOMP antigenic variation is a virulence mechanism. Recent phylogenetic studies on omp1 structures have supported the immune selection pressure for MOMP antigenic variation over evolutionary time, rather than any relationship between MOMP structure and disease pathogenesis (Stothard et al., 1998). Novel examples of continued diversification of the MOMP protein of *C. trachomatis* have been found amoung fresh clinical isolates from humans (Lampe et al., 1993; Brunham et al., 1994, 1995; Hayes et al., 1994, 1995; Bauwens et al., 1995; Dean and Millman, 1997) . In particular, a study using monoclonal antibodies to serotype large numbers of clinical samples identified many several serological variants (Lampe et al., 1993). The omp1 genes were sequenced and revealed single nucleotide changes that encoded amino acid substitutions in the

variable domains of the inferred MOMP primary structure (Lampe et al., 1993). Similarly, large numbers of variant omp1 genes containing nucleotide changes, the majority (90%) of which were found to encode amino acid substitutions in the variable domains MOMP, were found in *C. trachomatis* isolates from trachoma-endemic areas of Tunisia (Dean et al., 1992). Human convalescent serum antibodies, and serovar specific (anti-MOMP) monoclonal antibodies, while observed to be highly neutralizing for the homologous serovar, were found to be only weakly or non-neutralizing for new MOMP antigenic drift variants (Lampe et al., 1997). While escape from serum antibody or single monoclonal antibody binding directly supports the role of MOMP antigenic variation as a virulence factor, this does not rule out the possibility that the coexisting mucosal antibodies elicited at the local sites of infection may cross neutralize better. Mucosal antibodies are likely the most relevant for the evaluation of efficacy in protection against *C. trachomatis* infection (Campos et al., 1995). Measurement of serum antibodies in neutralization does not necessarily reflect the protective capacity of the mucosal immune system as it fails to account for protection provided by local memory B cell pools (Juby, 1992), and broadly reactive sIgA antibody (Waldmann et al., 1970; Shvartsman et al., 1977). Indeed, the latter may be responsible for the heterotypic immunity observed *in vivo* against antigenic drift variants of influenza A virus (Adderson et al., 1982) and *C. trachomatis* (Grayston, 1970).

The MOMP also undergoes "shift" antigenic variation. In particular, molecular analysis of the chlamydial MOMP gene in clinical samples has revealed a high prevalence of both nucleotide substitutions, representing point mutational drift changes in

MOMP variable domains (Brunham et al., 1994), and mosaic MOMP genes, that may have been created by omp1 VD recombination ("shifts") (Brunham et al., 1994, 1995; Hayes et al., 1994; Stothard et al., 1998). The mechanism for this is unclear although *C. trachomatis* was shown recently to possess a Rec A gene homolog (by complementation) (Zhang et al., 1995), and thus may be capable of homologous recombination to create combinatorial antigenic diversity in order to avoid immune recognition. Similar mechanism of antigenic variation are seen in pathogenic species of *Neisseria* sp. (Borst and Greaves, 1987), *Borrelia* (Bianca et al., 1994), and trypanosomes (Lu et al., 1993). These mechanisms may be controlled by global response elements involved in the regulation of virulence (Dorman, 1995). Collectively, these points show that the MOMP of *C. trachomatis* has undergone antigenic drift and perhaps antigenic shift and that the omp1 gene mutations likely represent successful antigenic escape variants.

MOMP antigenic variation is not universal among chlamydial species. In contrast to the high prevalence of MOMP variants among *C. trachomatis* isolates, the MOMP from *C. pneumoniae* has not been found to contain mutations (Jantos et al., 1997). This suggests that host antibody has not played a role in selecting for MOMP variation in this species as it has in *C. trachomatis*, which may reflect differences in tropism, pathogenesis, or outer membrane composition. This latter is supported by the fact that an *in vitro* neutralizing monoclonal antibody generated to *C. pneumoniae* maps to the LPS rather than to the MOMP (Peterson et al., 1998) , and the fact that polyclonal anti-sera reacts differentially with *C. pneumoniae* isolates despite MOMP conservation (Jantos et al., 1997). The above described LPS specific mAb reduced of the infectious titres of the

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homologous strain of *C. pneumoniae in vivo* when pre-reacted with whole EBs (Peterson et al., 1998) . This suggests that other molecules in addition to MOMP may be important for generating protection against chlamydial infection and has important ramifications in vaccine design. Surprisingly, although the LPS mAb bound to other strains of *C. pneumoniae* it exhibited no neutralizing effects showing disparity between immunochemical and biological specificity, and corroborating the differential reactivity observed with polyclonal sera. Alternative vaccine design for an LPS-based *C. pneumonia* vaccine will have to take cell-mediated immunity into account in a vaccine preparation.

Emerging data from genomic sequencing of *C.pneumoniae* has identified a large family of putative surface proteins (POMPs) which may be capable of phase variation and sterically cover the *C. pneumoniae* MOMP. This may also explain the mono-allelic character of the *C. pneumoniae* MOMP.

1.2.4. Major Outer Membrane Protein (MOMP) Structural Implications

The MOMP is a bacterial outer membrane porin protein (Wyllie et al., 1998). Consistent with this, the primary-position of the MOMP variable domains corresponds to the outer-surface loops found in the OMP F crystal structure. The primary sequence of MOMP is only distantly related to the *Escherichia coli* outer membrane porin protein OMP F (~18 % aa identity), for which the 3-D crystal structure has been determined. However, when compared directly using several secondary-structural prediction programs (PC-gene database) the positions of the MOMP variable domain loops and the known OMP F loops in the primary structure are flanked by identical secondary structure

(my own analysis). In particular the MOMP VD1 (amino acids 64-83), and VD4 (288-317) regions aligned very well in terms of position with known surface exposed loops L2 (66-82) and L7 (282-294) of the OMP F (Cowan et al., 1992). Analagous loops have been found in sequence comparison in several other bacterial porins including the *E. coli* OMP C and PHO E, the *Klebsiella pneumoniae* OMP K (Alberti et al., 1995).

The sixteen stranded beta barrel transmembrane spanning structure common to porins typically creates 8 outer loop structures (Cowan et al., 1992; Jeanteur et al., 1994). However, there are only four variable domains in MOMP. This discrepancy may be due to the highly disulfide cross-linked and "closed" MOMP structure found on EBs (Dascher et al., 1993). Indeed, the MOMP becomes highly cross-linked, via an unknown intracellular mechanism, during the last 24 hours of the growth cycle as RBs revert back to the metabolically inactive EB form (Newhall, 1987). Immunochemical studies support the existence of alternative forms of the chlamydial MOMP (Hackstadt, et al., 1985), and this may have limited access of host antibody to only the four known MOMP variable domain putative loop structures. The MOMP structure is reduced, and perhaps "open", on the intracellular non-infectious RB to facilitate porin properties. The RB is not accessible to antibodies and thereby avoids selection pressure for MOMP variants by antibodies. Consistent with this notion, that the other putative loops are only presented only on the cell interior, the other MOMP domains are highly conserved in sequence.

We have directly demonstrated the surface accessiblity of the serovar C-VD1 loop by using a monoclonal antibody that maps to this region to visualize magnetically immuno-purified chlamydial EBs and recombinant filamentous phage containing a conformation-

optimized C-VD1 epitope analoque (Zhong et al., 1997). This provided an independent method of showing surface accessibility and improved biological mimicry for this type of mAb. The 3-D structure of the native MOMP is unknown and elucidation of this may help locate broadly conserved epitopes that could then be used to guide the design of vaccines portraying native neutralizing epitopes more accurately.

The antigenic variation seen in surface exposed domains of MOMP suggests the sequence mutation and immune selection pressure over evolutionary time generated escape variants (Stothard et al., 1998). This is supported by the ability to selectively enrich chlamydial MOMP mutants *in vitro* from a mixed chlamydial clinical specimens using a neutralizing mAb specific to a serovar-specific determinant (Lampe and Stamm, 1994). This shows that the *in vitro* selection procedure is stringent enough to selectively enrich MOMP mutants from the population. However, de novo MOMP mutations are not selected *in vitro* which may mean that MOMP mutants are exceedingly rare in laboratory strains, and/or that certain *in vivo* conditions are necessary to induce an active chlamydial mutator mechanism.

1.2.4. Pathogen-Host Coevolution and the Antibody repertoire:

The specific host molecules involved in adaptive immune responses, namely the T cell receptors, the B cell receptors, and MHC molecules, all have constant domains interspersed with highly variable regions (Pincus et al., 1992). Similarly, the major surface antigens of many pathogens have variable and constant regions and tend to contain immunodominant and protective epitopes within the variable domains (Pincus et al., 1992). Antigenic variation helps to enable the pathogen to survive in the presence of the host immune system. It is not known what determines the immunodominance of some epitopes and not others. Surface exposure, high mobility, and charge are all believed to be important. Host molecules themselves may play a role in determining dominance by virtue of their respective optimal ligands. Indeed, Nayak et al (1998) have shown that the rapidity of binding by an antibody is more important then the length of time an antibody remains bound in determining the dominance of a given epitope.

1.3   THE HOST

1.3.1. *Immunity and Immunopathology*

1.3.2.

a) *Pathological and Protective Host Immune Responses.*

Early human trachoma vaccine trials with whole purified chlamydial EBs revealed that serovar specific protection was achieved, albeit of a limited timespan (Jawetz et al., 1965; Grayston and Wang, 1978). These same studies revealed, however, that break-through infection of immunized individuals resulted in worsened disease than seen in un-immunized controls. Animal infection models and basic clinical studies together suggest that individual chlamydial proteins are centrally involved in pathogenesis and protection (Brunham and Peeling, 1994a). The intense pathology caused by the pathological antigen(s) leads to fibrotic lesions, and has been associated with immune responses to the highly conserved chlamydial Hsp60 protein (Morrison et al., 1992; Brunham and Peeling, 1994a). This presence of this pathological antigen has since confounded the use of whole chlamydial organism as a vaccine. The paradoxical properties of chlamydial antigens to both create immunity and pathology combined with the inability to genetically transform *C. trachomatis* for attenuation, drove chlamydial vaccine research into the realm of subunit design.

b) Evidence for Protective Immunity

A large body of evidence supports the generation of natural immunity to chlamydial infection in diverse animal models. While antibody mediated neutralization of chlamydiae has not been directly demonstrated to be an essential mechanism for protection or resolution of chlamydial infection, several points support the conclusion that the antibody response to MOMP is an important mediator of immunity (Fan and Stephens, 1997): The serovar or biovar specific resistance to challenge (Schacter, 1985; Brunham et al., 1985, respectively); The polymorphic nature of MOMP (Stothard et al.,1998); The selection of MOMP antigenic variants in STD core populations (Dean et al., 1992; Brunham et al., 1996); The demonstration that anti-MOMP antibodies neutralize infectivity in vitro and in vivo (Zhang et al., 1987). The demonstration that pre-absorption of anti-EB antibodies with E. coli bearing recombinant MOMP abrogates neutralization of only the homologous serovar (Fan and Stephens, 1997); The correlation between local mucosal IgA in humans and resolution of infection (Brunham et al., 1983). Indeed, secondary challenge infection in animal models shows either decreased morbidity (Grayston and Wang, 1978;Cui et al, 1989; Cotter et al.,1995; Pal et al., 1996) and/or decreased mortality (Rank and Bavoil, 1996), in some cases with decreased recovery of the infectious organism. Indeed, immune protection following genital infection of mice with the MoPn strain can last up to 150 days (Rank and Bavoil, 1996). Protection against re-infection in monkey eyes is type-specific, as it was in early human vaccine trials, and reflects the classification of chlamydial types determined in mouse toxicity studies (Wang and Grayston, 1963; Alexander et al., 1967). Serological classification of

chlamydial variants later correlated with the MOMP variable domain sequence determination (Yuan et al., 1989). Interestingly, there are serologically dominant strains of chlamydia that naturally exhibit one-way serological cross-reactivity (Wang and Grayston, 1971) with serovars C and B being the dominant (or senior) strains in their respective serogroups. This supports the notion that type-specific immunity is at least in part antibody mediated and directed to the surface accessible MOMP variable domains of *C. trachomatis* as the other junior members of each serogroup are drift variant in these regions. Collectively these experiments support the findings in early human vaccine trials.

c) Molecular epidemiology

The existence of protective immunity in humans to *C. trachomatis* in humans is supported by molecular epidemiological studies of high risk populations (Brunham et al., 1996). The rate of same-omp 1 genotype reinfection was reduced and much lower than the prevalence of new infections caused by the homologous serovar, and moreover, the duration of prostitution was inversely correlated with risk of *C. trachomatis* infection. The frequency dependent immune selection of omp1 variants among chlamydia isolates from this population collectively suggest that immune barriers are acting to prevent re-acquisition of the same serovar (Brunham et al., 1996).

d) Correlates of Immune Protection

There is a large body of evidence to support the existence of natural and vaccine induced immunity to *C. trachomatis*. Currently, it is believed that a T_H1 polarized immune response with IFN-gamma and IgA are important in immunity (Yang and Brunham, 1998).

Little is known concerning the role of cellular immune responses in protection against human chlamydial infections (Cotter and Byrne, 1996). Due to this, animal models have been used to generate most of the data on cellular immune responses to Chlamydia. Mouse models have been extensivley developed with the goal of applying the knowledge to vaccine development and detailed reviews have been recently published highlighting the importance of cell-mediated immuniy in resolving chlamydial infection (Cotter and Byrne, 1996; Yang and Brunham, 1997). Some inferrence to cellular mechanisms can be made from lymphokine profiling studies in humans. Women with positive endocervical chlamydial cultures had increased levels of interferon-gamma in endocervical secretions compared with uninfected women but the levels did not correlate with quantitative recovery of organisms from tissue culture (Arno et al., 1990). Thus it is not know whether gamma interferon is specifically involved or if it is a non-specific indicator of inflammation (Arno et al., 1990). The cellular arm is likely to involve the triggering of dendritic cells that are important in the stimulation of IgA production (Fayette et al., 1997).

The cell mediated immune response is likely dominant for the resolution of

chlamydial infection (Su et al.,1996) in particular given the intracellular location of replicating EBs. The cell mediated immune response demonstrates both IFN gamma dependent and independent mechanisms as IFN-gamma knockout mice are able to clear infection (Perry et al., 1997; Lampe et al.,1998). It is thought, however, that both cell mediated and humoral host defences that can act at the mucosal surface will be important targets of a chlamydial vaccine (Brunham, 1995). This notion is consistent with findings of recent animal infection models that observed mucosal CMI and IgA to be associated with protective immunity to *C. trachomatis* in a gamma interferon dependent manner (Igietseme, et al., 1998). Most of the discussion will focus on antibody mediated protection as it is central to this thesis.

Chlamydial antibodies contribute to host defense. Collective studies on infection/whole organism induced *in vivo* protection, passive antibody protection in vivo, and vaccine induced protection *in vivo* support this. Animal models of chlamydial infections have directly demonstrated that chlamydial antibody alone can passively protect the naive animals from infection of the genital tract (Rank and Batteiger, 1989; Cotter et al., 1995; Sukumar et al., 1997), lungs (Williams et al., 1984;) and monkey eyes (Baranfanger and MacDonald, 1974). In particular, both conformational and linear epitope specific MOMP mAbs alone have been shown to be capable of preventing *in vivo* infection (Sukumar et a., 1997) which is relevant to the development of a chlamydial vaccine. Significantly, MOMP antibodies can be found in all cases of protective immunity to *C. trachomatis* induced by either whole organism or a candidate vaccine (Zhang et al., 1997) in immunocompetent hosts for at least some of the animals. Thus,

chlamydial antibody may play a role in providing immunity to infection if induced by protective vaccines.

While MOMP antibody responses are important, these studies do not indicate whether antibody is a required component for protection or clearance (Coter et al., 1995). Antibody is the most desired effector arm of immunity because of its unique ability to completely prevent infection (Ada and Ramsey, 1996). Despite this, B cell knockout mice are capable of becoming partially immune to challenge infection with *C. trachomatis* (Yang and Brunham , 1998). The data does , however, support the beneficial role of B cells in immunity through the observed increased morbidity among the B cell knockout mice compared to their normal litter-mates; Antibody was generated in the immunocompetent controls and functional B cells likely contributed to the observed differences in resistance through antibody production and/or as antigen presentation cells. The presence of compensatory immune reactions has not been ruled out and may have played a role in protection. Similarly, others have observed increased susceptibility of B cell deficient mice to reinfection compared to normal littermates, in the presence of partial immunity (Su et al.,1997). Results of this nature highlight the importance of passive protection studies because they allow the evaluation of the protective effects of antibody in isolation. Moreover, cell-mediated protection inherently requires infection to occur to allow the effector arms to clear them. Although active infection is not required for pathological sensitization (Brunham and Peeling, 1994a), the possibility that even low level replication, produced in a limited and self clearing infection, would exaccerabate pathology remains. Thus an ideal chlamydial vaccine should elicit both antibodies and

CMI in order to maximize efficacy and prevent chlamydial infection altogether in as many cases as possible via antibody while still priming the cell mediated immune defenses in order to clear an infection should it surpass the antibody.

1.3.2. *Chlamydial antibodies: Resolution of Infection and Resistance to C. trachomatis*

For scientists, understanding the role of antibody against intracellular pathogens is fundamental to immunology, and important to vaccine design (Casadavell, 1998). A vaccine capable of eliciting a powerful antibody response to protective antigens on *C. trachomatis* may reduce transmission and infection with *C. trachomatis* and hence chlamydial disease. As *C. trachomatis* enters and primarily infects host cells at mucosal sites, it will be necessary in the future for scientists to develop methods for better analysis of host antibody responses at mucosal surfaces.

Secretory IgA is the predominant immunoglobulin class found at mucosal surfaces in most species (Tomasi and Zigelbaum, 1963). The external barrier formed by immunoglobulin A (IgA) in mucosal secretions inhibits the attachment and penetration of microorganisms through the outer layers and into the mucosal epethelial cells (Lamm, 1997). Thus mucosal IgA represents the front line of host defense. The host produces more IgA than any other class of antibody (Mestecky and McGee, 199?). This is due to the fact that there is more mucosa-associated lymphoid tissue than systemic, as well as the fact that mucosal plasmablasts tend to make IgA antibodies (Lamm, 1997). In fact,

the majority of all host IgA is synthesized by plasmablasts localized within the mucous membranes (Lamm, 1997). The predominance of B cells in the local lymphocyte population of the female genital tract (Crowley-Norwick et al., 1995), which contains all of the effector components of the mucosal immune system (Kutteh and Mestecky, 1994), supports the dominant role of immunoglobulins in front-line defense.

The mucosal antibody system can be common to all mucosal surfaces. Antigen specific IgA antibodies can be induced in certain secretions, such as the female genital tract, not only by immunization in the proximity of the mucosal tissues that correspond to pathogens entry, such as the vagina (Wassen et al., 1996), rectum (Crowley-Norwick et al., 1997), but also by oral and intranasal immunization (Mestecky et al., 1997). This shows that scIgA expression is commonly directed to all mucosal sites and supports its role in repelling mucosal pathogens.

Paradoxically, the mucosal immune system can also produce highly compartmentalized B cell (McGee and Kiyono, 1993; Quiding-Jarbrink, et al., 1995) and T cell responses (Lee et al., 1998). For this reason, vaccines must ultimately be analysed in the target tissue, rather than in the systemic immune system alone, as this can be misleading (Putkonen et al.,1998). The segregation of IgA into the mucosal secretions reflects the compartmentalization of IgA plasma cells into the lamina propria and exocrine tissue away from the other plasma cells making antibodies in the spleen and peripheral lymph nodes (Cebra et al., 1976). The seeding of remote mucosal tissues with B lymphocytes initially induced at other mucosal sites (McDermott and Bienenstock,

1979) suggests that regulatory mechanisms exist to ensure production and concentration of secretory IgA throughout the mucosal surfaces (Burns et al., 1982). These mechanisms include the preferential recirculation of B cells activated in mucosal membranes to mucosal surfaces by memory mucosal lymphocyte homing receptors such as MADCAM1 and alpha-4 beta-7 (Williams et al., 1998), and the effects of polymeric immunoglobulin receptor (pIgR) on increasing local IgA concentrations. Indeed, Ghaem-Maghami et al, (1997) observed 8-25 times more chlamydia-specific plasmablasts in the urine of convalescent trachoma patients, in the absence of urogenital infections. This supports the notion that there is site specific homing within a common mucosal immune system. Appropriate elicitation of local sIgA and T cells to induce mucosal immune protection will likely depend upon methods of antigen delivery (McGee and Kiyono, 1993), and improved methods to measure local cytokine production (Villaverda et al., 1997). In some cases specific mucosal immune responses are recruited in the absence of measurable systemic antibody responses (Trentin et al., 1996; Rowland-Jones, 1997). The compartmentalized nature of some mucosal immune responses has recently been implicated to be important in host defense against the fatally infectious pathogen HIV-1 (Mazzoli et al., 1997; Kaul et al., 1998), whereas systemic infection seems to invariably lead to AIDS progression and death. Additional studies are needed to establish if these compartmentalized immune responses are strictly localized or merely appear to be due to the concentrating effects of the various local mucosal effector mechanisms. In the case of the latter, one would expect to find the same specific IgA in the sera but at very low concentrations.

a) IgA as a barrier

The beneficial role of appropriate IgA responses as an immune barrier is emphasized in populations deficient in mucosal antibodies. In human populations endemic for lethal mucosal pathogens, IgA responses are associated with long-term survival in people continuously exposed to HIV-1 through sex (Mazolli et al., 1997; Kaul et al., 1998). In suppport of this, IgA deficient individuals living in areas endemic for gastrointestinal pathogens have increased morbidity and do not thrive (Castrignano et al., 1993). Significantly, HIV-1 resistance is associated with HIV-1-specific scIgA in the cervix which occurs in the absence of stimulation of serum IgG antibodies (Kaul et al., 1998, submitted). This extreme example suggests that the barrier role of scIgA may be critical in order to sequester a pathogen from the systemic immune system by preventing initial colonization of the host. The barrier role of IgA in natural chlamydial infection is not clear but reports have demonstrated a correlation between increased levels of chlamydial antibody in secretions and either resolution of primary infection (Brunham et al, 1983; Rank et al., 1979) or immunity to reinfection (Murray et al. 1973; Rank and Barron, 1983).

b) Mucosal antibodies produced in natural infection

Previous studies have indicated that mucosal antibody have protective roles during chlamydial infection (Brunham et al.,1983; Barron et al., 1984; Baettiger and Rank, 1987; Brunham et al., 1987). Resolution of cervical infection correlates with the appearance of chlamydial IgA in secretions (Brunham et al., 1983). Recently, the suppression of chlamydia-specific IgA responses was postulated to have a pathological role in trachomatous inflammation (Ghaem-Maghami, et al., 1997).

Studies in animal models (Barenfanger and MacDonald, 1974;Cotter et al., 1995; Yang and Brunham, 1996; Sukumar et al., 1997) and with humans (Brunham et al., 1983) supports the contention that secretory IgA antibodies, found on the mucosal surface, represents the first line of immunological defense against *C. trachomatis* (Pal et al., 1997). For example, using the guinea pig model of inclusion conjunctivitis, Murray et al (1973) observed a correlation between immunity and chlamydial-scIgA in the eye, but not with serum IgG. In humans, protection is also serovar specific and correlates with MOMP diversity. Individuals challenged with the same strain used for immunization showed solid protection (Jawetz et al., 1965). However, they remained completely susceptible to reinfection with heterologous strains. The demonstration of serovar specific protection naturally focussed much attention on the MOMP (Cotter and Byrne, 1996). The MOMP elicits strong antibody responses during human infection, directed mainly to the surface exposed epitopes (Cotter and Byrne, 1996).

c) Heterotypic MOMP Immune Responses and Mucosal Immunity

While the physical characteristics of the scIgA molecule may play a large part in mucosal antibody mediated protection (Taylor and Dimmock, 1985) other factors of the local immune system are likely important. For example, the proximity of B cells and scIgA molecules to the site of challenge are likely critical components of mucosal host defense (Liew et al., 1984), in particular, the presence of a local, highly reactive, memory B cell pool is important in heterotypic immunity.

Secretory IgA has been observed to be more cross-reactive than serum antibody. Secretory IgA collected from nasal washings, has been observed to have broader reactivity with type A influenza viruses than serum antibody produced after lung infection (Waldman et al.,1970; Shvartsman et al., 1977). A similar finding was observed in self-limiting *C. trachomatis* infection of cynomolgus monkey eyes (Caldwell et al.,1987). Chlamydial IgA was generally more cross-reactive with chlamydial antigens than IgG in tears. The local IgG responses peaked at the same time as maximal inflammatory response suggesting that tear IgG was in part due to transudation (Caldwell et al., 1987), and not due to local B cell production. However, the concentration differences of IgA and IgG in thoses tears is not known.   Interestingly, eye secretions from humans with active trachoma passively neutralized the infectivity of homologous but not heterologous serovars in owl-monkey eyes in vivo (Barenfanger and MacDonald, 1974). The lack of heterotypic protection seen in Barenfager and MacDonalds (1974) study is consistent with the only distant MOMP relationship seen between serovars A and B . Serum antibody to trachoma strains failed to provide protection against challenge eye

infection when passively transferred from immune monkeys (Orenstein et al., 1973). This supports the importance of antibody localization to mucosal sites to prevent chlamydial entry and suggests that protective chlamydial immune responses may be compartmentalized as the donor monkeys, who received their initial immunizations via eye-infection, resisted eye challenge at the time their sera was collected (Orenstein et al., 1973). The relative roles of human IgG and IgA in local secretions remains to be examined but the high local concentration of antibodies locally is clearly an important component of mucosal immunity.

Natural infection of target mucosal sites specifically generates sIgA which is associated with superior cross-protection against re-infection by antigenic variants. Indeed, such heterotypic immunity has been shown in mice (Schulman and Kilbourne, 1965; Beare et al., 1968; Webster and Askonas, 1980) and in humans (Hoskins et al., 1976, 1979) infected with live influenza A virus but not in those receiving immunization with dead virus. Furthermore, in all cases where live vaccines were shown to be more effective than inactivated virus in prevention of influenza virus infection, they were introduced via the respiratory route (Schulman and Kilbourne, 1965; Beare et al., 1968; Hoskins et al., 1976, 1979; Scott and Sydiskis, 1976; Shvartsman et al., 1977; Webster and Askonas, 1980; Yetter et al., 1980b; Murphy et al., 1982;). The differences in immunity have been attributed to the induction of local scIgA in lung secretions via the stimulation of local B cell pools. Moreover, influenza specific scIgA is induced only after lung infection and not after immunization with live organism at other sites (Liew et al., 1984). Injections with much larger doses (10^5 more organism) of inactivated organism

still do not generate as effective cross-protection as is seen in response to live virus (Armerding et al., 1982) showing that differences immune responses can not be attributed solely to antigen mass. For resistance to influenza A infection, serum antibody and cytotoxic T cells induced in mice by live organism (stimulated via any route) were found to be important only in recovery from, and not in the prevention of, infection (Liew et al., 1984). Delayed-type hypersensitivity responses (DTH z-) responses in infected mice are found to be deleterious (Liew and Russel, 1983).

Heterotypic protection has also been observed between closely related chlamydial serovars. Partial protection was afforded both-ways to challenge mucosal infection among serovars A and C (highly related MOMP drift variants, both of C sero-group). This is despite one-way junior and senior antigenicity status respectively in MIF antibody assay (Grayston et al., 1970). Thus the MIF reflects only the antigenicity of a serovar, and reveals little about immunogenicity provided by local primary immune response to these closely related MOMP structures. Interestingly, heterotypic infections are associated with cross-reactive memory antibody responses. These are produced in immunized mice upon re-exposure to any chlamydial serovar (San pin Wang pers. Commun. See letter to verify). This suggests that cross-reactive memory B cells are involved in heterotypic immunity. We speculate that heterotypic antibody responses, produced by local memory B cells, play a role in providing a local barrier to infection through broadened recognition of neutralizing MOMP epitopes. Moreover, hapten studies suggest that the variable region genes that encode the antigen binding domains of immunoglobulin, in responding B cell may be successively mutated and selected for

broadened reactivity through exposure to variant epitopes during clonal selection to the most conserved epitopes (Fish et al., 1991). Thus the broadened protection provided by sIgA may be due in part to the maturation of local memory B cell pools.

Mucosal sites establish local memory B cell pools. The ability to induce a secondary scIgA responses is specifically associated with the initial and direct priming of the mucosal surface, which serves to drive the formation of local memory B cell pools (Wright et al, 1983; Lyck and Holmgren, 1987). While systemic immunization is capable of boosting local IgA responses in locally primed immune responses (Svennerholm et al., 1977) it does not induce significant antigen-specific scIgA or protective immunity in naive mucosal tissues (Mystecky, 1987;Holmgren et al., 1992).

d) *IgA produced in active immunization*

IgA is associated with protection of mucosal surfaces. In BALB/c mice orally immunized with live *C. trachomatis* a high titer IgA response was produced in all mucosal secretions, and the mice were protected against a subsequent intranasal or vaginal challenge as indicated by the absence of chlamydial antigen in lung and genital tissues.(Ciu et al., 1989; 1991) Furthermore, in animals that received intranasal challenge, chlamydial-IgA was associated with the absence of the development of a pneumonic process (Ciu et al., 1989). In a murine model of salpingitis and infertility, intravaginal inoculation with EBs of the MoPn biovar of *C. trachomatis* results in a significant reduction in the fertility rates of female mice (de la Maza et al., 1994). Using this model, it was recently shown that a single intranasal immunization with EBs of the

MoPn biovar of *C. trachomatis* protected the mice against salpingitis and infertility following direct challenge of the ovarian bursa (Pal et al., 1994). The immune mice had high serum and vaginal mucosal antibodies to chlamydia. Moreover the protection conferred by mucosal immunization was long-term (Pal et al., 1996). Although these studies are only associative, they support the contention that mucosal antibody may play a role in preventing chlamydial infection of mucosal surfaces. Direct measures of the role of antibody are discussed below.

There are limited studies on the antigenic specificity of chlamydial mucosal antibody in humans. Many previous studies have indicated that mucosal antibody has protective roles during chlamydial infection (Brunham et al.,1983; Barron et al., 1984; Baettiger and Rank, 1987; Brunham et al., 1987). Detailed analysis of the specificity of protective mucosal antibodies in humans are needed. MOMP specific antibodies can be measured in human mucosal secretion, supporting the immunodominant role of this antigen (Hayashi and Kumamoto, 1991). The antigen specificity of tear antibodies from self-limiting monkey eye chlamydial infection model is also informative (Caldwell et al.,1987). This study revealed that the MOMP, and LPS were the predominant surface exposed antigens recognized by IgA in immunized monkey tears. MOMP antibody was measurable by day 14 and persisted beyond the resolution of the infection (day 56). Interestingly, the mucosal IgA exhibited a stronger and broader reactivity for MOMPs and LPS for other members of the B serogroup (more crossreactive) than the mucosal IgG. Whether this is due to differences in concentration or in blot detection components, or represents a true difference in reactivity is not clear. The measurement of MOMP

specific IgA antibodies in local secretions by the use of western blot has been considered useful for the diagnosis of human chlamydial urogenital infection (Hayashi and Kumamoto, 1991). Others have found an association between specific serum IgA antibody and acute chlamydial infections in humans using enzyme immunoasssays (Miettinen et al., 1990).   Importantly, the titre of secretory IgA antibody to *C. trachomatis* in the cervical mucus has been inversely correlated with quantitative shedding of the organism from the cervix in humans (Brunham et al. 1983). This suggests that IgA plays a role in protection against chlamydial infection perhaps as an immune barrier. This is consistent with the notion that IgA1 protease production by co-infecting gonnococci (an extracellular bacterium) is responsible for an associated increased risk of chlamydial infection (Brunham et al., 1996) presumably by the proteolysis of scIgA on the genital mucosa. Lastly, Murray et al (1973) showed in a guinea pig model of inclusion conjuctivitis that immunity to a conjunctival challenge correlated with the presence of chlamydial scIgA in the eye but not with serum antibodies. Thus mucosal antibodies are readily observed in chlamydial infection and are immunobiologically important.

e) IgA in passve immunization

MOMP antibodies can be protective in vivo when passively administered. For example, passive transfer of convalescent human tear immunoglobulin partially protected owl monkey eyes against chlamydial infection (Barenfanger and MacDonald, 1974; Nichols etal., 1973) and supports the barrier role for chlamydial antibody.

The relationship between the presumed barrier function of chlamydial antibody, sIgA and *in vitro* mediated neutralization of infectivity are not clear.

Although *in vitro* infectivity neutralization assays have been shown to possess predictive value for antibodies to influenza virus (Couch and Kasel, 1983), in other cases, for example. antibodies to *C. trachomatis* (MoPn strain) and to Semliki Forest virus, and rotavirus, have failed to neutralize pathogen *in vitro* and were not predictive of their demonstrable *in vivo* protective effects (Cotter et al.,1995; Snijders et al., 1993; Burns et al.,1996, respectively). In contrast, others have observed that antibody to MoPn produces *in vitro* neutralization of cell infectivity and correlated with in vivo passive protection (Pal et al., 1997a). The study by Pal et al (1997a) in particular lends hope for the development of a MOMP specific chlamydia vaccine.

Monoclonal antibodies raised to native MOMP map mainly to the surface exposed variable domains (Brunham, 1994). The MOMP contains the principle neutralizing determinants on *C. trachomatis*. Many laboratories have investigated the *in vitro* neutralizing capabilities of antibodies to various chlamydial surface structures.

The barrier function of IgA serves to keeps pathogens that enter the host through mucous membrane out of the host. Early studies leading to the successful development of the live oral polio (Sabin) vaccine were targeted to interrupt the natural pathogenesis of the virus at the step of entry which, like chlamydia infection, also occurs at a mucosal surface (Lamm et al., 1997). Subsequent studies showed that oral vacination leads to the production of sIgA antibody that blocks the primary mucosal infection (Ogra et al., 1968; Onorato et al., 1991). Indeed, this prominent sIgA antibody response represents a

fundamental difference from the mainly systemic antibody response induced by parenteral immunization with the Salk vaccine (Lamm, 1997). Presumably the Salk vaccine confers some mucosal protection as well and / or acts to neutralize the virus at a different stage prior disease pathogenesis as both vaccines have excellent efficacy.

In a direct demonstration of the important role of IgA as a barrier, anti-IgA antibody, when passively instilled into the lungs of an immune mouse, completely abrogated infection-induced immunity to influenza A (Renegar and Small, 1991). This directly demonstrated the importance of IgA by interference with its normal function. Naive mice passively administered IgA antibody to the chlamydial MOMP were found to be protected from mucosal challenge infection with *C. trachomatis* MoPn (Sukumar et al., 1997). In another example, Cotter et al (1995) directly tested the ability of MOMP monoclonal antibody to protect against infection of the mouse genital tract with *C. trachomatis* using MOMP hybridomas in a backpack tumor model. Remarkably, MOMP IgA and IgG mAbs both significantly reduced the incidence of infection following vaginal challenge and consistently reduced vaginal shedding from infected animals. Significantly, these mAbs reduced the severity of inflammatory infiltrate in oviduct tissue of treated animals and had a pronounced effect on upper genital tract pathology. This is significant because in humans a similar disease sequelae is associated with PID and leads to involuntary infertility. While the numbers of animals tested by Cotter et al (1995) were small, the IgA producing hybridoma (which produced both monomeric and dimeric IgA molecules) did not protect the mice as well as the IgG mAb. This was suggested to be due to the effects of systemic rather than local release of IgA dimers which does not portray

the physiological production of dimeric IgA by mucosal plasmablasts beneath the lamina propria. Indeed, dimeric IgA released into the blood stream is picked up by liver hepatocytes and excreted into bile in some animal species (Russel et al., 1982). The fact that IgG was able to provide protection again illustrates the redundancy and flexibility of the immune system. A coincident and measurable increase in the albumin levels in the mucosal secretions of the mice receiving the IgG hybridoma indicated increased transudation of IgG from sera had also occurred (Cotter et al., 1995). Regardless, these studies show that MOMP antibodies are capable of passively protecting mice from the effects of chlamydial infection in vivo. We believe that studies of epitope specific immunoglobulin repertoires in the systemic immune systems are a representative model for the studying the MOMP antibody repertoire in Balb/c.

The demonstration of passive immune protection has , in the past, often been the key element in the development of successful active vaccines (Krause et al.,1997). Studies of this nature must be performed with caution however and should be limited to antibodies raised to the native organism, as passive immunization does not necessarily reflect on the ability of a vaccine candidate to de novo induce immunity. For example, a recombinant polio virus vaccine construct containing a linear MOMP epitope in an antigenic site was able to induce serum antibody in rabbits that neutralized *C. trachomatis* infectivity in *in vitro* cell culture (Murdin et al., 1993; 1995) and passively protected monkey eyes from infection *in vivo* (Murdin et al., 1993). However, the recombinant polio virus construct failed to produce de novo protection when used to immunize monkeys. Thus it is particularly important to keep the potential for biological

disparity in mind when comparing antibodies of the same immunochemical specificity (Barnett et al.,1996; Greenspan and Cooper, 1995).

f) IgA and Vaccine considerations

Inadequate local antibody responses may be responsible for the lack of efficacy of most putative chlamydial vaccines in vivo. For example, parenteral administration of a MOMP synthetic oligopeptide vaccine containing immunodominant MOMP B and T cell epitopes did not provide protection against vaginal challenge with *C. trachomatis* (serovar D) (Su et al., 1995). While the synthetic vaccine produced high levels of neutralizing IgG antibodies in serum, chlamydial IgA antibodies were not present in the sera or vaginal washes, and thus no conclusions on the mucosal efficacy of the vaccine can be made. In contrast, mucosal delivery of MOMP DNA vaccines created nearly sterile immunity against reinfection with *C. trachomatis* MoPn in mouse lung infection (Zhang et al., 1996a). Intramuscular MOMP DNA vaccination also provided significant protection from infection (Zhang et al., 1996b, 1997), however, mucosal immunization reduced the recoverable EB titer from mouse lung by an additional 2 logs below the recoverable titer from intramuscular DNA vaccination (Zhang et al., 1996a). These experiments highlight the importance of inciting MOMP mucosal immune responses for protection against *C. trachomatis.*

g) *Portrayal of pathogenic epitopes by vaccine candidate antigens*

The ideal vaccine should accurately portray the protective epitopes. Indeed, the lack of *in vivo* protection seen with most unsuccessful MOMP subunit vaccines, despite some *in vitro* assurances of immunogenicity such as whole EB reactivity (Murdin et al,1993; Zhong et al., 1994a) or neutralization of infectivity in cell culture (Murdin et al., 1993; Zhong et al., 1994a), do not indicate whether the vaccine induces an authentic anti-MOMP host response. In fact, the absence of protection argues that the subset of host defences that are aroused by these vaccine are either inappropriate, weak, and / or overlap poorly with defenses that are raised against native MOMP.

Methods are needed that can identify correlates of protective immune responses to pathogens for which vaccines are difficult to derive. A deeper understanding of the MOMP antibody response may help to guide (by comparison) the design of vaccines intended to biologically portray the pathogen. As this thesis deals with the potential use of antibody gene expression as a measure of the host response to MOMP epitopes, a brief review of B cells and immunoglobulin genes are necessary.

1.3.3. B Lymphocytes

The theory of clonal selection was first developed by Sir McFarlane Burnet (1959). He envisaged that a genetic randomization, of an initial common specificity, might create a large population of cells each expressing a distinct antibody specificity. This is essentially what happens in chicken immune diversification, however, in humans and mice B cells express distinct antibody specificities from the beginning (Rajewsky, 1996). Since this time it has been shown repeatedly that antigen does indeed select for survival and proliferation of antigen specific lymphocytes to proliferate and differentiate out of a diverse background of cells (Fish et al., 1991). By having the ability to somatically develop a diverse pool of antigen receptors on lymphocytes the host can combat organisms with life cycles and mutation rates many times faster than itself, at the single cell level. Clonal selection of B cells results in affinity maturation of the antibody response which along with immune memory comprises the basis of vaccination. The variable region gene repertoire is somatically assembled into functional antibody molecules and B cells are activated or anergized depending on their B cell receptor specificity. Activation leads to proliferation and differentiation of B cells from which antigen selects winner populations with the highest relative affinity (Vora and Manser, 1996) , in the context of secondary signal from helper T cells for T dependent responses. T dependent immune responses are the most efficient at inducing immune memory. In this fashion a diverse assortment of antibody binding domains are created and produced as distinct effector isotypes in order to ensure survival of the the host species.

a) The Antigen Binding Domains

The complete antibody molecule (IgG) is a heterodimeric glycoprotein made up of light and heavy chain polypeptides (Butler, 1987). The N-terminal variable domain of each chain is sequence variable and is responsible for antigen binding specificity and affinity. The C-terminal domain, or constant regions are highly conserved and encode effector functions such as complement activation or Fc receptor binding. Class switching occurs via DNA recombination events, whereas membrane versus secreted forms of immunoglobulins are controlled via alternative splicing of the same RNA transcripts (Max, 1993). Maximal disparity in amino acid sequences was found to be localized in three distinct " hypervariable " domains or complementary determining regions (CDRs) interspersed by relatively conserved framework regions (Max, 1993). These were predicted to be the sites of antigen contact and the diversity contained in these regions was thought to contribute to antigen specificity (Wu and Kabat, 1970). This has been supported by the more recent elucidation of the crystal structures of several antigen-antibody complexes, for both carbohydrate and protein antigens. The predicted critical contact points are found in the CDR regions (Mariuzza, et al., 1987; Stanfield et al., 1990). Changes in the amino acid residues in CDR3 of the heavy chain directly affect the affinity of the antibody for the antigen (Parhami-Seren et al., 1989). The CDR3 of the heavy chain (H3) contains the highest level of genetic (Tonegawa, 1983) and structural diversity (Chothia et al., 1989) of all six CDRs consistent with proposed dominant role of H3 for conferring antibody specificity (Stanfield et al., 1993). This diversity is reflected by the inability of computer modelling programs to accurately predict canonical

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structures for VH-CDR3 (Chothia et al., 1989). There are however, some exceptions to this whereby the light chain may be more important for binding to a particular antigen, or other CDRs of the heavy chain. For example the V_H- CDR1 region has been elegantly shown to be the critical contact domain for a haptenic antigen using oligonucleotide directed mutagenesis and recombinant antibody expression systems in vitro (Sompuram, et al., 1996). The fact that different CDR regions can be critical for binding to different antigens supports the multispecificity theory on antibody diversity, whereby a single antibody may be able to engage distinct antigens using alternative binding formats (Van Regenmortel, 1998). This contributes an as of yet unknown level of antigen binding diversity to the already diverse Ig repertoire. This suggests that structural commonalities found within this veritable sea of immunoglobulin molecules is significant.

b) The B cell Repertoire

B cells produce antibodies, which are soluble ligands important for the binding and removal of pathogenic microorganisms and their products as well and other environmental antigens. There are multiple classes of antibodies that have different effector functions associated with the C-terminal constant region domains. The seemingly infinite number of unique N-terminal antigen binding domains is not mirrored in the numbers of available B cells in a host (Coutinho, 1993). At any one time, only a portion of the total B cell repertoire is available to engage a pathogen. Thus it is commonly accepted that developmentally programmed rearrangements of antibody V-gene segments predispose the immune system with antibodies to pathogens (Sollazzo et al., 1989). The fact that there is biased expression of only a portion of the total antibody repertoire at all

stages of development, as well as the recurrent usage of particular antibody genes in a species to target some epitopes on pathogens (Cassadeval et al., 1997), in some cases without the need for affinity maturation (Roost et al., 1995) would seem to support this.

B cell ontogeny occurs in a specific order. During the pre-B cell stage, in the bone marrow, the immunoglobulin genes undergo an ordered rearrangement which ultimately results in the display of membrane bound forms of antibody called the BcR (B cell receptor). In vivo, the heavy (H) chain is usually rearranged before any light chain (Alt et al.,1984). The first DNA rearrangement occurs between the D_H and J_H elements, and the resulting $D_H J_H$ element is then joined to a V_H element to form the $V_H D_H J_H$ segment, which, if in frame, will encode the variable domain of a heavy chain (Yu et al.,1998). The intervening DNA, between these elements, is looped out and lost to that B cell. Aberrant, non-productive rearrangement leads to D-J_H rearrangement on the other allele (on the other chromosome). Next, a Vk rearranges next to a Jk. Again if this is aberrant the other allele initiates rearrangement. If no Kappa alllele is produced the same begins at the lambda locus. Successful recombination of both a heavy and light chain gene is required for the expression of antibody on the cell surface. Although all V genes carry an upstream promoter, only one V_H and one Vk is succesfully expressed in a given B cell at one time (Alt et al., 1987). This is termed allelic exclusion (Adams, 1980; Colecough, 1983) and forms the basis of the clonal selection theory. Only the promoter of the assembled V genes are actively used to produce functional transcripts.

1.3.4. The Immunoglobulin Genes

How does the host create antibodies to bind to every potential antigen it may encounter over it's lifetime? This fundamental question was answered for the most part by Tonegawa (1983). It is counter-intuitive to imagine that an antibody gene exists for every possible epitope as there would be more antibody gene DNA then there is total DNA in the human genome. However, the host immune system deals with this problem creating a diverse and huge potential repertoire by combinatorial assembly of gene minicassettes that encode antigen binding domains of B and T cell receptors.

The ability to produce VDJ region recombination is highly evolutionarily conserved among all jawed vertebrates (Thompson et al., 1995). It is mediated by recombinases that act in a tissue and cell line specific manner. T cell receptor genes and not B cell receptor genes are only rearranged in T cells, and vice cersa. The recombinase system probably arose from invasion of the ancestral host genome with a pathogen derived transposon (Spanopolou et al., 1996) that became inserted into a host gene that encodes a host surface receptor involved in self/non-self recognition (Thompson, 1995). This is thought to have precipitated rapid evolution in T and B cell receptors, via gene duplications from an ancestral gene (Ohno, 1970). This is theorized to have given rise to the large multigene families that we observe today. Thus, despite the general similarities in the immune repertoire of most vertebrates, divergent evolution of the receptors and their chromosomal location continues (du Pasquier, 1994; Thompson, 1995, and references therein). Differences in the other additonal mechanisms of genetic diversification, such as somatic hypermutation further differentiate the species.

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The repertoire of immunoglobulins is heterogeneous in all species (du Pasquier, 1993) and can vary between members of the same species (see below). While the elements involved in the build up and assembly of immunoglobulin genes are highly conserved, the actual number and location of the genes differ from species to species (du Pasquier, 1993). The assembly process for expression of V-gene segments creates somatic changes in all species.

1.3.5. Pathogen-Host Coevolution II

Contrary to some dogma in immunogenetics (Weiss, 1993), natural selection may influence the expansion of host antibody alleles. Despite evidence to suggest that allelic (purifying) selection seems to have been weak or absent for the immunoglobulin locus (Gojobori and Nei, 1984), as well as conceptual difficulties for the soma to germline feedback loop (Steele et al., 1995), there may be allelic selection for some specific variable region alleles (Weiss, 1993; Steele et al.,1995). In particular, virulent pathogens that exert stringent and immediate selection may favor survival of individuals who are resistant. In this situation, individuals whose antibody molecules are inherently focused non-protective epitopes would clearly not thrive. Alternatively, individuals whose immune repertoire frequently includes antibodies to protective epitopes would have a selective advantage. The natural expression of antibodies to common childhood pathogens, early on in life, and the tendency for "early" protective antibodies to be encoded by germline (unmutated) V-genes (Ben-Aissa-Fennira, et al. 1998) or of some antibodies to apparenty not require affinity maturation (Kalinke et al., 1997) supports the

notion of pathogen-host coevolution of the antibody repertoire. Indeed, a careful examination of the sequences of PCR isolated germline V-gene segments (David et al., 1992) reveals the presence of Wu and Kabat-like variablility in germline V-genes CDRs (Steele et al., 1993; Walter et al.,1995). This suggests that global selection for diversity of the antibody repertoire may not entirely be the case (Hood et al.,1975). In fact, the data strongly suggest that each germline V segment has been subjected to powerful antigenic selection pressure (Steele et al.,1995). This supports the notion that germline V elements have been directly ligand selected during evolution in direct proportion to the predictability of various bacterial antigens, and senescent host components (Steele, 1979; Langman and Cohn, 1993). In addition to this, most peripheral B cells in mice appear to have been ligand selected (Gu et al.,1998) and some B cells naturally undergo somatic mutations and entry into the memory B cell compartment without overt immunization (Schittek and Rajewsky, 1992). This suggests that a programmed somatic diversification of immunoglobulin genes may take place, perhaps to ensure the production in a of protective V-genes. It has been suggested that environmental or self antigens may guide early repertoire development (Gu et al., 1998).

A direct test of this hypothesis is to sequence the immunoglobulin genes used to target the an epitope of a common pathogen. This has been acheived through molecular analysis of single separated epitope specific B cells (Jena et al.,1996) and through the analysis of epitope specific immortalized B cell hybridomas (Berek et al., 1984; Kalinke et al., 1997). This can determine if some alleles are used significantly more frequently than others, or more than their frequency in the general population (Weis, 1993). Given

the multispecific nature of V-gene paratopes, it is concievable that immunoglobulins capable of providing immediate protection to the host from more than one pathogen would be selected. Survival from intial exposure would allow fine tuning of affinity through the maturation processes. Patterned usage of immunoglobulin genes further suggests purifying selection.

1.3.6. The Control of B Cell Responses Neccesitates Repertoire Skewing

By combinatorial assembly of V-genes in individual lymphocytes, the host immune system maximizes germline diversity. This feature allows for the most efficient storage of many antigen binding domains (the complementary determining regions) held together by relatively conserved scaffolds (the framework regions). This potentially enables the host to generate protective antibody assemblages new and to historically common pathogens. In mice the genes encoding the heavy chain and the kappa light chain (which accounts for more than 90% of all expressed light chains in the mouse) are found on chromosomes 12 and 6 respectively. There is considerable polymorphism of the V-genes and the numbers of genes between individuals This type of diversity necessitates some form of control to prevent harmful anti-self antibody responses from occuring. Most of this control is probably due to clonal deletion of self reactive specificities early-on in B cell ontogeny (Nossal et al., 1994) in the bone marrow via apoptosis (Fang et al., 1998). This contributes to the underrepresentation of certain antibody genes in the periphery of both mouse and human (Decker et al., 1995; ten Boekel et al., 1997). However, this represents only one of several factors which bias the

expressed antibody repertoire (see below). When deleterious B cells arise in the periphery, they now appear to be salvaged through receptor editing, a process whereby a new upstream heavy chain V-gene is recombined proximal to the DJ region and expressed (Fanning et al., 1998). It remains to be seen if receptor editing creates the pool of naturally occuring somatically mutated memory B cells (Schittek and Rajewsky, 1992). Peripheral B cells that continue to produce anti-self specificities face apoptosis (Lebecque et al.,1997) or are anergized (turned "off") (Fang et al., 1998). This may also create holes in the antibody repertoire may in part explain the requirement for maintaining a vast germline and combinatorial diversity. Loss of other antibody specificites may be partially made up for through the aditional mechanisms of multispecificity, alternative heavy and light chain parinings, and somatic mutations. Key to this control system is the helper T cell which regulates the proliferation of T cell dependent B cell responses through T cell receptor- MHC interaction.

1.3.7. The Germline Repertoire and V Gene Allelic Polymorphism

There are a large number of antibody V-genes that contribute to the antibody repertoire. Whether the usage of genes is a stochastic process or is developmentally regulated is still controversial. Most recent data is in favor of an antibody repertoire that is biased at al stages by skewed expression of particular V-genes.

In the newborn mouse the repertoire is restricted by the absence of N-region diversification and the preferential recombination of certain V-gene elements (Rajewsky, 1996). Variation in germline V_H-genes directly effects the diversity of the antibody

repertoire, and may profoundly effect patterns of V_H gene utilization (Milner et al., 1995).

There is allelic polymorphism of individual minicassettes found in the mouse and human immunoglobulin locus. Germline V-gene polymorphisms in humans and mice can be attributed to differences in nucleotide sequences between allelic VH genes (Berek et al., 1997; Ulrich et al., 1997), D sequences, and J_H segments (Solin and Kaartinen, 1992; Mattila et al., 1995) as well as differences in the number of V-genes (Blankenstein et al.,1987; Adderson et al., 1993; Milner et al., 1995). Indeed, some genes are unique to individuals which suggests that the antibody gene repertoire is in constant flux (Juul et al., 1998), and in this regard resembles the HLA locus (Mattila et al., 1995). Additional differences in restriction fragment length polymorphism (RFLP) patterns have been found in ethnic groups (Sasso et al., 1995) who were at one time geographically isolated. Over evolutionary time these V_H-gene loci have undergone enormous selection and diversification by virtue of exposure and coevolution with infectious pathogens. It is possible that certain allelic variants in the V locus, for example, have particularly high binding affinity for the antigen. Individuals with such a variant allele could in principle be more efficient at recognizing the antigen and in provinding protection against disease (Weiss, 1993). Zinkernagel recently presented data showing the presence of B cells producing high-affinity, germline encoded protective antibodies to a cytopathic virus in mice, without the need for affinity maturation (Roost et al., 1995; Kalinke et al., 1996). The antibodies found early and late in the immune response to an epitope in the VSV glycoprotein have the same affinity. The early antibodies, produced on day 4 post-infection, are encoded by germline variable region gene assemblages. Furthermore,

optimal antibody mediated protection against *Streptococcus pneumoniae* is provided by germline anti-phosphorylcholine antibodies (Claflin and Berry, 1988). Collectively, this observations suggest that affinity maturation is not always necessary for immune protection and that there has been selection for protective genes for survival/resistance to common pathogens. Alternatively, the immune repertoire may be vast enough that these high affinity receptors may have been created by chance. However, evidence exists to support the former. We might logically expect that if there has been selection for protective V-gene alleles, than V-gene polymorphism should be associated with differential resistance to a pathogen. Indeed, VH-gene polymorphism has been correlated with altered disease susceptibility in humans (Feeney et al., 1996). Some pathogens elicit genetically restricted antibody responses to their protective epitopes, and these systems are model for studying the effects of V-gene polymorphisms. For example VH3 alleles and VkA2 in humans are critical for encoding protective *Hemophilus influenzae* type B capsular-polysaccharide antibodies to the native organism and to vaccine preparations (Adderson et al., 1991; Silverman and Lucas, 1991; Carroll et al., 1992; Newkirk and Rioux, 1995). The aboriginals of North America, in general, have a much higher incidence of *H. influenzae* type b infection (Siber et al., 1990). Upon examination, a defective Vk A2 allele was found in aboriginals and may play a role in increased susceptibility of aboriginals to *H. influenza* disease (Feeney et al., 1996). Moreover, *H. influenzae* type b conjugate vaccines have poor efficacy in aboriginals (Ward et al., 1990; Siber et al., 1990). Similarly, in animal models, the presence of particular V-gene segments in one murine strain mouse strain associated with reduced susceptibility to

disease by *H. influenzae.* (Adderson et al., 1993). These repertoire differences likely arose as a result of evolutionary selection pressure (Klinman and Linton, 1988), perhaps in relation to geographical isolation. The significance of individual V-gene differences is not yet clear. Thus V-gene polymorphism impacts upon the antibody repertoire and represents one of the underlying genetic reasons for differential susceptibility to some pathogens (Paige and Wu, 1989). Collectively, these observations help to explain the high incidence of *H. influenzae* type b infection some human populations and support the notion that individal V-genes may have undergone selective evolution due to microbial burden.

The heavy chain immunoglobulin locus in the most diverse. In the mouse, there are approximately 60-1000 V_H genes (Brodeur and Riblet, 1984), 14 D_H (Kurosawa and Tonegawa, 1982), and 5 J_H (Gough and Bernard, 1981) genes that are found in the antibody locus on chromosome 12. These genes have been loosely organized into 15 families based upon sequence relatedness (figure 3). Members of a family have at least 80% homology and ususally no less that 70% homology. V-genes that do not meet at least 60% homology with a known family are termed miscellaneous.

There are about 140 murine kappa chain V-genes located on chromosome 6 (Kirschbaum et al., 1996). These can be organized into approximately 20 Vk gene families depending on the classification scheme used. The Vk genes show degrees of relatedness that are more like incremental distinctions rather than discrete steps (Max, 1993).

The murine V-genes are in general more clustered and less interdigitated than the V-genes in humans. While there are differences between murine and human V-gene organization, there are individual V-genes that appear to pre-date the rodent-primate species divergence (Max, 1993). While the human immunoglobuin gene locus has been sequenced in its entirety, the mouse counterparts remain a black box. However, deletional mapping (Mainville et al.,1996) and YAC (yeast artificial chromosome) positional cloning (Kirschbaum et al,. 1996) has provided some knowledge of gene order for the heavy chain and kappa light chain loci respectively (figures 3 and 4, respectively).

Legend for Figure 3 (facing page): A schematic map of showing the relative locations of the known murine VH gene families. Positions were identified based on deletional mapping studies (modified from Mainville et al., 1996). The individual VH families have a clustered organization although there is interspersion between some adjacent families. Thus far in the mouse there have been 15 different VH gene families identified on chromosome 12.The relative location of the D H (Kurosawa and Tonegawa, 1982; Atkinson et al., 1994; Paul, 1993) and J H regions (Gough and Bernard, 1981; Atkinson et al., 1994; Paul, 1993) region locations are from several published sources . Theproposed number of D H segments are shown in brackets (Atkinson et al., 1994). The symbol JHΘ represents a known pseudogene (Gough and Bernard, 1981). VH family nomenclature has been adapted from Dildrop, 1986 and the Kabat data base at the National Institutes for Health in the United States.

Family[a]		Complexity[b]
VH5	(7183)	12
VH2	(Q52)	15
VH7	(S107)	2-4
VH14	(SM7)	3-4
VH4	(X-24)	2
VH11	(CP3)	1-6
VH3	(36-60)	5-8
VH9	(VGAM3.8)	5-7
VH13	(3609N)	1
VH12	(CH27)	1
VH6	(J606)	10-12
VH10	(DNA-4)	2-5
VH15		1[c]
VH8	(3609)	7-10
VH1	(J558)	60-1000
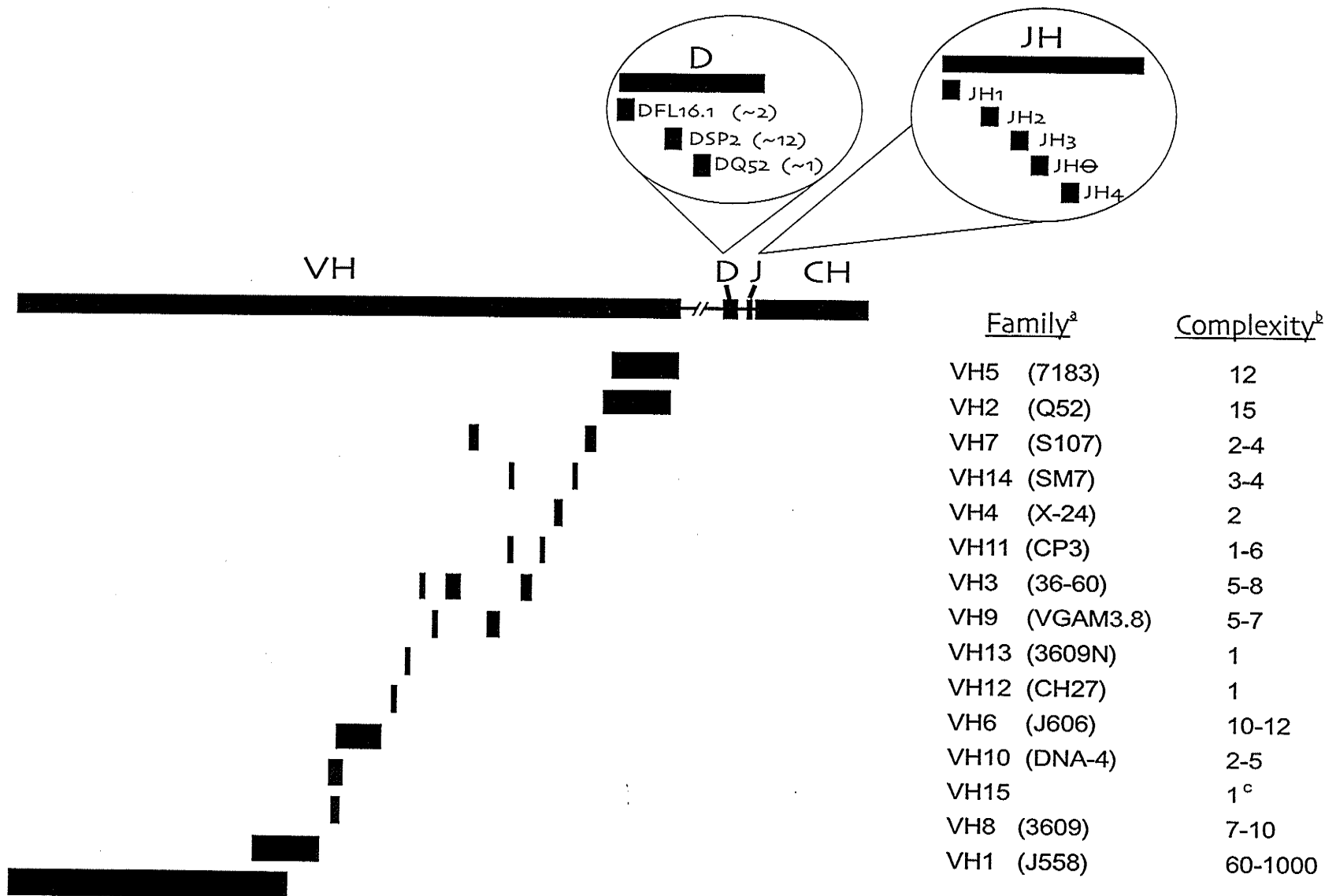
[a] VH Gene family nomenclature adapted from Dattamajumdar et al., 1996 and Paul, 1993) Family names are in brackets and typically represents the name of the myeloma/hybridoma clone from which the gene was first identified.
[b] The complexities were obtained from Kofler et al.,1992, Kelsoe, 1991, Brodeur et al., 1988; Brodeur and Riblet, 1984; and represent the estimated number of VH genes per family in the germline.
[c] VH15 has only recently been identified (Mainville et al., 1996).
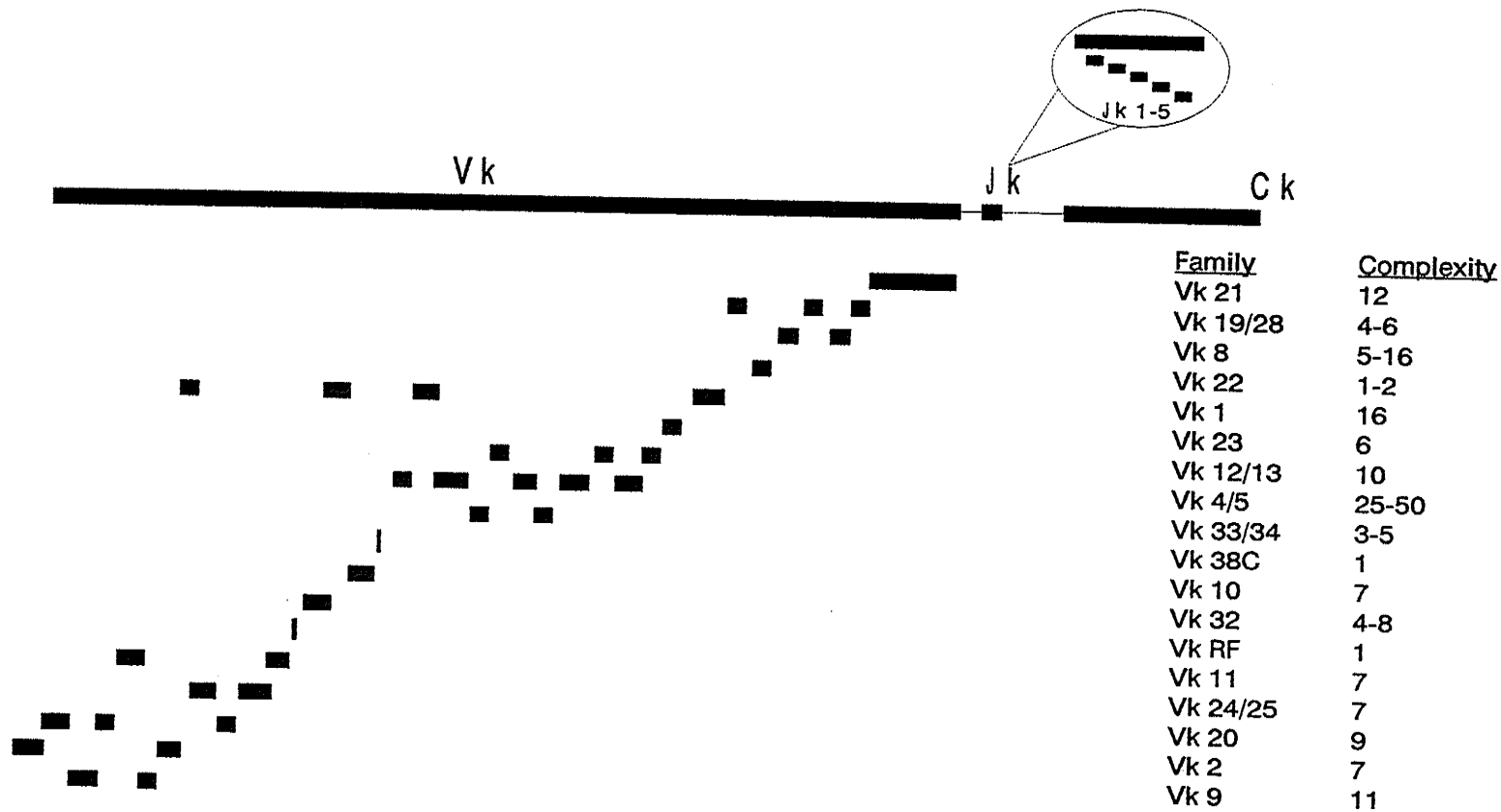
Figure 4: Schematic map of the murine kappa light chain locus located on chromosome 6. The map shows the relative positions of individual kappa variable region gene families relative to one another. There are approximately 140 variable region genes and 5 joining region segments. The estimated complexity of individual Vk gene families are given on the right. Many members of individual Vk gene families are interspersed among other Vk genes. This figure was compiled from data contained in Kirschbaum et al. (1996) and references therein.

Family	Complexity
Vk 21	12
Vk 19/28	4-6
Vk 8	5-16
Vk 22	1-2
Vk 1	16
Vk 23	6
Vk 12/13	10
Vk 4/5	25-50
Vk 33/34	3-5
Vk 38C	1
Vk 10	7
Vk 32	4-8
Vk RF	1
Vk 11	7
Vk 24/25	7
Vk 20	9
Vk 2	7
Vk 9	11

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1.3.8. Developmental Expression and Pre-immune Repertoire Bias

There is a non-random, pre-immune expression of V genes. The usage of individual V-gene families has been assessed in a number of ways. For example, the amount of V-gene family specific RNA found in total fetal liver and spleen during development has been measured (Paige and Wu, 1989). Strikingly, the most JH-proximal VH families, namely VH5 and VH2, are the most frequently used early in B cell development, in several mouse strains including BALB/c (Yancopolous et al.,1984; Reth et al., 1986; Alt et al., 1987; Jeong and Teale, 1989; Malynn et al., 1990). This was suggested to be the result of preferential rearrangement of the 3' located V-gene families (Freitas et al., 1990; Malynn et al., 1990).

Transcription is intimately involved with V-gene usage. Several mechanisms contribute to the bias in the "newly-generated" repertoire (Kofler et al., 1992), as it exists prior to antigen selective forces. V-gene usage in the newly generated repertoire depends on whether V-gene selection by the recombinase enzymes RAG-1 and RAG-2 (Schatz et al., 1989; Oetiger et al., 1990, respectively), the enzyme complex that mediates V-gene rarrangement, is a random process. Recombination of a given V-gene site is always preceeded by the production of sterile transcripts for V gene recombination and for class switch recombination. Furthermore, there are cell specific differences in local chromatin structure (Stanhope-Baker, et al., 1996). Whether transcription is necessary for allowing the RAG enzymes to access the DNA , or a side effect of improved access to the chromosome is not clear. The fact that biases exist in the expressed antibody repertoire, and that V-gene transcriptional promoters and recombination signals have varying

strength (Bemark et al.,1998; Fitzsimmons et al., 1998; and Yu et al., 1998, respectively), between even closely related genes, supports V-gene recombination as being a developmentally regulated process. There are also cis-acting strain-dependent differences in the utilization of certain V gene elements. For example, mouse D_H gene segment DFL16.1 is strain dependent and under-represented in BALB/c pre-B cell repertoire compared to C57BL/6 (Atkinson et al., 1994). Thus the antibody repertoire is encoded by polymorphic germline V_H -genes and the expression of these genes are further skewed by biased developmental expression systems.

1.3.9. Assembly of the minigene elements creates Junctional Diversity

Additional diversity is generated during the physical joining of the minicassettes through several ways. Junctional diversity (Tonegawa et al., 1983), is incurred through the imprecise joining of V, D, and J regions Examples of precise joins are rarely observed but it is not known if this is the result of imprecise joining per se or the effects of other changes like somatic mutations. The developmentally regulated expression of terminal deoxynucleotide transferase (TdT) is limited to the pre-B cell and is responsible for the addition of small stretches of new nucleotides (N-region addition) (Alt et al., 1984; Rajewsky, 1996). Small di- and tri- nucleotide inversions have also been observed at the junction of the joining and have been termed P region divesification (Sanz, 1991; Rajewsky, 1996). The sequences that arise from junctional diversity are not found in the un-rearranged copies of the same alleles. The possibility remains that they could be templated by very short regions of homologous DNA via some as of yet unknown gene

conversion like method, as they are too short for use as meaningful probes. All of these changes can lead to different translation products. CDR3 of the V_H (H3), which is encoded by the V_H-D_H-J_H join, has the most genetic and functional diversity. It has the most variation in size as well which precludes meaningful structural prediction studies (Chothia et al.,) . This is consistent with the H3 having the most intimate antigen contacts of all the CDRs (Mariuzza et al., 1987). Many examples exist in the literature where precisely the same junctional diversity has arisen in unique monoclonal antibodies selected to the same epitope, even among individual animals. Mechanisms that may contribute to the recurrence of particular junctional sequences have been proposed (Wysocki et al, 1986; Manser, 1990).

1.4.0. The antibody response

In mice, the available B cell repertoire consists of about 5 X 10^7 cells. B cells initially display receptors of the IgM constant region class and go on to coincidentally express IgD class receptors on the cell surface which importantly carry the same rearranged variable region on the N-terminii (Radbruch et al., 1986). More than three quarters of the available repertoire are dual IgM^+IgD^+ mature B cells which are the main source of B cells involved in primary antibody responses (Rajewsky et al., 1996). Other lineages make up the remaining population including long lived pools of recirculating memory B cells, B-1 cells, and possibly other as of yet unknown lineages. The V-region genes of the pre-immune B cell population are largely unmutated. The mature B cell responds to antigen, in the appropriate setting, by differentiating and proliferating to

become antibody producing cells (blasts) and/or memory B cells (DeFranco, 1993). Blasts can be found in various locations including the the bone marrow in humans and maroow and spleens in mice (Slifka et al., 1998) and are responsible for the production of circulating antibody.

B cell blasts are responsible for antibody production. After specific antigen encounter and receiving the appropriate second signals from T cells (in a T dependent antibody response) some B cells develop into blasts. The blasts account for the majority of circulating antibody produced in an immune response (Ahmed and Gray, 1996). The B cell changes its morphology dramatically in order to facillitate the massive production of secreted immunoglobulin. The cell volume increases several fold to accomodate the greatly enlarged endoplasmic reticulum and nucleus to accomodate the massive upregulation in immunoglobulin protein and gene expression via upregulation of the translation and transcription of antibody molecules respectively (Yuan and Tucker, 1984). Memory B cells on the other hand may also home to the marrow and periodically cycle through the periphery for surveillance purposes. Memory B cells are responsible for the production of secondary antibody responses which are functionally important in immunization.

1.4.1 Bias in the secondary (available) antibody repertoire

The primary repertoire is altered through selection and somatic variation to form the secondary antibody repertoire. Clonal selection ensures that despite preexisting bias high affinity B cells can be expanded to to deal with the diverse epitopes found on pathogens. In the adult mouse there appeared to be a roughly stoichiometric relationship between expression and the size of a given VH gene family (Dildrop et al., 1985; Paige and Wu, 1986; Rajewsky et al., 1987; Yancopolous et al., 1988) .Thus biased expression of V-gene families, found early in B cell development, appears to decline as mice mature (Marshall et al.,1996). In contrast, a recent detailed analysis of the V-gene repertoire in purified human pre-B, immature B cells from bone marrow, and mature B cells from the periphery revealed that there is biased expression at all stages (Kraj et al., 1997). Cellular selection may also play a role in the prevalance of individualB cells (Gu et al., 1991). Furthermore, biased expression of the individual V-genes for given families was observed in the IgM+ IgD+ B cell subset. It was concluded that biased expression of individual members of V-gene families could create the global perception of random V-gene usage in the other studies, wherein the expression of individual family members were not examined (Gu et al., 1991). Therefore it appears that developmental and regulatory bias may create the over-representation of certain genes in the early stages of B cell diversification, whereas ligand mediated clonal selection may be responsible for the bias observed in mature B cells (Gu et al., 1991).

The patterns of immunoglobulin VH gene expression reflect the diversity a host's available immunoglobulin repertoire (David et al., 1996). There is a huge amount of B cell death that occurs through the loss of cells with non-functional light and heavy chain pairings, tolerogenic mechanisms, antigen mediated expansion and apopotosis of B cells that fail to be immunologically selected in germinal centers (Lebecque et al., 1997). Clearly this contributes to the bias seen at later stages. Given that the mouse turns over about 5×10^7 B cells every day (Osmond, 1986), the B cell compartment is probably renewed several times a week (Pauge and Wu, 1989). Some V gene combinations are frequently created. Despite the high turnover in the B cell compartment, the patterns of expression are stable over time (Kohsaka et al., 1996). The extent to which the repertoire turned-over is unclear, as are the mechanisms that act to maintain individual specificites. However, the stability may bedue in part to the existence of long-living B cell populations (Ahmed and Gray, 1996; Slifka et al., 1998) as well as frequent re-creation of identical heavy and light chain Ig rearrangements in newly released B cells (Seidl et al.,1997).

1.4.2. Affinity maturation and antigen selection

Antibodies with increased affinity for the target antigen emerge in secondary response (Eisen and Siskind, 1964). Affinity improvements in antibody molecules relies upon two mechanisms (Andersson et al., 1998). The first molecular, that involves the targetted introduction of somatic mutations into rearranged immunoglobulin genes. The second is cellular and invlolves clonal expansion of B cells expressing surface

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immunoglobulin of higher affinity for the cognate antigen compared to their precursors. In the early stages of a primary immune response mainly low avidity antibodies are produced. Somatic hypermutation of V region germline genes and preferential selection of high-affinity B cells by antigen, especially declining antigen concentrations, leads to an increase in the overall average avidity of specific antibodies for most antigens. The mechanisms responsible for this increased affinity are discussed below.

Clonal selection is highly stringent and dominant B cell specificities emerge. Despite the complexity of antigens such as whole bacterium, antibody responses are focussed to relatively few immunodominant epitopes. Many B cells of diverse genetic makeup engage an antigen early on in a primary response (Kelsoe et al., 1989; Kelsoe, 1991). However, when the level of antigen declines affinity maturation occurs in germinal centers. Follicular dendritic cells present antigen in an unprocessed form on their cell surface and B cells compete for antigen binding. B cells are selected into the antibody secreting plasmablast pathway on the one hand and in to the germinal centre pathway on the other (McHeyzer-Williams et al., 1993). Elegant studies have been done using single cell molecular analysis and have directly assessed the mutational status of cells from both of these pathways. Somatic mutations being around 7 days following antigen exposure and is most extensive in the germinal centre (Kelsoe et al., 1991; McHeyzer-Williams et al., 1993). B cells bearing receptors with the best fitness for antigen binding recieve second signals from nearby helper T cells to prevent apoptosis prior to undergoing somatic mutation (see repertoire drift below) (Lebecque et al. 1997). Clearly, these selective processes can result in a biased expression in V genes used in

epitope specific antibody responses. Both somatic hypermutation of V genes (repertoire drift) and the use of new V genes (repertoire shift) contribute to affinity maturation of immune responses (Berek and Milstein, 1987).

a) Repertoire Drift

Somatic hypermutation is the mutational change in the DNA sequence of immunoglobulin variable region genes observed after stimulation by antigen(Wagner and Neuberger, 1996). It is a T cell dependent reaction (Manser, 1987) and occurs mainly within the germinal center (Jacob et al., 1991). The antigen present on the folicular dendritic cells generally selects for clones incurring mutations which improve affinity for antigen. These centrocytes recieve anti-apoptotic second signals and enter the long lived population (Lebecque et al., 1997). Hybridoma studies have shown that somatic hypermutation significantly expands the antibody repertoire (Kim et al., 1981; Gearhart and Bogenhagen, 1983; Griffiths et al., 1984; Manser et al., 1985; Clarke et al., 1985). Somatic hypermutation occurs in a stepwise fashion corresponding to the stepwise affinity maturation (Wysocki et al., 1986;Sharon et al., 1989; Rajewsky , 1996) and is localized to the CDRs (antigen contact domains) regions. It is still not entirely clear whether mutations are site specific and directed specifically to the CDR regions or if they are intially random, and incurred throughout the variable regions, and those B cells selected for survival by improved mutations that are found in the CDRs (Wagner et al., 1995). However it has been shown that the CDR sequences of human germline V-genes are encoded by codons which are more susceptible to replacement mutation than a random sequence (Chang and Casali, 1995). Consistent with this the codons of

framework regions utilize codons that are less susceptible to replacement mutation. Furthermore, modifying the sequence of an immunoglobulin passenger transgene altered the resulting pattern of hypermutation (Goyenechea and Milstein, 1996). Thus affinity maturation may involve the incorporation of point mutations to mutation-prone hot-spots found in the CDR regions.

Heavy chain constant region class switching associates a particular antigen-binding specificity, the V-domain, with a series of different effector functions encoded by the various CH regions (Davis et al., 1980). Somatic hypermutation occurs independent of class switch (Griffith et al., 1984; Rudikoff et al., 1984; Sieskevitz et al., 1987) and can be observed in un-switched mRNA of the IgM class in humans (Insel et al., 1994). Class switch begins to take place between 7-10 days post immunization, but may be effected by adjuvants (Griffiths et al., 1984). De novo somatic hypermutations may beseen as early as 5 days but are difficult to distinguish from pre-existing mutations (Schittek and Rajewsky, 1992) without comprehensive evaluation of V-gene status pre and post antigen exposure. Somatic mutation appears to co-opt mis-match repair to instruct correction of the parental sequence to match the mutated one (Cascalho et al., 1998). The mechanism is still not entirely clear but may involve transcriptional apparatus (Tumas-Brundage and Manser, 1997). All types of changes are observed including additions, deletions, and substitutions (Wagner and Neuberger, 1996). The encoded amino acid alterations that result from somatic mutational mechanisms can lead to increases or decreases of antibody affinity (Griffiths et al., 1984; Manser et al., 1985;Claflin et al., 1987), as well as changes in antigen specificity (Rudikoff, et al., 1982;

Diamond and Scharff, 1984; Clarke et al., 1985). Initial evidence to supoort the occcurence of somatic hypermutation in germinal centers was obtained by PCR amplification and sequencing V genes from populations of purified germinal center cells (Berek et al.,1991) or from histological sections (Jacob et al., 1991) suggesting that clonal expansion and stepwise selection of beneficial mutations was indeed occuring. Unfortunately, it has not yet been shown yet that the B cells examined were actually clonal. However, germinal center reactions are founded by only a few (1-3) responding cells that mature in an autonomous fashion (Vora and Manser, 1997). Somatic hypermutation and antigen selection complements germline and junctional diversity, by further altering the structure of the expressed V gene repertoire (Walter et al., 1995). In some cases however the identical somatic mutations have been identified in individual B cell clones ab\nd have been associated with increased affinity (Sharon et al., 1989). This supports the role of affinity for antigen for positive selection of B cells in the process of affinity maturation.

b) Repertoire Shift

In addition to the somatic drift observed in affinity matured V gene sequences, new V-gene assemblages emerge over time in epitope-specific antibody responses to haptens (Griffiths et al., 1984;Berek et al., 1985; Berek et al., 1987; Berek and Milstein, 1987) and to pathogenic epitopes (Kalinke et al., 1996). The term "repertoire shift" has been defined as the phenomenon observed where the V-gene repertoire against an antigen changes with repeated immunizations (Andersson et al., 1995). For example the immune response to the VSV glycoprotein has been well characterized with respect to V-

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gene usage during affinity maturation (Kalinke et al., 1996). Here the dominating V_H and Vk genes of the primary antibody response, a V_{H2} and a Vk-19-28 alleles respectively, are exchanged to other V-genes in secondary and tertiary immune response. All the new V-genes have somatic mutations and bind to antigen with a high affinity (Roost et al., 1995; Kalinke et al., 1996). While novel V gene specificities are continually emerging in naive B cells as they exit the bone marrow, naive B cell responses are repressed in the presence of circulating antibodies (Janeway and Travers, 1995). Receptor editing was implicated as a potential mechanism of increasing the expressed antibody diversity years ago (Alt., 1986; Kleinfeld et al., 1986; Reth et al., 1986) . While it is currently unclear how new V-gene combinations arise in the presence of high titre antibody responses they do nevertheless arise (Kalinke et al., 1996). Receptor editing (Radic and Zouali, 1996; Fanning et al., 1998), could conceivably play a role in the recycling of low affinity or deleterious B cells already previously stimulated by antigen. Receptor editing can generate novel antibody gene assemblages and generate new specificities. This takes place in a distinct subset of mature B cells, that may not be susceptible to Fc receptor induced repression. Because the intervening DNA is lost upon rearrangement, receptor editing can only swap for V-genes that remain upstream in the DNA. This may in part explain the early biased expression of 3' V-genes in some antipathogen responses as this leaves most of the upstream diversity intact should the B cell require recycling. Affinity maturation of the immune response ensures that high affinity antibody is produced to immunodominant epitopes. B cell memory maintains these antibody specificities for future encounters.

1.4.3. The Memory pool

Antigen specific B cells are selected into the memory B cell pool by antigen on the basis of affinity (Vora and Manser, 1997). This may also occur in the germinal center the site of somatic hypermutation. Little is known about lymphocyte memory pools and much remains to be elucidated. For instance, why do some antigens instill lifelong memory and protection, while others require frequent boosting? It is for this reason that the kinetics of memory responses must be examined for each antigen. It is known however that memory B cells have several unique properties (Ahmed and Gray, 1996): They have usually undergone class-switch and produce antibodies of isotypes other than IgM and no longer express surface IgD; Memory B cells are far more reactive and may require less T cell help. The BcR on the B cell acts to uptake and concentrate specific antigen for processing and presentation T cells in a MHC restricted manner with high efficiency in antigen experienced animals (Lanzavechia, 1985); The antibody response occurs for a much greater duration during a secondary response.

Memory B cells can respond to antigens in the presence of circulating antibody, whereas naive B cells cannot. Memory B cells also express different cell surface markers which may be used for analysis of specific B cell subsets. Blasts on the other hand are surface Ig negative as all of the mRNA is alternatively spliced for secretion. Memory B cell activation is responsible for the rapid induction and recall of antibody seen in secondary responses to the same or cross-reacting antigen and are the basis of vaccination. Thus B cells undergo selective expansion of expressing antibodies with

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improved affinity produced by point mutational changes as well as the the expression of alternative V-gene rearrangements. Memory responses ensure that the host is protected against future encounters with the same pathogen.

1.4.4. Chlamydial Vaccines

a) Chlamydial subunit vaccine development

Many attempts have been made to create a chlamydial vaccine based upon the early success of whole EB trials. The presence of the pathological antigen required scientists to consider methods of immunization with protective antigens alone. There have been several attempts at genetic transformation either through physical means or through bacteriophage mediated tranduction. Unfortunately we are still awaiting the generation of a reliable procedure to create attenuated chlamydial organisms for use as a vaccine. At the outset of this thesis work the most significant vaccine advances had been made with minimal based vaccines either as synthetic antigens (Su and Caldwell, 1992; Zhong et al., 1993; Qu et al., 1994), or in live replicating vectors such as recombinant polio virus (Murdin et al., 1993; 1995) or *Salmonella* (Hayes et al., 1991). The recent successes of DNA based MOMP vaccines (Zhang et al., 1997) and the successful cloning of potentially immunogenic whole recombinant MOMPs (Koehler et al., 1992; Fan and Stephens, 1997) merit comment.

The use of recombinant DNA technology to create recombinant MOMP has been explored. The MOMP is an integral membrane protein and thus has been difficult to generate and have it retain immunogenicity. Indeed, the full-length MOMP was found to

be toxic in many *E. coli* hosts resulting in the creation of MOMP fragments (Toye et al.,1990) and MOMP-LamB fusion protein (Hayes et al., 1991). Subsequent attempts have sucesfully cloned the full length MOMP from the guinea pig inclusion conjunctivitis strain (GPIC) (Dascher et al., 1993) and serovar B Manning and Stewart, 1993). In both cases however the proteins were not translocated to the surface of the host cell, suggesting that protein folding was inaccurate and the constructs would be poorly immunogenic. Another attempt by Koehler et al (1992) utilized a vector designed for the overexpression of toxic products. Induction was indeed lethal, however, successful translocation of the recombinant MOMP to the surface of the *E. coli* host was indicated by MOMP mAb binding studies. Subsequent immunogenic analysis of rMOMP by Fan and Stephens (1997) has shown that the rMOMP contains some of the conformational and neutralizing epitopes that are found on the native molecule. This suggests that a rMOMP protein may have value in subunit vaccination or in combination with other components. Unfortunately, most recombinant subunit vaccines do not trigger identical immune responses compared to those elicited by the native antigen (Eisenstein and Engleberg, 1986).

The use of anti-idiotype vaccines for chlamydial disease have also been explored. Brossay et al (1994) examined the immunogenicity of anti-idiotypic antibodies bearing an internal image of the species conserved epitope,TTLNPTIA, in mice. Immunization

with a polyclonal anti-idiotypic antibody preparation induced an antibody response directed against the VD-4 peptide and neutralized in vitro infectivity of EBs. There is also some evidence to suggest that T cell responses can also be generated to idiotypic vaccines in a number of experimental systems (Brossay et al., 1994). Similarly, anti-idiotypes to linear epitopes of viral pathogens has shown that an area of sequence homology can be observed between the V_H chain of the anti-idiotypic antibody and the protective determinant (Pride et al.,1992). One main advantage to idiotype vaccines is the ability of the antibody paratopes to mimic the three-dimensional structure of an epitope (Fields et al.,1995). Nearly identical combining site residues were utilized by the anti-idiotypic antibody and lysosyme, which it portrayed. Mimicry was confirmed by the induction of a lysosyme reactive antibody response by the anti-idiotypic antibody. This suggests that anti-idiotypic vaccines may be designed to portray neutralizing conformational epitopes of pathogens. These vaccines provide a molecular structural basis for the retainment of the internal image and have potential as vaccines to induce protective immunity against *C. trachomatis*.

b) DNA Vaccines

Studies using DNA immunization/expression systems (Wolff et al., 1990; Tang et al., 1992) have revealed that MOMP is the major protective surface antigen (Zhang et al., 1996b). The omp1 gene, but not the gene for chlamydial cytidine synthetase induced partial protective immunity from infection in mice (Zhang et al., 1996b). Indeed, either intramuscular (Zhang et al., 1997) or mucosal (Zhang et al., 1996a) immunization with

omp1 produces protection from reinfection from the MoPn strain of *C. trachomatis*. At the present time the DNA vaccne strategy represents the best candidate for a successful vaccine against chlamydial infection.

1.4.5. Synthetic Peptides as Chlamydial Subunit Vaccines

Synthetic peptides have been considered as potential vaccine components for some time (Sutcliffe et al., 1983; Arnon, 1991; Arnon and Van Regenmortel, 1992). These vaccines contain a relatively small peptide or peptides which have been shown to comprise epitopes of the organism that elicit a protective immune response (Arnon and Van Regenmortel, 1992). While it is difficult to imagine how antibody body binding sites, which on average bury 1550 square angstroms of protein including more than 20 amino acid contacts (Chothia, 1997), could be accurately mimicked by a tiny synthetic peptide, studies of peptide-antibody binding indicate that only six or seven amino acides are sufficient for binding to occur (Geysen et al., 1984, Getzoff et al., 1988). Peptides can be immunogenic and in some cases the antibodies they raise neutralize the infectivity of microbes in *in vitro* assays (Emini et al., 1983). Indeed, in some cases in vivo protection or partial protection can be shown. Examples where peptides have produced some protective immunity in vivo include, live challenge vs *Plasmodium falciparum* (Patarroyo et al.,1988) influenza A (Friede et al., 1994), foot and mouth disease virus (Bittle et al.,1982) and canine parvovirus (Langeveld et al.,1994). In the initial studies of synthetic peptide immunogens using Tobacco Mosais Virus (Anderer and Schlemberger, 1965) and bacteriophage MS2 (Sela, 1969) infectivity neutralizing antibody responses

were observed. Interestingly, neither of these microbes encounters immune responses in their normal habitat (Rowlands, 1992).   Despite some early successes, most peptide vaccines have low efficacy (Bittle et al., 1982; Langeveld et al.,1994;Schodel et al., 1994; Obeid et al. 1995; Robinson et al., 1995). Examples of successful peptides vaccines are only those that ultimately work for the host they are intended for. For example, a recent study pointed out that a canine parvovirus peptide vaccine was the first such example where protection of the real host was found (Langeveld et al., 1994). For the most part, these peptides are of relatively low immunogenicity in most hosts (Cox et al., 1988) which may be simply due to the structural simplicity of these antigens. A dominant peptide or a recombinant protein, may produce antibodies that react poorly with the native antigen conformation. While there have been examples of peptide-structural mimics that resemble native conformational epitopes (Bidart et al., 1990), they are not easily identified. Methods to attempt to improve peptide immunogenicity have included the use of adjuvants (Miller et al., 1992; Derfoot et al., 1992; Beckers et al., 1993) and immunogenic carrier molecules (Bessen and Fischetti, 1988; Charbit et al., 1988; Newton et al., 1989; 1995; Auvinen et al., 1993; Benito et al.,1996), increased peptide length (Kaumaya et al., 1992), increased structural complexity (Derfoot et al.,1992; Reynolds et al., 1994; Mahale et al., 1996), optimized epitope organization (Manca et al.,1985; Cox et al.,1988; Golvano, et al., 1990), MHC promiscuous T cell epitopes (Kauyama et al., 1993; Nayat et al., 1996) and conformational constraints (Lee et al., 1989; Muller et al., 1990; Leonetti et al., 1995).

The chlamydial MOMP contains many identified B and T cell epitopes. To

identify the surface exposed B cell epitopes, antisera was adsorbed with whole homologous EBs (Baehr et al., 1988;Stephens et al.,1988; Conlan et al., 1989; Zhong and Brunham, 1991). The study by Zhong and Brunham (1991) identified several immunodominant epitopes in the MOMP of serovar C using rabbit immune-sera. One of these was found in VD-1 and is a serovar specific epitope (-DVAGLQND-), the other, located in VD-4, is species conserved (-TTLNPTIA-). The serovar C VD-1 epitope is also the target of neutralizing antibodies in mice (Zhong et al.,1994a), and homologous anti-sera in convalescent humans also recognizes these peptides (Jones et al.,1992). Recent mapping studies by Battieger et al., (1998) suggest that there may be subtle differences in the exact identity of the linear MOMP B cell epitopes recognized by humans compard to mice. Although there was great serological variation in this study, the human anti-peptide epitope response clearly requires additional study. Synthetic peptides representing these epitopes were used to characterize and identify antibodies in this thesis.

Several laboratories have undertaken immunogenicity tests of chimaeric VD-peptides. For example, Su and Caldwell (1992) observed that a chimaeric serovar A VD-1 and T cell epitope produced high titre nuetralizing antibody in six different strains of inbred mice. This peptide contained a promiscuous T cell epitope (Su et al., 1990b) which apparently contributed to the immunogenicity of this construct. Experience in our lab has shown that high titer neutralizing antibodies are not always attainable *de novo* with synthetic constructs (Zhong et al., 1993). The analagous VD-1 containing peptide from serovar C was used in tandem with the VD4 peptide epitope and a promiscuous

helper T cell epitope in a lipidated form and used to immunize inbred strains of mice. Consistent with the observations of Su and Caldwell (1992), a high titer antibody response was observed to the peptide constructs. Moreover this sera reacted with the analagous variable domain synthetic peptides on pins. However, in contrast to Su and Caldwell (1992), antibody titers to the whole EBs were 10-100 fold less than to the peptides, and neutralization 500 fold less than whole EB reactivity. These studies suggested that the geometry of the peptides was perhaps less than optimal (Brunham 1994) and that structural constraints may be desirable in order improve complementarity of fit.

1.4.6. Immunological optimization of VD-1 peptide structure

The MOMP variable domains are proposed to be surface loops. Comparisons of the MOMP structure to other bacterial porins (Baehr et al., 1988; this thesis) revealed that the MOMP VDs are likely contained within putative loop structures (Brunham, 1994). Conformation is known to effect the immunogenicty of peptides (Gras-Masse et al., 1988; Manca et al., 1985; Jemmerson and Hutchinson, 1990). In some cases the introduction of conformation has improved the immunogenicity of peptide epitopes (Schulze-Gahmen et al., 1986; Williams et al, 1991;Lee et al., 1989; Muller et al., 1990; Leonetti et al., 1995), while in other it has not (Carmarero et al., 1993). Structural constraints that improve immunogenicity can be designed rationally, based upon some initial knowledge of the structure of the native target (Kobs-Conrad et al., 1993) or through randomization with some form of selection. In order to find MOMP peptides

with improved immunogenicity we created random structural constraints around a VD-1 borne on filamentous bacteriophage (Smith, 1985; Scott and Smith, 1990). The VD-1 peptide epitope contained the VD-1 epitope of serovar C, flanked by cysteine or serine residues, flanked by random residues. The phage-peptide library was panned with an anti-VD-1 monoclonal antibody with unique epitope conformation requirements (Zhong et al., 1990a). From these libraries we selected comformationally constrained VD-1 phage-borne peptide mimics for further characterization (Zhong et al., 1990b; Appendix 1). Sequence determination of the selected phages revealed preference for the C-VD1 epitope in a putative disuplhide looped structure with a distinct motif preference for flanking residues. Several of these phage-borne peptides were grown up and purified and tested for their immunogenicity. The phage-81 clone with a looped strucutre had an improved immunogenicity over that of linear peptide construct controls (Zhong et al., 1994b). Unfortunately, the peptide was not able to raise neutralizing antibodies in the context of the phage carrier (See Appendix 1). Serological analysis revealed the recombinant peptide to be a relatively minor immunogen on the phage particle (data not shown) which may explain the paradoxical ability of this construct to boost, but not de novo prime, for neutralizing EB antibody responses (See Appendix 1). Clearly, maintaining the immunogenicity of a peptide in the absence of the native MOMP molecule can be a serious challenge.

Although a chlamydial peptide may be identified as dominant for T (Su et al., 1990) or B cells (Baehr et al., 1988; Zhong et al., 1990a; 1990b; 1991) in the immune response generated against the native oganism, immunogenicity may be different on the

vaccine construct (Nayat et al.,1998, and references therein). For this reason each peptide construct must be rigorously tested after it is designed. Indeed, even the knowledge of the three-dimensional structure of a protein does not guarantee an efficacious peptide (Rowlands et al., 1992).

Using information learnt from our earlier studies, we synthesized co-linear synthetic peptides containing looped and linear VD-1 epitopes with a promiscuous helper T cell epitope (Su et al., 1990b). This was in order to evaluate the immunogenicity of the optimized looped structure in the absence of the phage carrier. These studies are also summarized in Appendix 1 of this thesis. Additional studies were performed with these peptides in order to study V-gene expression to synthetic peptide immunogens. We predicted that antibody repertoires elicited by immunogenic subunit vaccines will overlap with those against the native epitopes based largely upon the ability of haptenic epitopes to elicit the same V-genes when conjugated to different carrier molecules (Fiah and Manser, 1987).

1.4.7. Epitope Specific Recurrent Antibody Responses

Antibodies bind to a spectrum of antigens. Individual antibody molecules can bind to a spectrum of antigens with varying affinities (Rocca-Serra et al., 1983; Kabat and Wu, 1991). This may be accomplished by using unique CDR folding arrangements, alternative antibody-antigen physical orientations, or through differential usage of paratope subsites (Van Regenmortel, 1998). Thus, it cannot be expected that a perfect correspondence exists between B cell diversity and immunoglobulin gene antigen

specificity, as a given V-gene can be assembled somatically with an assortment of D_H and J_H regions in various Vk and Jk combinations (Tonegawa, 1983). This contributes to the redundacy observed between some antibody responses.

The analysis of epitope specific antibody repertoires is fundamental to immunology. Some epitope specific antibody responses are encoded by a diverse repertoire of antibody variable region genes (Schilling et al., 1980; Clarke et al., 1990; Wang et al., 1991), perhaps reflecting the redundancy of the antibody gene locus. For example, Wang et al. (1991) found the antibody response to a common carbohydrate epitope is encoded by diverse V_H genes. However, closer examination of the V-genes utilized by these anti-carbohydrate antibodies reveals patterned usage of some genes. Indeed, 6 of 15 Id+, groove-type antibodies utilized the same V_H-D_H combination along with the same V_k gene. Thus, even genetically diverse antibody responses can show patterned usage of particular V-gene asssemblages.

Despite the apparent redundancy in V-genes used in some antibody responses, other antibody responses to microbial epitopes reveal the patterned usage of certain V-genes (Claflin and Berry, 1988; Kavaler et al., 1990; Casadevall et al., 1994; Kalinke et al., 1996). A non-random usage of the same or very similar antibody genes are found to encode antibodies to the same epitope. The many and diverse publications in the literature on epitope specific antibodies has forced us to produce a working definition of V-gene recurrence. V-gene recurrence describes a range of similarities in antibody structures elicited to the same epitope. A recurrent response is encoded by either a predominant, or recurring, or restricted, set of V-genes segments. An example of

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predominant would be a case such as in Zenita et al (1990) where 48 of 50 monoclonal antibodies used the same V-gene structure. Cases where low numbers of antibodies were examined, for example less than 5, or where low numbers of antibodies were isolated from the same animal and thus may be clonally related, are not defined as recurrent. Patterned recurrence means a repeated finding of the same V-gene strucutures, encoding antibodies to the same epitope, in multiple individual animals/humans (Kavaler et al., 1990; Solin et al., 1992; Newkirk and Rioux, 1995; Kalinke et al., 1996) Restriction is an extreme form where the same Vk chain (Akolkar et al., 1987; Scott et al., 1989; Adderson et al., 1992; Mo et al., 1993; Casadevall et al., 1994; Patera et al., 1995), same VH chain (Pascual et al.,1992) or both (Griffiths et al., 1984; Kaartinen, et al., 1984; Seidl et al., 1997; Ikemetsu et al., 1993, 1998) are predominantly found to encode antibodies to the same epitope (Kofler et al., 1992). A predominant antibody responses could later be classified as restricted if epitope specific B cells from another individual animal/human at the same or different immunization conditions are found to be encoded by the same limited set of V-genes. Likewise, a predominant antibody response could be termed a patterned recurrence if while the same V-genes are found in another individual amoungst other novel combinations. In all of these cases, these types of responses are termed "recurrent". For example the B cell response Trypanosoma cruzi is biased to VH5 and VH7 genes in the chronic phase of the disease (Minoprio et al., 1989). Several notable examples of antibody responses that meet our criteria of recurrence based upon their molecular data are listed in table 2. The explanation for variable gene recurrence is not entirely clear, but may depend upon aspects of both the antigen; epitope mobility/

rigidity, complexity (Andria et al., 1990) ; the host germline or somatic diversity (Fish et al., 1991), tolerance, developmental control (Nicoletti et al., 1991), MHC; or on both host and pathogen co-evolution / mutation. Limitations on joining region diversity may also contribute to recurrence (Feeney et al., 1988). As well, differences in the germline V-gene framework 3 structures may contribute to restricted antibody responses and to the antigen specificity of individual V-region gene families as groups (Kirkham et al., 1992).

Recurrent usage of antibody V-gene structures is reproducibly expressed by all members of a species. For example, the usage of particular V gene combinations, to target the same hapten was found to be a heritable trait in 10 strains of mice (Kaartinen et al., 1991). Similarly, the phosphocholine epitope on *Streptococcus pneumoniae* is recurrently targetted by antibodies encoded by germline V-genes. Furthermore, somatic mutation worsens the affinity of these neutralizing antibodies suggesting that the germline sequence of these genes is already optimal for protection against this pathogen (Claflin and Berry, 1986). A similar phenomenon was seen recently in the predominant primary V-gene configuration used in the anti-VSV

Table 2 (facing page): Epitope specific V-gene recurrence. Representative recurrent antibody responses to both synthetic and pathogenic epitopes are listed. The V-gene segment structures that were commonly found are also given. Most of the data has been derived through hybridoma immortalization which has resulted in the vast majority of studies being performed in mice.

Immunogen	ANTIGEN/EPITOPE	Recurrence	Significance	Methodology	References
Hapten	phosphatidylcholine	VH11 +JH1 ; Vk9 + Jk2/4	found in 3 strains	Hybridoma	Seidl et al., Int. Immunol. 9, 689, 1997
Hapten	phenyl-oxazolone	VH2/11/13 ; Vk45.1 (25/29 mAbs); VH2 + JH3	found in 10 strains; independent methodology	Hybridoma; PCR/cDNA analysis	Griffths et al., Nature 312, 271,1984.; Kaartinen et al., Eur. J. Immunol. 21, 2863, 1991; Delassus et al., J. Immunol. Mth. 184, 219-229, 1995.
Hapten	Ars-KLH/ Ars-Brucella. abortus	VH1 + JH2; VkIdCR +Jk1	epitope elicits same V genes	Hybridoma	Fish and Manser, J. Exp. Med. 166, 711,1987.
Peptide	TG4 peptide and polypeptide TGAL / TGB5 Id+	VH10 + JH2/4; VK1 + Jk2	mimic elicits same V genes	Hybridoma	Giorgetti et al., J.I. 152, 136, 1994
VSV	glycoprotein / conformational	VH2 + JH4; VH5 + JH2 ; VH1 + JH2	neutralizing	Hybridoma	Kalinke et al., Immunity, 5, 639, 1996
Influenza A	HA/Cb - conformational	VH1 + JH1/3; Vk4/5 + Jk5	neutralizing	Hybridoma	Kavaler et al., J.I. 145,2312, 1990
	HA/45-IdX	VH5 + JH4; X + Jk2	neutralizing	Hybridoma	Caton et al., EMBO 5, 1577, 1986
C. neoformans	capsular polysaccharide (CNPS)	VH4 + JH3 ; VL1or2 + JL1or2	same H3 aa. sequence	Hybridoma	Casadevall and Scharff, J. Exp. Med. 174, 151, 1991
Tumor Antigen	ganglioside	VH1 + ?	cancer - associated	Hybridoma/ northern	Zenita et al., J. Immunol. 144, 4442, 1990
**C. trachomatis	MOMP + VD1 peptide / VD1	VH5/VH6 + JH2; Vk21 + Jk2	multistage L + H recurrence + same H3 aa. sequence ; neutralizing	Hybridoma/PCR/cDNA analysis	Berry and Brunham, manuscript in preparation
H. influenza type b	capsular polysaccharide	VH3b + JH4	Protective - human	Hybridoma	Adderson et al., J.I. 147, 1667, 1991

synthetic

pathogenic

response in mice (Kalinke et al., 1997). Recurrence is due to V-gene encoded, paratope-recognition of the epitope, as recurrent antibody responses were found to the hapten arsonate, whether conjugated to keyhole limpet haemocyanin or to the whole bacterium, *Brucella abortus* (Fish and Manser, 1987). Indeed, the fact that the same V_H/V_L combination could be found independently in murine hybridomas created from separate mice supports the notion that an immunogenic epitope mimic should engender antibody responses encoded by the same V-genes used against the native target (see hypothesis below). Many studies that have examined antigen specific antibody responses at the level of an individual epitope have found reccurent usage of partcular V genes. However the use of a particular set of V gene assemblages may correlate with usage for a particular pathogen. There is also growing evidence to suggest that similar recurrent variable gene usage can be found in the analysis of epitope specific human antibody responses (Scott et al., 1989; Pascual et al., 1992; Newkirk and Rioux, 1995; Silverman, 1995; Andris et al., 1997; Ikematsu et al., 1998).

Clonal expansion may skew epitope specific V-gene expression. The first step in the B cell response is the selective activation of B cell clones. These clones, expressing certain V-gene combinations, proliferate and differentiate. This results in an immediate bias in the responding B cell population for the expression of those germline configurations in the available B cell repertoire for higher affinity receptors and a coincident common frequency (Berek and Milstein, 1987; Roost et al., 1995). Muliple

factors likely contribute to the perceived presence of high affinity germline V-genes, including: cellular properties of different lymphocyte lineages (Freitas et al., 1986; Gu et al.,1991; Nicoleti et al., 1991; Schittek and Rajewsky, 1992); lymphocyte trafficking (Williams et al., 1998); compartmentalization (Mazzoli et al., 1997; Kaul et al., 1998) repertoire bias (non-random expression) either germline or developmental (Kaplan et al. 1988; Steele et al., 1993; Seidl et al., 1997; discussed above); antibody networks and self- ligand selection (Jerne, 1974; and Gu et al., 1991 respectively); somatic mutations (Berek et al., 1988; Schittek and Rajewsky, 1992); and differential activation properties of naive and memory lymphocytes (Rennick et al., 1980). Because even the most dominant B cell clonotypes are only lowly represented in the total repertoire, any structural commonalities in a B cell response are likely significant (Klinman and Linton, 1988). An estimation of a particularly common clonotype, T15, used to target the bacterial membrane phospholipid, phosphocholine (PC) antibody responses, was 1 anti-PC antibody forming cell /50,000 B cells (Klinman and Linton, 1988, and references therein). This is many fold higher than most antibody clonotypes. Indeed, using even conservative estimates of germline VH diversity, Tarlinton et al. (1988) estimated that the probability of two V genes recombining with the same DH element is extremely low (1:1000-1:5000). However, it is now clear that even combinatorial recombination is in fact a skewed process and V-gene alleles recombine differentially (Nadel et al., 1998b). This is consistent with earlier studies on primary anti-phosphocholine responses. For example, the high frequency of expression of T15+ clones was apparently the result of frequent recurrence of these clones within the generative cell pool as opposed to clonal

expansion of B cells bearing this specificity (Klinman and Linton, 1988), as the same

number of PC-antibody forming cells could be found per B cell in the spleen and the

bone marrow. Collectively, this suggests that predominant clonotypes may in fact be due

to frequent recurrence of certain V-gene segment combinations being formed in the

generative pool, in particular in responses to common microbial surface antigens.

Whatever, the cause, epitope-specific biased expression seems to become "locked-in",

even when the repertoire is not initially limited (Benjamini et al.,1988).

1.4.8. Pathogen-Host Coevolution (revisited)

The host has evolved a complex arsenal of receptor molecules encoded by many

related genes of the immunoglobulin superfamily in order to repel, recognize and or

eliminate foreign antigens. It follows then that particularly common environmental

antigens or pathogenic epitopes have likely provided a major evolutionary selection

pressure for the maintenance of a diverse germline immunoglobulin repertoire (Steele et

al., 1995). While extrinsic antigen contact does not appear to effect the ordered

expansion of the B cell repertoire (Klinman et al., 1976) the mechanisms that govern the

formation and expression of the functional antibody repertire are biased at all stages. One

possible explanation for this is that epitopes on common pathogens have shaped the

immune repertoire through evolutionary antagonism.

Intuitively, an epitope mimic for example in a vaccine, should portray the host

epitope structure maximally in order to elicit antibody that recognizes the native structure

in an infection encounter. Our earlier studies revealed what appeared to be a restricted antibody repertoire that mapped to the linear C-VD1 epitope (Zhong et al., 1994b). At the molecular level, the immunoglobulin V-genes used by neutralizing antibodies may be useful as immunological markers of a protective host antibody response. Many foreign epitopes have been found to be targetted recurringly by the same variable region genes or mini-gene elements (Caton et al., 1986; Kofler et al.,1992, and references therein; Solin et al., 1992; Kalinke et al., 1997; Seidl et al., 1997; above). This thesis also extends our earlier studies in the characterization of antibody genotypes used by BALB/c mice to target the VD1 epitope of serovar C.

1.5. HYPOTHESIS

A vaccine candidate that biologically portrays a native epitope will have the ability to elicit antibodies that are encoded by the same immunoglobulin variable region genes (or a subset therof) used to target the native epitope structure. Most of the evidence to support this hypothesis is indirect and suggests that the epitope itself is key in selecting B cells with particular V-genes to predominate. Evidence is mainly derived from the study of haptens, which are the simplest single epitopes to study, or from idiotype studies. For example, a haptenic epitope that produces an antibody response with limited genetic diversity, or is "restricted", was found to elicit antibodies with the same V-genes whether conjugated to keyhole limpet hemocyanin or to whole *Brucella abortus* bacterium (Fish and Manser, 1987) . Idiotypic network studies have shown that antibodies are capable of bearing an internal image of linear epitopes of the reovirus, to which they are raised which has supported the study of anti-Id antibodies in vaccine design (Bruck et al., 1986). Indeed, studies performed with anti-idiotypic antibodies (Garcia et al., 1992) and in antibodies to a peptide that portrays a meningococcal carbohydrate determinant (Hutchins et al., 1996) have directly shown that the same repertoire of V-genes can be recruited to the mimic. Because the average probablility of two Ig heavy chains being encoded by the same VH- DH combination is excessively low, around 1:1000 - 1:5000 (Tarlinton et al., 1988), this suggests that any structural commonalities found among antibodies raised to the same epitope are significant. Thus, these examples (table 2 ; and above), where a surrogate antigen recruits antibodies with sequence structure homology to the V-genes used against the original antigen, directly

support our hypothesis.

1.6. OBJECTIVES AND RATIONALE

In order to test the hypothesis we had to perform several objectives defined below:

1.   Characterization of the Balb/c polyclonal antibody response to MOMP variable domain -1 (VD-1) epitope.

2.   Characterization of the Balb/c monoclonal antibody response to a MOMP peptide epitope as induced by the native antigen.

3.   Examine the relationship between chlamydial antibody responses and recurrent immunoglobulin gene expression during the maturation of the immune response..

4.   Test of Hypothesis -Correlate expression of V-gene markers in response to immunogenic synthetic peptide epitopes as compared to that observed with the native antigen.

To first guage the breadth of the antibody repertoire to the MOMP we performed prime-boost experiments in mice immunized with antigenically variant strains of *C. trachomatis*. The polyclonal antibody reactivity was used to examine whole EB cross-reactivity and peptide epitope fine-specificity in vitro. In order to test the ability of a vaccine epitope to upregulate expression of the same antibody variable region genes that

are used against the native MOMP, we had to first examine the profile of antibody genes used against the MOMP variable domain 1. The variable region gene expression profile in MOMP variable domain-1 monoclonal antibodies was evaluated using hybridomas from BALB/c mice. Following this, we had to directly evaluate the relationship (if any) between recurrent expression of specific antibody genes, identified in the MOMP specific mAbs, and antibody responses to the native MOMP. The recurrent and predictable expression of particular V-genes may be useful as markers of MOMP epitope specific antibody responses. The effects of immunization on specific antibody gene mRNA expression (identified in Objective 2) was measured using a reverse transcriptase - polymerase chain reaction with biotinylated oligonucleotides (Delassus et al., 1995). This assay is capable of measuring relative amounts of mRNA expression in a B cell response. Affinity selected conformational peptides representing the VD-1 epitope of MOMP were tested for their immunogenicity in H-2 disparate mice. Following this the ability of the peptides to trigger expression of the same antibody genes was evaluated.

II. MATERIALS AND METHODS

2.1. Growth, Purification, and strains of Chlamydiae

Five mammalian cell lines were tested for their relative susceptibility to *C. trachomatis* infection. The cell lines examined include: Hela 229 (ATCC CCL 2.1, Human cervical carcinoma); Hak (ATCC CCL 15, Syrian hamster kidney); Vero (ATCC CCL 81, African green monkey kidney); L929 (ATCC CCL 1, murine connective tissue derived); and CHO-K1A (ATCC CCL 61, Chinese hamster ovary, proline auxotroph). All of these cell lines support chlamydial infection and form evenly distributed confluent monolayer when plated at appropriate density. However, each cell line displayed differential susceptibilities to infection by *C. trachomatis* EBs as shown by different infectious titres between cell lines for the same serovar. HeLa cells were generally the most sensitive cell line to infection by all serovars tested in our assay system (data not shown). This is consistent with the results of others who earlier tested eleven cell lines for chlamydial infectivity with serovar C EBs and found that the HeLa 229 cells were the most sensitive (Croy et al., 1975).

Serovars A (UM1/OT); B (TW5/OT); C (TW3/OT); H (UW43/Cx); K (UW31/Cx); and L2 (L2/434/Bu), were grown in HeLa 229 monolayers and the elementary bodies were harvested and purified on renograffin density gradients as reported previously (Maclean et al., 1988). Briefly, there are four standardized steps for the growing *C. trachomatis* in mammalian cells in vitro: a) Propagation of the host cell lines. b) Infection and growth of C. trachomatis. c) Preparation of EBs for inoculum stock. d) Gradient purification of EBs.

a) Propagation of the Host Cell Lines:

Clean HeLa 229 cells (American Type Culture Collection, Rockville, MA) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (Rehatuin), and 2 mM L-glutamine (GIBCO).The media is adjusted to pH 7.2 with sodium bicarbonate. This media is called MEM. Cells are split daily and grown overnight to confluence in the ratio of one flask to two in order to maintain logarithmic growth. The short growth periods obviates the need for strong buffering systems. Spent media is poured out of a flask (175 cm^2, Corning) with an established monolayer. The cells are gently rinsed with 10 ml of GKNP solution to remove extraneous protein and cell debris. A second rinse is performed with 3 ml of trypsin (O.1% in PBS) and than another 1 ml of trypsin is added and the flask is incubated for 2 minutes at 37°C. The monolayer will star to detach and this is further facilitated with a firm pat on the side of the flask. Next, 10 ml of MEM is added and used to disperse the cells. Half of the suspended cells are transferred into a new flask and another 30 ml of MEM is added to each flask for optimal growth overnight at 37°C.

b) Infection and Growth of *C. trachomatis.*

Serovar A-K and mouse pneumonitis have traditionally been grown in cell lines that are pre-treated with DEAE-dextrans (anionic exchanger, ie. is positively charged). This is thought to improve chlamydial infectivity by changing the charge of the host cell proteins. A confluent, fresh (24 hour old) HeLa 229 monolayer is washed with 10

ml of Hank's balanced salts solution (HBSS) and than incubated for 20 minutes while bathed in another 10 ml of HBSS containing 30 ug/ml of DEAE-dextrans (Pharmacia). The DEAE-dextrans is poured off and 1 ml of stock inoculum chlamydial EBs (see below) are added. The flask is incubated for two-hours at 35°C with rolling every 15 minutes. (For LGV serovars, it is not necessary to include DEAE-dextran incubation as they are more infective). The inoculum is removed and the monolayer rinsed with 10 ml of HBSS. Fresh MEM (30 ml) is added containing cycloheximide (2 ug/ml) for optimal bacterial growth in the absence of host growth and cell division. The monolayers are incubated for 72 hours at 35°C, or 48 hours at 37°C for LGV strains.

When trying to recover a low-titre EB stock we have had success with a step-wise incremental expansion of the bacterium from a HeLa cell monolayer in a single well of a 96-well cell culture plate, to a single well of a 24-well cell culture plate and finally into a small 25 cm^2 cell culture flask. From this point the EBs can be grown in large flasks. This process is very delicate and must be performed painstakingly.

c) Preparation of EBs for Stock Inoculum:

Ideally, prior to harvesting the chlamydial organisms, large round vacuolar inclusions of "swarming" (brownian motion) organisms can be seen filling the cytoplasm in nearly 100% of the cells of a monolayer. Organisms harvested from one such 175 cm^2 infected flask can be used as a stock inoculum and be used to re-infect at least 10 new monolayers in the same fashion. The organisms are harvested as follows: The spent media is disposed of into an autoclavable waste container. Next 10

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ml of cold HBSS is added along with 30 sterile glass beads (2 mm diameter). The cells are gently rolled off of the culture flask and transferred to a 50 ml disposable centrifuge tube (Falcon) on ice. The flask is washed twice with 10 ml of cold HBSS, which is added to the tube (total volume is now 30 ml). The cell suspension is sonicated three times for 45-60 seconds while on ice, with 1 minute of cooling between each sonication. Cooling is critical as sonication produces heat, to which chlamydial EB infectivity is sensitive. Next, the suspension is centrifuged at 500 X g at 4°C for 15 minutes to remove cell debris (Beckman, Table-top centrifuge). Discard the pellet and centrifuge the supernatant containing chlamydia in a 30 ml polycarbonate centrifuge tube at 30,000 X g at 4°C for 30 minutes (Beckman, JA-20 rotor). Resuspend the pellet in 10 ml of SPG using a brief sonication at a low power percentage. Culture the stock for sterility on a blood-agar petri plate. Divide the chlamydial inoculum stock into 1 ml aliquots and store at -70°C.

d) Gradient purification of EBs:

Gradient purification is usually done in larger scale (10-20 infected flasks) so that the preparation can be used for many independent experiments and thus avoid effects due to batch variation. The spent media is disposed of into an autoclavable waste container. Next 10 ml of cold HBSS is added along with 30 sterile glass beads (2 mm diameter) to each flask. The cells are gently rolled off of the culture flask and transferred to a 50 ml disposable centrifuge tubes (Falcon) on ice. Flasks are washed sequentially with the same 10 ml of cold HBSS 2 X (to minimize volume). Sonicate

the cell suspension 3 X for 45-60 seconds on ice. with 1 minute of cooling between

each sonication. Next, the suspension is centrifuged at 500 X g at 4°C for 15 minutes

to remove cell debris (Beckman, Table-top centrifuge). Discard the pellet and

centrifuge the supernatant containing chlamydia through a 35% renograffin layer (10

ml) (Hypaque, Johnson; in 0.1% HEPES buffer) in a 40 ml plastic soft ultra-

centrifuge tube at 30,000 X g at 4°C for 60 minutes (Beckman Ultracentrifuge,

Swinging bucket SW16 rotor). Resuspend the pellet in 10 ml of HBSS using a brief

sonication at a low power percentage. Set-up the appropriate number of 3 level

gradient tubes for the size of the harvest. The gradient tubes consist of three layers

loaded from the bottom of the soft tube via 5 ml glass pipette: 13 ml of 40 %

renograffin is loaded first ; 8 ml of 45% renograffin is loaded second ; and 5 ml of

52% renograffin is loaded to the bottom of the tube last. Layer the suspended EBs in

HBSS on top of the 40% layer. The gradient is centrifuged at 35,000 X g for 90

minutes at 4°C under vacuum with no braking (Beckman Ultracentrifuge, Swinging

bucket SW16 rotor). Two bands are visible following this spin; the top band (at the

40-45% interface) contains RBs; the bottom band (at the 45-52% interface) contains

the EBs. Carefully remove the layers above the 45% level with a 10 ml pipette and

discard. Draw up the EB band using a sterile 1 ml pasteur pipette and deposit into a

30 ml polycarbonate centrifuge tube. Pool all EB bands into a single tube. Wash the

EBs by filling the remaining space in the tube with HBSS. Centrifuge at 30,000 X g

for 30 minutes at 4°C (Beckman, JA-20 rotor). Resuspend the pellet in 10-20 ml of

SPG using a brief sonication at a low power percentage, and freeze at -70°C in 100 ul

aliquots. The purified EBs are titred for infectivity (infectious units, IFU) in HeLa 96-well plate monolayers, and cultured for sterility on a blood-agar petri plate.

2.2. *Original Antigenic Sin Immunization and Bacterial Strains.*

Female BALB/c mice were obtained from Jackson Laboratories (Bar Harbour) at 5-6 weeks of age. *C. trachomatis* serovars used in these experiments include A, B, C, H, and K. Serovars C, A, H, and K belong to the C serogroup and serovar B to the B serogroup. Figure 2 shows the primary amino acid sequence of MOMP for each of these serovars in the variable domain I (green) and variable domain 4 regions (pink) (Yuan, Y., et al., 1989). The computer modelling of another bacterial porin, OMP F from E. coli, was performed using RASMOL modeling software freely available on the world wide web. The position of VDs 1 and 4 aligned with known exterior loops of the OMP F and are marked in colour for modelling purposes.

Twenty mice were inoculated intraperitoneally (IP) with 0.2 mL of an equal mixture of *C. trachomatis* EBs (containing 5×10^5 inclusion forming units [IFUs]) in SPG, and CFA (H37Ra, DIFCO). The mice received an identical inoculation of EBs in IFA 14 days later. In a separate experiment we had established the decline of anti-MOMP titers to be around 5-6 months after an initial inoculation as described above (data not shown). At 6 months, and prior to challenge, mice were bled and then challenged with homologous or heterologous EBs (5×10^5 IFUs IP in SPG [sucrose-phosphate-glycerol, pH 7.4]). Each group was composed of four mice and received either serovar C, A, K, H, or B. Sera was obtained seven days after challenge and 100

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ul of sera from each mouse was pooled for analysis according to group. Twenty naive mice were divided into groups of four and given 0.2 mL of an equal mixture of *C. trachomatis* EBs (containing 5×10^5 IFUs in SPG) and CFA. This was performed to measure the de novo IgG titre at 7 days in naive mice as a control for the recall antibody response in mice that received priming with serovar C. In all cases the 7 day serum IgG titre to C-VD1 in groups of naive mice immunized with each chlamydial serovar was less than 1/150 (data not shown).

2.3.   *Synthesis and cleaning of Pin-peptides*

Synthesis of support-coupled peptides Pin-peptides was performed on solid polyethylene rods by using a commercially available kit (Cambridge Research Biochemicals, Cambridge, United Kingdom) (Geysen, M.N., et al., 1987) as described previously (Zhong and Brunham, 1990a). Three blocks of peptides were used. The first set consisted of 8-mer peptides found in serovar C VD1, DVAGLQND. The second set consisted of 8-mer peptides based on the species conserved epitope found in VD4, TTLNPTIA. The third set of peptides represented the serovar C-VD1 peptide epitope VAGLQND, in which each residue was sequentially replaced with each of the 19 alternative amino acids. Derivatized F-moc amino acid residues (Cambridge Research Biochemicals and MilliGen) were activated in HObt (Aldrich). The quality of the pin-peptides were assessed by using monoclonal antibodies that mapped to the variable domains in a previous study (Zhong et al., 1994a), (data not shown). 94 identical copies of the pin were generated on each block along with the two control pins

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(GLAQ and PLAQ). Cleaning and regeneration of the pin-peptides was done in a sonic cleaner (Branson 3200) in 60°C SDS buffer made according to commercial instructions.

2.4. Micro immuno-fluorescence (MIF) Assay

The diagnostic micro immuno-fluorescent antibody (MIF) assay was performed in the laboratory of Dr. Rosanna Peeling (Laboratory Centre for Disease Control, Winnipeg) for the determination of serovar specificity and titre of IgG in mouse immune sera. Mouse sera were diluted 1:8 in PBS and assayed in 2-fold dilutions for IgG antibody against purified formalinized EBs of *C trachomatis* serovars A, B, C/J, F/G, H, K in the MIF assay (Wang and Grayston, 1974). All sera were titrated to end-point. Antibody titers were expressed as the reciprocal of serum dilutions yielding distinct fluorescence of *C. trachomatis* EBs.

2.5. Parameters of OAS Pin-Peptide ELISA

Serial dilutions of pooled anti-sera from each group of mice were tested on the immobilized peptides by enzyme linked immunosorbent assay (ELISA) as described earlier (Zhong, G., et al., 1994a).

The MOMP antibody response cross-reacts with synthetic peptide epitopes of the homologous MOMP structure based upon sequence relatedness. Our earlier experiments showed us that the development of antibodies to individual MOMP variable domain epitopes could be measured in polyclonal sera from rabbits (Zhong et

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al., 1990a, Zhong et al., 1990b), and mice (Guangming Zhong, Ph.D. thesis, University of Manitoba, Winnipeg, Manitoba, Canada). Representative serovars L2 and C , from the two major serogroups were used to prime groups of mice.

Results of the detection of peptide crossreactive antibody ELISAs were expressed in reciprocal values of the highest dilution with signal > 5 X over background optical density (O.D.). For the amino acid substitution analysis, a dilution of 1/800 of immune sera in 2% BSA in phosphate-buffered saline and tween-20 (0.05%) (SIGMA) was used for analysis of all sera except the B-challenged group and pre-challenge primed-sera, which was performed at 1:400, because of low, unboosted C-VD1 responses. The results were expressed in percentages of O.D. values relative to parental control peptides as detailed previously (Zhong, G., et al., 1994a). The solid phase peptides were reused after the rods were freed of bound antibody by sonication for 45 minutes at 60°C in a solution containing 1% sodium dodecyl sulfate, 0.1% 2-mercapto-ethanol, and 0.1 M sodium phosphate (pH 7.2).

2.6. Site Specific Murine mAb Production:

Naive, 3-4 week old BALB/c mice (Jackson Laboratories) were immunized i.p. with 10^6 IFU of highly purified serovar C EBs in an equal amount of Freund's complete adjuvant (H37Ra, DIFCO). On day 7, mice destined for the early hybridoma fusion received a boost with 0.5 X 10^6 IFU i.p. and 0.5 X 10^6 i.v. in PBS (Phosphate buffered saline, pH 7.2) 12 hours prior to their spleens being removed for fusion. Mice used for the mid-fusion were immunized i.p. with 10^6 IFU, did not receive a day

7 boost, but instead received a booster immunization with 0.5 X 10^6 IFU i.p. and 0.5 X 10^6 i.v. in PBS boost on day 20, 12 hours prior to fusion on day 21. Mice from the "Late" fusion were immunized on day 1, 28, and 150, with 1 X 10^6 IFU i.p. in IFA. Thirty days later, on day 180 the "Late" mice received 0.5 X 10^6 IFU i.p. and 0.5 X 10^6 i.v. in PBS and 4 days later their spleens were removed for fusion. The "Late" protocol was designed to have responding B cells undergo significant affinity maturation while B cells in the "Early" protocol would undergo minimal affinity selection (see Figure 5 for schedule). Hybrid myelomas were generated in a protocol modified from a previously published method (Lane, 1985). Spleen cells were gently washed out of the spleen with DMEM-0 (0 means containing no additives) using a 10 cc syringe and a 25 gauge needle (M. Ma, University of Maryland). Cells were filtered through a fine screen and washed three times successively by centrifugation and resuspending the cells in 37°C DMEM-0. All equipment and media were kept at 38°C during the entire procedure. Polyethylene glycol 1500 (Sigma) was used as a fusogen in the presence of DMSO (Sigma) and was added over one minute of gentle mixing. After a further minute of swirling the PEG was diluted out. Pre-warmed DMEM-0 (15 ml) was added over 5 minutes with constant swirling. The murine myeloma SP2/0-Ag14 (obtained from Dr. Mike Butler, Department of Microbiology, University of Manitoba) had been cultured continuously over several months prior to fusion. Sp2/0-Ag14 myeloma cell line makes no endogenous antibody polypeptides (Schulman et al., 1978). The cells were large and refractile when viewed under inverted microscope, and were fused at 1 myeloma cell for every 5 spleen cells. Hybridoma were selected by

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A) Immunization Schedule:
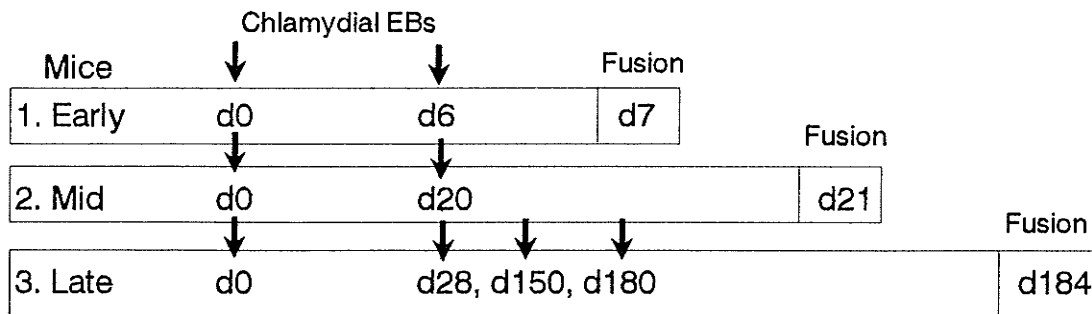
B) Paratope Hunting: An immunodominant epitope is used to identify mAbs.
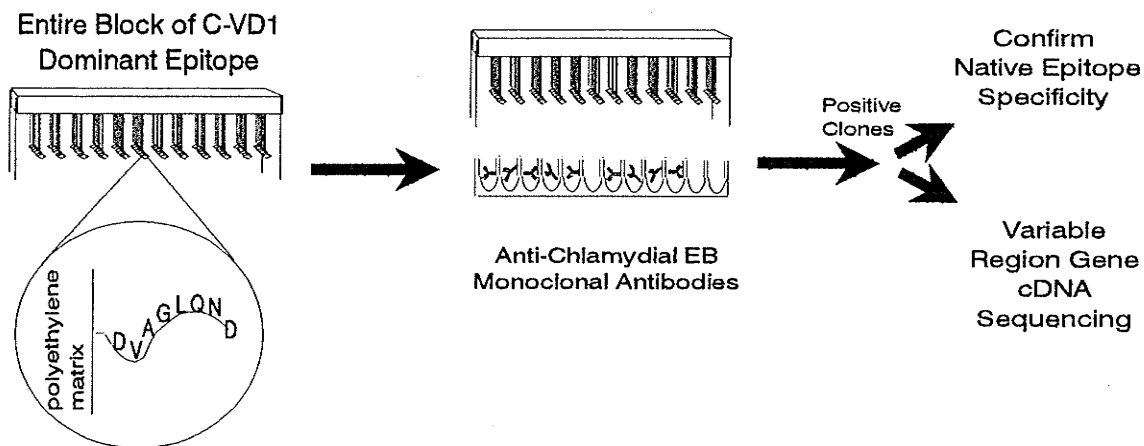
Figure 5: Immunization and screening for antibodies with a predetermined specificity to C-VD1. A) Mice (BALB/c, 3-4 weeks) were immunized with EBs in either an early, mid- or late fusion schedules. This was in order to generate antibodies from different stages of affinity maturation. Splenic B cells from Mice from the early schedule were fused to myeloma cells on day 7; from mid-shedule on day 21; and from the late schedule on day 150 or higher. B) Paratope Hunting: The use of pin-peptides as a primary screen for antibody specificity. After about two weeks of growth under HAT selection, hybridoma culture supernatants were screened in a pin-peptide ELISA by transferring neat supernatant into a 96 well ELISA plate. The supernatants were reacted with whole blocks of pin-peptides bearing the DVAGLQND peptide corresponding to VD-1 of serovar C MOMP. The wells that corresponded to the pins with IgG positive ELISA signals were subcloned and rescreened on whole EBs. Sequencing of the mAb variable region genes is required to determine the variable region genotypes used to bind to the epitope.

HAT (Sigma) in 96 well cell flat-bottomed culture plates (Corning) in DMEM
containing 20% fetal calf sera (Rehatuin, Intergen), and commercial OPI additive
(SIGMA) used according to manufacturers suggested concentration. Hybridized cells
were distributed to 10-12 96-well cell culture plates. They were grown at 37°c under
a 7% CO_2 atmosphere. Two spleens per time point were collected, pooled and fused.
All animals were used humanely in accordance with University and Federal
government animal handling regulations (Ref # 94-44).

a) *Screening for mAbs of a predetermined specificity:*

Two weeks after fusion, tissue culture fluid was screened for mAbs that
cross react with the pin-peptides DVAGLQND from serovar C immune splenocytes in
an indirect pin-peptide ELISA format as described previously (Zhong, et al., 1990b)
except as follows: The primary hybridoma screen is set up in an ELISA format by
transferring 150 ul of neat supernatant, after one to two weeks of growth under HAT
selection, directly to corresponding wells of a 96 well round bottom flex ELISA plates
(Falcon, cat# 3911). Pin-peptides, blocked with 4% BSA (SIGMA) in PBS, and then
reacted in the wells of the plate taking note of the orientation. Supernatants were
reacted on the pins for two hours at 37°C , or alternatively, 4°C overnight. The pins
were washed in three times in 500 ml PBS-Tween (0.05%) for 15 minutes each time
followed by a rinse with double distilled water. A goat anti-mouse IgG-horseradish
peroxidase conjugate (Pierce) was used as the secondary antibody [1:10000 in 2%
BSA in PBS-tween (0.05%)]. The pins were reacted for 1 hour at 37°C, and washed as
above. The colour is developed with 2,2-azino-bis-(3-ethyl-benz-thialozine-6-sulfonate)

in the dark and absorbance was read at 405 nm with a reference wavelength of 655 on an automated ELISA reader (BIORAD 3550). Wells corresponding to the positive pins were examined and expanded by limiting dilution at less than one cell per well in 96-well round bottom plates (Corning) (figure 5). Pin-peptides were regenerated by sonication in an SDS-bath at 60oC with 0.1% 2-mercaptoethanol. The pins have been reused repeatedly with no noticeble change in reactivity or sensitivity. Confirmation of native epitope specificity was done by standard immunological tests including immunoblot, radio-immunoprecipitation, and in vitro neutralization assay. Ascites was produced in BALB/c mice and the mAbs purified and isotyped as described below.

b)   Monoclonal Antibody Isotype Determination and purification:

The mAb supernatants were isotyped according to an antigen specific protocol which allowed for the use of whole chlamydial EBs as antigen, in a commercial murine mAb isotyping kit (Pierce, 37501). All were IgG subtypes with kappa light chains. Monoclonal antibodies were bulk precipitated with saturated $(NH_4)_2SO_4$, dialysed, and protein A / G column purified (Zhong et al., 1994a), followed by an additional overnight dialysis in PBS at 4°C to remove tris buffer.

c) Polyclonal Murine Antibody Preparation

Polyclonal anti-chlamydial antibody were prepared in hyperimmunized mice and used for positive controls by the polyclonal ascites method (Lacy and Voss, 1986). Briefly, pristane primed 8 week old BALB/c mice (The Jackson Laboratory, Maine)

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were injected with 10^6 ifu of serovar C or L2 i.p. in an equal volume of IFA. The

mice received identical boosts at two week intervals. The third and fourth boosts

consisted of 10^6 IFU, i.p. in an equal amount of PBS. Seven days after the fourth

boost mice were injected i.p. with 2.5 X 10^6 viable Sp2/0-Ag14 non-producing

myeloma cells. Ascites was collected within 1-2 weeks following injection of the

myeloma and purified on protein A/G columns. The eluted antibodies were dialysed

against two changes of PBS over two days at 4°C.

2.7.   *Immuno-dotblot, Immunoblot (western), and Radio-immunoprecipitation:*

The immunoblot assay for detecting mAb and immune sera binding capability

to membrane-immobilized protein from chlamydial serovar C EBs was carried out as

previously described (Zhang, et al., 1987) except for the followings: 2 X 10^5 IFU

serovar C EBs were dotted onto the nitrocellulose membrane in 50 ul SPG. The heat

treatment of EBs was carried out by heating the EBs at 56 $^{\circ}$C for 30 min. The

membrane was blocked with 4% bovine serum albumin and 0.05% Tween-20 in

PBS(PH7.4) (Blocking solution). Both mAbs and iodine conjugates were diluted with

2% bovine serum albumin and 0.05% Tween-20 in PBS (incubation solution). A

mixture of goat ant-mouse IgG conjugated with ^{125}I (Cat.# 68075, ICN Biochemicals,

CA) and ^{125}I-protein G (ICN Biochemicals, CA) was used to visualize the mAb

binding since we found that the goat anti-mouse IgG preparation preferably bound to

IgG1 isotype while the protein G preparation failed to bind to IgG1 isotype under the

experimental conditions that we used. The nitrocellulose membrane (Schleicher and

Schuell, cat# BA-S 83) was presoaked in sterile distilled water and the dot-blot

apparatus set up as directed (BIORAD BIODOT Apparatus). Whole chlamydial EB

cells were coated onto the nitro cellulose membrane wells at 1×10^4 IFU/well in SPG

(sucrose-phosphate-glutamic acid buffer [pH 7.4]) for two hours at room temperature.

The wells were rinsed with PBS-tween (0.05%) two times before blocking with

200ul/well of 4% BSA overnight at 4°C. Hybridoma culture supernatants containing

monoclonal antibodies were reacted for two hours at 37°C in the presence of 5% CO_2

to prevent gross pH change of the bicarbonate buffered media. Antibodies were

removed by vacuum, and wells washed four times with wash buffer (PBS-tween,

0.2%, [pH7.3]). Alternatively 100 ul/well of goat-anti-mouse-IgG- horseradish

peroxidase enzyme conjugate (above) was added at 1:5000 in 2% BSA-PBS followed

by Napthol color development. After reacting for 45 minutes at 37°C the second

antibody was removed and the membrane washed four times with wash buffer. The

membrane was removed from the apparatus and air dried for 10 minutes. Wrap the

membrane in saran wrap as it is still moist and we don't want to contaminate the X-ray

film case. Membranes were exposed for 1 hour at -70°C and developed.

For SDS-PAGE a 12% separating and a 4% stacking gel were used for

immunological visualization of antibody reactivity. Solubilized *Chlamydia trachomatis*

EBs protein antigens were transferred to nitrocellulose membranes using a BIO-RAD

mini-protean trans-blot transfer apparatus in the method of Towbin et al (1979). A

standardized amount of protein was loaded for each type of antigen mixture via the

Lowry method. Blotted membranes were blocked with 4% BSA for two hours at room

temperature prior to reaction for 1 hour at 37°C with 1:500 dilution of anti-serum in 2% BSA. Blots were developed as described previously (Zhong et al., 1994a).

Chlamydial EBs were harvested and purified following metabolic labelling with 35-S trans-methionine (ICN Biochemicals) and RIPs were performed as described previously (Zhong et al., 1994a).

2.8.   *In vitro Neutralization Assay:*

In 1992 a great deal of effort was spent in the attempt to standardize an in vitro chlamydial infectivity neutralization assay in the form of a workshop in Hamilton, Montana (Byrne et al., 1993). Representatives from many laboratories interested in the study of anti-chlamydial antibodies attended. Lessons learned from this workshop were translated back to our Winnipeg chlamydial lab by Dr. Guangming Zhong (University of Manitoba). The binding capability of mAbs to live EBs were assessed in a HAK cell line (Hamster kidney cells) *in vitro* neutralization assay as detailed at the international workshop described above (Su and Caldwell, 1991; Su et al., 1991). Briefly, neutralization assays are performed on HaK cell monolayers. HaK cells are grown to confluence in 175 cm2 tissue culture flasks in Dulbecco's modified Eagles media (DMEM, GIBCO) supplemented with 0.2% glucose, 0.75% Na bicarbonate, and 10% fetal calf serum (Rehatuin). Monolayers are washed with HBSS, and than rinsed with 4 ml of trypsin (O.1% in PBS). Another 4 ml of trypsin is added to the flask and incubated for 10 minutes at 37°C. Trypsinized cells are diluted to 10^6 viable cells /ml in medium (trypan blue exclusion principle), and 100 ul of cells was added to each

well of a 96 well cell culture plate (Corning). The cells are than incubated for 24 hours at 37°C in a 5% CO_2 overlay. Antibodies are assessed by preparing serial two-fold dilutions of purified IgGs in SPG. A mAb with unrelated specificity, or a preimmune sera, is similarly diluted and used as a control. An equal volume of EBs (EBs were titrated and adjusted to 2×10^4 IFU per ml in SPG) is mixed with an equal volume of antibody dilutions in a 96-well round bottom culture plate (Falcon) and incubated at 37°C for 30 minutes on a slowly rocking platform (Nutator) shaker. A 50 ul volume of the mixtures was then inoculated in triplicate onto 24-hour HAK cell monolayers in 96-well flat bottomed plates (Costar), and incubated at 37°C for 2 h. The inoculum is removed and the monolayers washed with 100 ul/ well of pre-warmed (37°C) HBSS. The cells are fed with 200ul of DMEM containing 1 ug of cyclohexamide per ml and incubated for 48 hours at 37°C in a 5% CO_2 overlay. After methanol fixation of the monolayers, chlamydial inclusions are detected by histochemical staining with a rabbit (different species than test mAb) anti-chlamydial serovar L1 and L2 polyclonal antisera (1:1000) as the first antibody, horseradish peroxidase conjugated goat anti-rabbit antibodies as the second antibody and 1-chloronaphthanol (Sigma Chemicals) as substrate. IFU are quantitated by counting 5 fields at a magnification of X200 using an inverted microscope. A mean IFU number per field was calculated from three plates, and the results are expressed as percent reduction in mean IFU compared with the control wells.

2.9.   Growth and Purification of Recombinant Filamentous Phage:

The procedures outlined below are essentially as described in a freely distributed laboratory manual prepared by Dr. George Smith (University of Missouri, Columbia). Cloned *E. coli* (K91) colonies containing C-MOMP VD1 monoclonal antibody selected recombinant phage-peptides (Zhong et al., 1994a) were picked and amplified in 200 ml of SB broth [(30 g tryptone (DIFCO) 20 g yeast extract, 10 g MOPS) per litre; pH 7.0] with 20 ug / ml tetracycline and 1 mM IPTG. The cultures were shaken overnight at 37°C. Bacteria were removed by a centrifugation step at 4000 RPM for 40 minutes (Beckman JA-10 rotor) at 4°C. Soluble phage particles were precipitated three times with 0.15 volumes of PEG/NaCl solution (polythylene glycol (8000 mW) 16.7%; NaCl 3.3 M) and centrifugation at 5000 RPM for 80 minutes at 4°C; the final centrifugation was in a sterile Oakridge tube at 12000 rpm (Beckman JA-20) for 40 minutes at 4°C. The final pellets were re-suspended in 2 ml TBS (50 mM tris base, 150 mN NaCl, pH 7.5) and stored at 4°C until used. Phage were titered in starved K91 cells in NAP buffer (80 mM NaCl, 50 mM $NH_4H_2PO_4$, pH 7.0) on SB-tet (100 ug/ml) plates. There was no growth by K91 or K91 kan bacterial strains on SB-tet plates in the absence of phage infection.

2.10.   EM Visualization of chlamydial EB and Phage Borne-peptide / antibody Interaction:

Commercial magnetite beads (10mg) precoated with polyclonal Goat anti-mouse IgG Fc-specific antibodies (BioMag, PerSeptive Diagnostics) were washed in

sterile phosphate bufferred saline (pH 7.2) and blocked in 10% dialysed BSA for 2 hours at room temperature. The blocked particles were next reacted with an excess of murine monoclonal antibody C1.8 ascites in a 2% BSA and PBS solution for 4 hours at 4°C followed by 40 minutes at 37°C. The beads were washed two times with 10ml of PBS-tween (0.05%) and separated each time using a cable magnet (Manitoba Telephone System).

Phage particles [1 X 10^9 transforming units (TU)]or chlamydial EBs [(1 X 10^7 infectious units (IFU)] were added to the antibody coated beads and reacted overnight at 4°C on a nutator shaker. The beads were washed as above and magnetically separated twice (as above) prior to direct visualization under electron microscope. The authors are aware that the electron dense iron of the beads makes for a less than optimal viewing substrate under EM due to high stigmatism (Dr. Paul Hazelton, University of Manitoba, Winnipeg), however the pictures were suitably clear (Zhong et al., 1997; data not shown).

2.11. *VD-1 Peptide Solid Phase Synthesis and Immunizations:*

In order to directly evaluate the immunogenicity of VD-1 peptide epitope without the interference of carriers, we created synthetic peptides analogous to the serovar C VD-1 of MOMP. We designed synthetic peptides to represent both a B cell neutralizing site and a conserved T cell epitope. An optimized VD-1 epitope ligand was previously determined to contain the flanking residues -DCL-VD1-WCW- (Zhong et al., 1994b). A conserved T-cell epitope, A-8, also found in the MOMP has been

identified as capable of presentation by multiple MHC class II alleles and has served as a promiscuous T-cell helper site in other vaccine constructs (Su and Caldwell, 1992). Using the sequence information derived from the filamentous phage system and from T cell mapping studies, synthetic oligopeptides containing both the serovar C VD-1 MOMP B-cell epitope and the A-8 MOMP T-cell epitope were synthesized (figure 6).

The general structure of these peptides from a vaccine perspective are:

NH3 - promiscuous T cell epitope- B cell neutralization epitope - COOH

The oligopeptides have a polylysine tail (3 residues) at their N-terminii improve their solubility. Residues 3-28 contain the A-8 TH epitope that has been characterised by Dr. H. Caldwell (Su et al., 1990, Su and Caldwell, 1992). In addition to promiscuity in multiple murine MHC alles, A-8 was chosen because it also functions as a TH epitope in non-human primates (Dr. H. Caldwell, unpublished data). The A-8 peptide represents a sequence conserved region of MOMP and is found in the MOMP of all known *C. trachomatis* serovars. The A-8 T cell epitope was linked to either a linear (B3-red, B-6) or looped (B3-ox) VD1 epitope. Three complex oligopeptides were sucessfully synthesized that are 47 amino acids in length. Peptides were generated with an N-terminal polylysine tail (3 residues) for improved solubility, and purified using high pressure liquid chromatography. Soluble peptides were prepared and purified by Dr. Bob Hodges (Canadian Bacterial Diseases Network, University of Alberta, Edmonton, Alberta).

B3-PEPTIDE (OXIDIZED, LOOPED)

KKKALNIWDRFDVFATLGATTGYLKGNSDC̲LS^D

```
KKKALNIWDRFDVFATLGATTGYLKGNSDCLS D V
                                 I        A
                              W C           G
                                W             L
                                N             Q
                                  T T  D    N
                                      P
```

B3-PEPTIDE (REDUCED, LINEAR)

KKKALNIWDRFDVFATLGATTGYLKGNSD̲C̲L̲SDVAGLQNDPTTNW̲C̲W̲

B6-PEPTIDE (LINEAR CONTROL, CYS to ALA SUBSTITUTION)

KKKALNIWDRFDVFATLGATTGYLKGNSD̲A̲L̲SDVAGLQNDPTTNW̲C̲W̲

Figure 6: Oligopeptide constructs structures. Peptides were synthesized and HPLC purified for conformation by Dr. Bob Hodges (University of Alberta, Edmonton). The N-terminal triple lysines were added for improved solubility. The chlamydial MOMP sequence that encodes the C-VD1 linear epitope is shown in blue text. Residues 4 to 28 contain the A-8 T-helper epitope that has been characterised by Dr. H. Caldwell (Su et al., 1990; Su and Caldwell, 1992).

2.12. Standard ELISA and mAb Avidity Measurements:

Microtiter plates (CORNING, modified flat bottom) were coated overnight at 4 °C with 1 X 10^6 IFU of native serovar C EBs , 1 ug/ml *Mycobacterium tuberculosis* (DIFCO, H37Ra), 1 X 10^6 *Pseudomonas aeruginosa* (A gift from Dr. James Karlowsky, University of Manitoba) in 100 ul of SPG or 0.1 ug/well of peptides in 100 ul of PBS. The plates were washed with 200ul of SPG or PBS once and blocked with 200ul/well of a 4% BSA in PBS blocking solution at 37°C for 90 min. Serial dilutions of antibody preparations were made in solution and added to the plates with 100ul/well. The plates were incubated at 37°C for 60 minutes or overnight at 4^0C. After washing four times with 200 ul/well of wash solution (0.05% Tween-20 in PBS, PH 7.4), a goat anti-mouse IgG-peroxidase conjugate (Pierce) at 1:3000 dilution in 2% BSA-PBS solution was added with 100 ul/well and the plates incubated as before. After washing as before, 100 ul of substrate [2.2-azino-bis-(3-ethyl-benzthiazoline-6 sulfonate) in citrate buffer (pH 4.5) in the presence of H_2O_2] was added to each well. The enzymatic reaction was developed for 15 minutes in the dark at room temperature and the absorbance was measured at 405 nm.

The relative binding affinity or avidity of the mAbs were estimated using whole EB ELISA. The mABs were serially diluted from a known starting concentration and reacted on whole EBs as described. The relative affinities were defined as the consentration of antibody (ug mAb/ml) required to yield an optical density (OD) value of 0.5 at 405 nm in whole EB ELISA. Similar measurements of affinity have been used to estimate antibody avidity to Tula virus (Lundkvist et al.,

1996).

2.13. Complementary DNA (cDNA) and Cycle Sequencing Reagents:

Superscript II reverse transcriptase, Taq enzyme, TdT enzyme, and dNTPS were purchased from GIBCO BRL. Oligo-dt primers were obtained from Boehringer Mannheim. PRIME-RNase inhibitor was purchased from 5-PRIME to 3-PRIME (Boulder, CO).

2.14. Oligonucleotide Primer Design:

In order to determine the nucleotide sequence of the immunoglobulin variable region genes we designed and used oligonucleotide PCR primers based on the sequences in the database of Kabat et al, (1991). Leader region primers were constructed from information available on the murine heavy and light chain leader region codon sequences. The RACE generic 5' primers, Q_1, Q_o, and Q_N, were modified from Frohman (1994). Primers MHcL4 (murine heavy chain leader-4), and MKL4 (murine kappa leader-4) were modified from published oligonucleotides MH-SP-ALT.1, and IgKVL5'-B respectively (Coloma et al., 1991; Lan et al., 1996). Isotype specific antisense primer sequences, and MHC1 and 9 were obtained from Barbas and Burton (1994). MVL (Zhong et al., 1994) was modified from Huse et al (1989). Primers MVL and MHC (1 & 9) anneal to the FR-1 of kappa light chain and heavy chain v-regions respectively. Oligonucleotide primers that correspond to the aberrantly rearranged endogenous SP2/0-Ag14 gene sequence were used to survey the hybridoma

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clones for co-expression of the abberrant transcript. No specific amplification was seen suggesting very few if any aberrant transcripts were present. Additionally, when added to PCR reaction mix containing primers for amplification of full length light chains, no reduction in full length signal was seen (data not shown). If the aberrant chain primer was annealing internal to the outer pair a reduction in signal would be expected, even if truncated products were not visible.

Primers are listed in Table 3 and their respective annealling sites are shown in the accompanying figure 7. Oligonucleotides were either made on a BECKMAN OLIGO 1000 DNA synthesizer or ordered through commercial sources (GIBCO, Custom Primers). They were removed from the column as suggested by the manufacturer, and no additional purification was performed prior to use.

2.15. Total RNA purification, cDNA Preparation, and Homopolymer Tailing of CDNA:

For each hybridoma cell line 1 X 10^6 logarithmic phase, and for whole spleens 1 X 10^7 viable cells (trypan blue exclusion principle) were collected, washed, than lysed (in 0.5 ml of 4 M guanidium isothiocyanate, 1% beta-mercaptoethanol, pH 7.0). Total RNA was isolated using RNeasy total RNA kit (QIAGEN). About 20 ug of total RNA was used for cDNA production for each cell line. Briefly RNA, oligo p(dT)15 (0.8 ug), and water (to total of 41 ul), were combined and heated at 70°C for 10 minutes than transferred briefly to ice, than to 37°C for 10 minutes. Next, 8 ul of the

Table 3
Oligonucleotide Primers

#	Name	Sequence	Use
1	IgG1N	5'-AGG CTT ACT AGT ACA ATC CCT GGG CAC ATT-3'	RACE-1; IgG1 cDNA
2	IgG2a/b	5'-GTT CTG ACT AGT GGG CAC TCT GGG CTC-3'	RACE-1; IgG2a/b cDNA
3	IgG3	5'-GGG GGT ACT AGT CTT GGG TAT TCT AGG CTC-3'	RACE-1; IgG3 cDNA
4	IgKappa	5'-GCG CCG TCT AGA ATT AAC ACT CAT CCT GTT TGA A-3'	RACE-1; IgKappa cDNA
5	QG .	5'-ATG AGT CCA GCA ATG ATT ATG TCA AGC GGG GGG GGG GGG GGG GG-3'	RACE-1
6	QO	5'-ATG AGT CCA GCA ATG ATT ATG TCA AGC-3'	RACE-1
7	Q1	5'-ATG ATT ATG TCA AGC GGG GGG G-3'	RACE-2
8	MHG3	5'-GAC CAA GGG ATA GAC-3'	RACE-2; IgG3 cDNA [a]
9	MH125	5'-GGC CAG TGG ATA GAC-3'	RACE-2; IgG1,2a/b cDNA [a]
10	MKC1	5'-GGA TAC AGT TGG TGC AGC-3'	RACE-2; IgKappa cDNA [a]
11	MHcL-1	5'-ATG GAC TT(GCT) G(GAT)A (CT)TG AGC T-3'	IgH cDNA [a]
12	MHcL-2	5'-ATG GAA TGG A(GC)C TGG (GA)TC TTT CTC T-3'	IgH cDNA [a]
13	MHcL-3	5'-ATG AAA GTG TTG AGT CTG TTG TAC CTG-3'	IgH cDNA [a]
14	MHcL-4	5'-ATG (GA)A(GC) TT(GC) (TG)GG (TC)T(AC) A(AG)C T(TG)G (GA)TT-3'	IgH cDNA [a]
15	MKcL-1	5'-ATG AAG TTG CCT GTT AGG CTG T-3'	IgKappa cDNA [a]
16	MKcL-2	5'-ATG GAC TTT CAG GTG CAG ATC T-3'	IgKappa cDNA [a]
17	MKcL-3	5'-TTG CTG TTC TGG GTA TCT GGT A-3'	IgKappa cDNA [a]
18	MKcL-4	5'-ATG GAG ACA GAC ACA CTC CTG CTA T-3'	IgKappa cDNA [a]
19	MHC1	5'-AGG TCC AGC TGC TCG AGT CTG G-3'	IgG cDNA [a]
20	MHC9	5'-AGG TII AIC TIC TCG AGT C(TA)G G-3'	IgG cDNA
21	MVL	5'-GTG CCA GAT GTG AGC TCG TGA TGA CCC AGT CTC CA-3	IgKappa cDNA
22	mIL2-5'	5'-GCA CCC ACT TCA AGC TCC ACT TCA AGC TCT-3'	mIL-2 cDNA
23	mIL2-3'	5'-TTA TTG AGG GCT TGT TGA GAT GAT GCT TTG-3'	mIL-2 cDNA
24	MG1-3Seq	5'-AGA TGG GGG TGT CGT TTT GGC-3'	IgG1 cDNA [s]
25	MG2a/b-3Seq	5'-GAC (T/C)GA TGG GG(C/G) TGT TGT TTT GGC-3'	IgG2a/b cDNA [s]
26	MK-3Seq	5'-TAC AGT TGG TGC AGC ATC AGC-3'	IgKappa-cDNA [s]

[a] Amplification and Sequencing
[s] Sequencing

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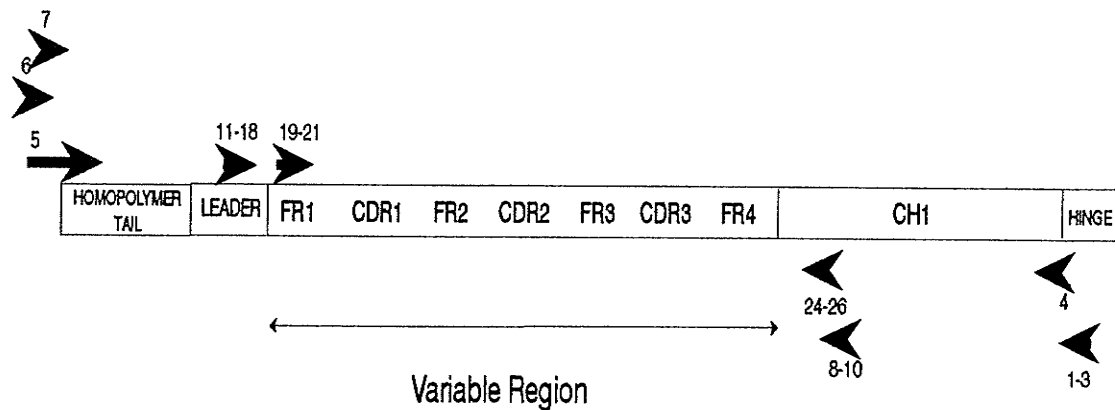
Figure 7: Graphical representation of the oligonucleotide priming sites on a representative immunoglobulin cDNA. The numbers correspond to primers listed in table 3. Primer 4 (Barbas and Burton, 1994) is the kappa light chain constant region reverse primer and is shown on the same cDNA for completeness.

following master mix was added: 4 ul of 5 X reverse transcriptase buffer, 2 ul 0.1mM

DTT, 1ul 10 mM dNTP stock, 1ul PRIME RNA inhibitor was mixed per sample. The

reaction was performed at 37°C for 60 minutes after the addition of 1ul (200 units) of

Superscript RT/ tube, followed by 90°C for 5 minutes. RNASE-H (PROMEGA) 1ul

(1.5 units) and RNASE-A (SIGMA), 0.2ul, (1.25 units or 2.5ug) were added to

destroy RNA by incubation at 37°C for an additional 20 minutes. An aliqout of cDNA

was purified (to remove dNTPs, NTPs, and oligo-dt) on a sephadex TE-MIDI SELECT

D-50 spin columns (5' to 3', Boulder, CO) which retains oligonucleotides <72 bp in

length. The sample was recovered and precipitated overnight at -20°C, washed once

in 70% ETOH, and resuspended in 10ul of 1X TE (tris-edta) buffer. Allele specific

PCR was performed directly upon splenic cDNA at this point (without further

manipulation) for the variable region gene analysis by run-off blot (see Results,

Objective 3).

Oligo d(C) homopolymer tails were added to the 3' end of aliqouts of purified

first strand cDNA as described briefly (Frohman et al., 1988; Frohman, 1994). We

adapted the method of Frohman for the addition of poly-C tails as follows: The 20ul

C-tailing reaction volume consisted of 10 ul of cleaned up cDNA; 0.7 ul of dCTP (100

mM, GIBCO); 1ul TDT enzyme (15 units); 4 ul of the 5 X TdT cacodylate buffer

(GIBCO); and 4.3 ul ddH$_2$0. We used an approximate value of 1 ug of cDNA (even

though we started out with <0.6ug amount of mRNA) in our calculations as 1 ug

provides 3.125 pmol of "ends" (assuming about 1200bp total length of cDNA and the

average molecular weight of a dNTP is about 320 daltons). We realize that the starting concentrations of RNA were likely far from optimal, however they were more than adequate for our purposes. Commercial instructions suggested 20-25 pmol of the dNTP species to be added per pmol of cDNA "ends". Thus we used 22.5 X 3.125= 70 pmol dCTP, or 0.7 ul of a 100mM dCTP per tailing reaction. The mixtures were incubated at 37°C for 30 minutes, heated to 65°C for 5 minutes to inactivate the TdT enzyme, and diluted to a final volume of 300 ul with 1X TE buffer. Murine IL-2 cDNA was created from RNA harvested from phorbol myristic acid stimulated EL4-IL2 mouse thymoma cells (ATCC #TIB 181) and was used as a control PCR amplification due to its known expression of IL-2 (Ma, M. et al., 1984). IL-2 mRNA produced a specific band of approximately 450bp. Several different amplification protocols were used in these experiments. Specific modifications are discussed in the Results section.

The first amplification round of RACE (designated hereafter as RACE-1) was performed as follows. Sephadex G-50 column purified, and 3'-poly-C tailed cDNA (1 ul) was added to 90 ul of master PCR mix (see above). Upstream primers, Q_o/Q_G were added at a volume of 0.5 ul of Q_o (60 pmol) and 0.25 ul of Q_G(40 pmol) respectively. RACE-1 began with three cycles of 96°C for 20 seconds, a annealing step of 60°C for 2 minutes, and an extension step at 72°C for 5 minutes. The next 20 cycles were as follows: A denaturation step at 96°C for 20 seconds, an annealing step at 58°C for 2 minutes, an extension at 72°C for 2 minutes. The final 20 cycles of RACE-1 involves two-step temperature cycling from 96°C for 20 seconds, to 60°C for 2

minutes. Again, a final extension at 72°C for 15 minutes was added for complete extension. RACE-1 products were analysed on agarose gels and diluted accordingly.

The second round of RACE amplification (designated hereafter as RACE-2) was performed on appropriately diluted RACE-1 products (unpurified). RACE-2 amplification was performed utilizing RACE-1 amplified cDNA with 5' primer Q_1 and 3' nested isotype specific primers (MH125 for IgG_1 and $IgG_{2a,b}$; MHG3 for IgG_3, and; MKC1 for Kappa light chains). Briefly, 1ul of diluted RACE-1 product was mixed with 90 ul of master PCR mix (above), 0.5 ul Q1 primer (70 pmol), 60-100 pmol of the corresponding nested 3' antisense primer . Water was added according to a volume calculated to give 100ul after Hot start addition of approximately 2.5 units of Taq polymerase (GIBCO). RACE-2 began with 35 cycles of denaturation at 96°C for 25 seconds, annealing at 48-55°C for 2 minutes, followed by extension at 72°C for 1 minute.

2.16. *Immunoglobulin Leader region primer PCR amplification*

Unpurified RACE-1 amplified products were diluted and used as template for leader PCR. Analysis of 20 ul of RACE-1 product on a 2% agarose analytical gel determined the dilution of template for leader PCR according to the following criteria: If there was no specific band visible at 400-600 bp, the RACE-1 procut was diluted 1/20 in dH2O. If a weak but specific band was observed, the product was diluted 1/200 in dH2O. If a strong and specific band was observed, the products were diluted 1/800 in dH2O. For leader PCR 1 ul of these diluted products were used as template. A

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standard cycling protocol for cDNA amplification was used to compare the efficacy of raw, purified, and RACE-1 amplified cDNA. It was determined that a single round of RACE amplification followed by leader primer PCR with a nested isotype specific oligonucleotide was most efficient for cDNA amplification (data not shown). This protocol was used for the sequence determination of the RACE-1 preamplified templates.

The leader PCR protocols were set up as follows: A master PCR mix consisting of 100 ul 10 X PCR Buffer (No Mg, GIBCO), 8 ul dNTPs (25 mM each, GIBCO), 30 ul of 50 mM $MgCl_2$, 764.5 ul of double distilled water was used for PCR amplifications. Template cDNA (1 ul of appropriately diluted samples) were added to 90 ul of a master PCR mix with 60-100 pmol of each primer (one leader primer, numbers 11-18 (Table 3), with a respective isotype specific 3'-primer; MKC1 for kappa light chains; MH125 for IgG1, 2a/b; MHG3 for IgG3) in a total volume of 2 ul, and water was added to a calculated total of 100ul. Samples were vortex mixed and overlayed with 2 drops of white mineral oil (Mallinckrodt) before a final brief spin and hot start with approximately 2.5 units of Taq polymerase. All samples were amplified for 35 cycles. The cycles consisted of a 30 second denaturation step at 96°C, a 2 minute annealing step at 51-55°C, and a 2 minute extension step at 72°C. All amplification protocols were completed with a final extension for 10 minutes at 72°C. Following completion of PCR 20 ul of each sample was added to 4 ul of 6 X sample buffer (Maniatis,) and run on a 2% agarose gel containing) 0.001% ethidium bromide for 1 hour at 100 volts. Gels were visualized and photographed under U.V. light. In

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all cases, RACE-1 preamplified template produced a good amplification for all 14 VH
and Vl cDNAs, with at least one of the leader primers. Thus RACE preamplification
improved the ability of leader primers to amplify monoclonal cDNA.

2.17. cDNA sequence Determination

The amplified immunoglobulin cDNAs were sequenced directly with a
commercial cycle sequencing kit (GIBCO BRL, Missisauga, On, Canada). Two
independent reverse transcribed samples for each clone were sequenced. Recalcitrant
cDNAs were cloned into the PCR 2.1 TA vector (Invitrogen) and sequenced with
primers annealing to the vector. In all cases more than two clones were sequenced to
produce a consensus sequence. Briefly, on a conventional sequencing apparatus, using
4-6% polyacrylamide gels containing 6 M urea. Oligonucleotide primers were labelled
with 32 or 33-P gamma labelled ATP and were not purified prior to use. The gels
were dried onto filter papers for 2-3 hours at 80OC under constant vacuum. The dried
gels were exposed on KODAK XOMAT film for 24-72 hours depending on the signal
intensity. The sequences were analysed on the Kabat antibody databan and the
Immunogenetics database which are freely available databases on the internet. There
were some inherent limitations to the RACE method as not all of the products
produced readable sequencing products despite good amplification of correct sized
products. Possible explanations include internal homology with sequencing primers,
sequence specific secondary structure and PCR jumpimg which can produce artifacts.
The most reliable method was cloning.

2.18. PCR Run-off Reactions for Variable Gene Expression

Twelve, six-week old female BALB/c mice (Jackson laboratories, Bar Harbour, Maine) were immunized intraperitoneally with 5 X 10⁵ IFUs of *C. trachomatis* serovar C EBs in incomplete Freund's adjuvant (DIFCO) in a total volume of 200 ul per mouse. A group of 5 mice recieved 200 ul of Complete Freund's adjuvant (H37 Ra; DIFCO) intraperitoneally without other antigens (containing *M. tuberculosis* antigens). Another group of 3 mice recieved 5 X 10⁵ colony forming units of whole viable *Pseudomonas aeruginosa* bacterium, intraperitoneally, in IFA to a total of 200 ul per mouse. (The *P. aeruginosa* was a cloned mucoid clinical isolate donated by Dr. James Karlowsky, University of Manitoba, Canada). A total of 6 naive mice were bled and sacrificed, and the spleens removed for RNA isolation. All immunized mice were tail bled on day 24 and sacrificed. The spleens were removed for RNA isolation. Total RNA was isolated using the RNeasy® kit (as outlined above) from the same number of viable cells (5 X 10⁶) per mouse spleen. Complementary DNA was prepared as outlined above. Immunoglobulin V-gene allele specific PCR and the linear run-off blot was performed as follows: For each reaction, 1 ul of cDNA was combined with 90 ul of a PCR master mix (as described above), 60 pmol of a 5' allele specific framework 3 (VH6-like, designed from the VH of hybridoma clone 4B6, ACA TTG ACT GTA GAC AAG TCC TCC AG; VH5-like, designed from the consensus of the VH genes of hybridoma clones C1.1-C1.6, CGA TTC ACC ATC TCC AGA GAC/G AAT G) or framework-1 primer (UmIgVH, a pan heavy chain variable region primer, TGA GGT GCA GCT GGA GGA GTC; UmIgVk, a pan kappa light chain primer, GAC ATT

CTG ATG ACC CAG TCT; Vk21-like, designed based upon the Vk21 light chain sequences of the early and late hybridoma clones, GAC ATT GTG CTG ACA CAG TCT CCT GCT TCC), 60 pmol of a 3' isotype specific primer (IgG3', GTG GAT CCT GCA TTT GAA CTC CTT G; Ig-kappa-3', GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A), and water to a calculated total of 100 ul following hot start addition of 2.5 U of Taq polymerase. The UmIgVH and UmIgVk primers were made based upon the design of Dattamundjar et al (1996), and the IgG-3' was based upon the identical primer used by DeLassus et al. (1995). Thus five individual sets of PCR amplifications were performed upon the cDNA of each mouse. All of the PCR reactions produced bands of the correct size representing Ig V-gene cDNAs (807 bp for the heavy chain cDNA produced with framework 3 primers and the IgG-3' primer that anneals to the 3' end of CH2 of all IgG isotypes; 600bp for the light chain cDNA with framework-1 primers and the Ig-kappa-3' primer that anneals to the 3' end of CH1) (Appendix 2). This confirmed the success of the RNA isolation and cDNA production for all mice, and that the PCR products all contained amplified V-gene cDNAs (albeit of unknown assemblage), prior to J-region run off assay. Excess primers and dNTPs were removed with TE-Midi G50 sephadex spin columns (5 Prime to 3 Prime, Boulder, CO, USA) prior to run-off. The Run-off reactions utilize only a single primer in a one-way polymerization, to create 5'-biotinylated, single stranded, anti-sense DNA copies of the V-gene. The Run-off assay is designed to only detect the products of this secondary run-off step. The Run-off reaction was performed as follows: 2 ul (1/50th) of the cleaned-up amplified product then subjected to a Run-off

elongation (Pannetier et al., 1993). The product was combined with 100 pmols of 5'

biotinylated J-region primer (JH2-biotin, Biotin-GG AGA CTG TGA GAG TGG TG;

or Jk2-biotin, Biotin-GTC CCC CCT CCG AAC GTG T; GIBCO CUSTOM

PRIMERS, Baltimore, ML, USA), 90 ul of PCR master mix (as above), and water to

a calculated total volume of 100 ul following hot-start addition of 2.5 U of Taq

polymerase. Primer extension started with a denaturation step of 1 min at $96^{\circ}C$,

followed by 20 cycles of 30 s at $96^{\circ}C$, 30 s at $60^{\circ}C$ and 25 s at $72^{\circ}C$, and finally a 4

minute step at $72^{\circ}C$. 20 ul of the products of the elongation were size separated on

mini-gel polyacrylamide gels (BIORAD) using gels with sequencing gel composition.

The gels were cast as follows: (The following mixture made enough gel for 2 X 7%

separating gels.) 6.4 ml of dH2O was combined with 1 ml of 10 X TBE (tris-borate-

edta, pH 8.4; Maniatis), 2.5 ml of 30% polyacrylamide/bis-acrylamide (19:1 ratio), 50

ul of 10% ammonium persulfate (Anachemia), and 5 ul TEMED (Mallinckrodt). Once

poured the gels were overlayed with water-saturated butanol until firmly cross-linked.

A small stacking gel was added to focus the DNA and consisted of (for two gels): 6.6

ml of dH2O, 2 ml of 5 X TBE (tris-borate-EDTA, pH 6.2), 1.4 ml of 30 % acrylamide

/bis (19:1), 60 ul of ammonium persulfate, and 10 ul of TEMED. The combs were

added until just before sample loading. A 1 X TBE buffer was used for a running

buffer. The gels were run for 1 hour and 15 minutes, or 2 hours, for heavy and light

chain Run-offs respectively (both at 125 volts). This is because light chain Run-off

products span the Fr-1 to Jk region and are much larger than the heavy chain products

which essentially only span CDR3 (~300 and ~170 bp respectively). Gels were NaOH

denatured and transferred to nylon membrane as described below (Southern transfer of DNA).

Blotted membranes were blocked with 4% BSA in PBS for 2 hours at room temperature. The membranes were rinsed 1 X in dH2O and incubated with avidin conjugated horseradish peroxidase (Pierce) at 1/3000 in 4% BSA-PBS for 1 hour at room temperature. The membranes were washed 3 X gently in 500 ml of PBS-Tween 20 (0.2%) with shaking, for 10 minutes each wash (Do not overwash). The membranes were agains rinsed 1 X in dH2O. Color develoment was carried out for 15 minutes in a solution containing 5 ml of 1% 2-chloronapthol (FISHER), 15 ml of dH20, and 10 ul of hydrogen peroxide. We determined that the linear Run-off reaction, following 20 rounds of elongation, had a maximum sensitivity of 0.05 ng of starting material using a monoclonal cDNA template, which represents a 20 fold improvement over EtBr staining (data not shown).

For the evaluation of the effects of peptide immunization on variable region gene expression levels, the identical PCR amplification and Run-off procedures was performed as above and was run in tandem. RNA and sera were collected 24 days following immunization of female BALB/c mice (6weeks). Three groups of 8 mice received 25 ug of peptide per mouse emulsified in 200 ul of IFA. The peptides were B3-ox, B3-red (VD1 looped or linear peptides, see figure 6) or the similarly sized linear control peptide 0921 (NH3 -ATKKEVPLGVAADANKLGEIEALKAEIEALK AGGDEQFIPKGGEIEALKAEIEALKA-COOH; courtesy of Dr. Bob Hodges, University of Alberta, Edmonton, Canada). The synthesis and initial immunochemical

characterization of these peptides has already been described in detail (Appendix 1).

2.20. Southern Transfer of DNA

PCR amplified cDNAs were vertically transferred from 1% agarose gels or 7% polyacrylamide mini-gels to nylon membranes by capillary blotting or southern transfer. The gels were initally soaked for 20 minutes in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) in order to create single stranded DNAs with improved binding to the nylon of the membrane (Hybond N+, Amersham). The gels were neutralized in neutralization buffer (1.5 M NaCl, 0.5 M tris-HCl, pH 7.0) for 20 minutes. The transfer apparatus was set up using the southern wick stand and a piece of 3M Whatman filter paper cut to size. Nylon membranes were cut to the sizeof the gels and wet in 10 X SSC (1.5 M NaCl, 0.15 M Na-citrate, pH 9). Membranes were placed on top of the gels using excess buffer to remove air pockets. Absorbent paper towels were cut to size and piled up on top of the nylon membrane to facillitate capilary action.Transfers were carried out for 18-20 hours. Following this the nylon membrane was rinsed in distilled H2O and denatured in 0.4 M NaOH for 30 s, followed by a neutralization step for five minutes in 10 ml of 0.2 M tris-HCl, pH 7.5. Single stranded cDNA was covalently bound to the membranes by baking at 80°C for 30 minutes. Prehybridizations and hybridization reactions were performed at Tm-4°C, and wash steps were performed at Tm-2°C. Oligonucleotide probes were labelled using a commercial kit (T4 polynucleotide Kinase, GIBCO-BRL). Hybridization was performed according to standard protocols (Maniatis, 1988) using a hybridization oven (VWR).

III. RESULTS

OBJECTIVE 1:   *Characterization of the Balb/c Polyclonal Antibody Response*

(I) Rationale

We initiated serological studies of the *C. trachomatis* secondary B cell response

in Balb/c mice in order to guage the breadth of the B cell compartment that responds to

the VD-1 epitope of MOMP. The *C. trachomatis* MOMP is ideal for the study of

crossreactive memory B cell responses as the different serovars are natural antigenic

variants. Wang and Grayston initially recognized that reinfection with a *C. trachomatis*

serological variant (serovar), different from the original infecting serovar, recalled

antibodies to the priming serovar, a phenomenom termed original antigenic sin (OAS)

(Wang and Grayston, 1970; 1982; 1984). The OAS was initially observed in sero-

epidemiologic studies of influenza A virus infection (Davenport et al., 1953; Fazekas de

St. Groth and Webster, 1966), and is believed to be a common feature of infectious

agents capable of antigenic variation and that thereby confer only strain specific

immunity.

Secondary infection or immunization rapidly recalls B cells from the memory

pool (Wright et a., 1983; Quiding et al., 1991). Memory responses are of higher

magnitude, longer duration and can occur very rapidly (Janeway and Travers, 1996). This

is due to several important properties of memory B cells. Memory B cells have

successfully undergone affinity maturation and antigen selection in a primary response

prior to their recruitment into the memory compartment (Ahmed and Gray, 1996). The

response of memory B cells dominates over the response of naive B cells (Brown et al.,

1980) mainly due to the differential activation of memory B cells mediated via the the cγRII-B1 receptor (IgG receptor) on the B cell surface. This ensures that co-engagement of the Ig receptor and the Fc receptor on a naive B cell, but not a memory B cell, leads to an inactivation signal (Janeway and Travers, 1996). Thus secondary antibody responses are more amenable to study due to the recall of high titre IgG.

Memory B cells can be recalled by structurally variant epitopes. In fact memory B cells, and not naive B cells, are specifically activated by a structural haptenic analogue of a priming antigen (Eisen et al., 1969; Fish et al., 1989; 1991). Boosting antigens recall only a small fraction of the B cell population that participated in the priming response (Fish et al., 1989), suggesting that affinity maturation selects from a diverse set of B cells mobilized during the primary response and matures them into the memory compartment. Thus the primary B cell repertoire to a single antigen is diversified through somatic mutation and antigen selection creating a pool of highly responsive memory B cells, some of which may have acquired improved reactivity for structural antigenic variants (Fish et al., 1991).

OAS responses classically produce cross-reactive antibodies which have higher avidity for the priming strain antigen than to the related recall antigen (Janeway and Travers, 1996). Serological studies have revealed that the portion of B cells that respond in an OAS response are those cells which recognize antigens common to both the priming and boosting strains of influenza A (Janeway and Travers, 1996). Consistent with this, molecular analysis of OAS monoclonal antibodies have demonstrated that boosting with a structurally variant antigen recalls only a portion of the B cell population that

participated in the initial B cell response (Fish et al., 1989). These heterologous monoclonal antibodies have unique antibody specificities which are not predominant in homologous immunization (Suga et al., 1995). The emergence of these rare specificities in secondary responses implicates the existence of a large and heterogeneous pool of antigen specific memory B cells with a spectrum of reactivities. For it is from this heterogeneous pool that cross-reactive antibodies are rapidly recalled after boosting with structurally related antigens. Similarly, we postulated that an OAS antibody response to an individual chlamydial epitope would thus demonstrate the existence of a spectrum of epitope specific B cell binding specificities.

We used polyclonal antibody binding patterns as a surrogate measure for individual B cell receptor specificity. The polyclonal antibody recall response was compared, pre- and post-boost, in mice primed with serovar C EBs but boosted with either serovar C or other chlamydial serovars. Dominant binding characteristics of a polyclonal antisera reflect the pooled binding characteristics of the multiple B cells that best recognize the immunizing antigen. We assume, based upon our previous observations of B cell pleiotrophy in binding to an epitope found in VD-1 of MOMP (Zhong et al., 1994a), that the immunochemical characteristics of polyclonal antibody binding are predictive of differential activation of different B cell clones. Thus, the polyclonal antibody binding patterns are a measurable proxy for individual B cell receptor specificity activated in the secondary antibody response. We predicted that an OAS antibody response to the VD-1 epitope would be accompanied by broadened serological reactivity in a secondary response of identically primed and heterogously

boosted mice, as cross-reactive antibody specificities would be enriched in the high titre circulating antibody population produced in the resultant memory response. Furthermore, we hypothesized that phenotypic differences in OAS binding specificities would suggest the existence of genetic diversity in the Ab response. We compared the polyclonal antibody recall response, pre- and post-boost, in mice primed with serovar C EBs but boosted with either serovar C or other chlamydial serovars.

1. Kinetics of the serum IgG Antibody Response to Whole EB and to MOMP Peptide Epitopes

A.. The Effects of Heterologous Priming on Chlamydial Antibody Responses

The de novo antibody responses produced to whole chlamydial EBs was evaluated. Naive mice were immunized with chlamydial EBs from either serovar A, B, C, H, K, or L2. Seven days later the mice were bled and the serum IgG reactivity to the serovar C VD-1 pin peptide DVAGLQND were measured. In all cases the reactivity was less than 1/100 (data not shown) indicating that measurable IgG responses to C-VD-1 are absent in naïve mice at this early time point.

Next, two groups of mice (n=3-4) were primed with either serovars C and L2 and rested for 159 days prior to boost with the homologous serovar on day 14. mice were bled on days 14, 28, 150, and 180 (see figure 8, A and B). The whole EB reactivity of both serovar C and L2 primed groups followed very similar kinetics with both reaching maximum IgG titres on day 28 of 1/31250 and 1/156250 respectively. Following this period of rest time, these two groups of mice received identical boosters with serovar C EBs (on day 180), and were bled seven days later.

A. C Primed, C boosted Mice.

B. L2 Primed, C boosted Mice.
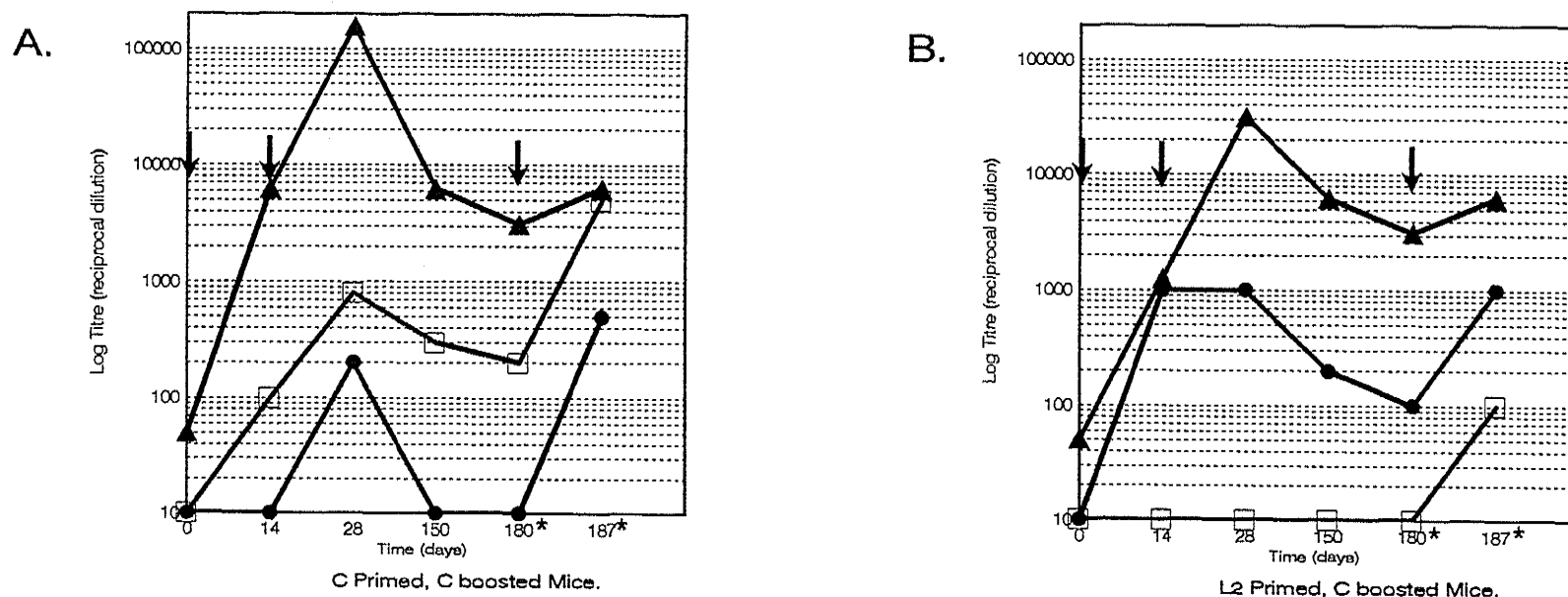
Figure 8: Heterologous priming and serum antibody responses to *C. trachomatis*. Evaluation of *C. trachomatis* specific serum IgG response, and cross serogroup priming. Whole EB ELISA was performed on Serovar C EBs (▲), as described in Methods . Peptide-pin ELISAs were performed on serovar C-variable domain 1 (□), and the species conserved variable domain 4 (●)continuous protective MOMP epitopes. (A) Anamnestic serum IgG response to serovar C in serovar C primed animals. Memory B cells specific to both MOMP epitopes are rapidly recalled and parallel the whole EB response. (B) Anamnestic serum IgG response to serovar C in serovar L2 primed animals. Memory B cells specific for the species conserved epitope (VD4) are recalled in L2 primed animals, but not to C-VD1, as this epitope is antigenically variant in L2. An asterix beside a time point denotes MIF was performed and is in table 1.

This was performed to observe the effects of heterologous priming on antibody reactivity to C-VD-1 and the species conserved VD-4 epitope, TTLNPTIA, as well as whole EB (serovar C) reactivity. The serovar L2 primed mice produced a high titer VD-4 antibody response 7 days after booster with serovar C, but had only backgroud levels of C-VD-1 reactivity (figure 8, B). In contrast the C-primed mice recalled high titer IgG antibody to both epitopes in responses to serovar C booster. Indeed, the C-VD1 titer rose from 1/200 to 1/5000, and the VD4 titer from 1/10 to 1/500 in 7 days (figure 8, A). There was a concurrent rise of IgG titres to whole organism measured on day 187 which demonstrates the boosting effect of reexposure to EBs in a memory response. Serovar C primed mice recalled a high titre C-VD1 response and a lower VD-4 response. While L2 primed mice produced a primary C-VD1 response at 7 days post-boost, and had a strong recall of the VD-4 antibodies. This demonstrates that the pin-peptide assay is MOMP specific, and can be followed as it develops in whole sera. This also demonstrates the existence of a epitope specific memory B cell populations in primed mice. Additionally, priming with L2 does not effect the specificity of the de novo antibody response to the serovar C-VD-1 epitope.

B. *The Effects of Heterologous Serovar Boosting on MOMP Antibody Responses*

The above experiments demonstrated the serovar specificity of anti-MOMP C-VD1 antibody responses as measured on the surrogate pin-peptides. Next, we set up experiments designed to observe the effects of heterologous booster immunization on identically primed mice. This was performed in order to compare the fine specificity of

polyclonal antibodies recalled by structurally variant VD-1 epitopes. Groups of mice (n=4) were primed with serovar C EBs and rested for 150 days. Mice were bled and then each group received either serovars A, C, H, K, or B EBs as a booster immunization. Seven days later the mice were bled, the serum pooled and tested for IgG reactivity to whole EBs, and to MOMP peptide epitopes C-VD-1 and VD-4 (below). Pooling the sera from 4 individual mice was done in order to reduce the potential effects on fine specificity due to individual variations in available antibody repertoires.

1. Whole EB Reactivity

Whole EB reactivity was measured in the microimmunoflouresence (MIF) immunoassay (Table 4). Six months after priming with serovar C, the mice had IgG reciprocal antibody titers of between 128 and 512 against serovar C and between 32 and 512 against serovar B in the MIF assay. The MIF response against serovars A, H, F/G and K ranged from zero to 128 consistent with their antigenic status as junior serovars (Wang and Grayston, 1970). The intergroup variation in the pre-boost titers was unexpected as all groups were primed in an identical fashion. This may be due to differences in the decay rates of circulating antibody titers to epitopes found on the whole organism as a result of minor differences in antigen depots or other variations in the vaccination process. After homologous boosting, the mice showed an 8-fold

Table 4: Original antigenic sin antibody responses analysed by microimmunofluorescence (MIF). Patterns of pre and post challenge serum IgG on whole chlamydial organism (EBs). Shown are the reciprocal tires of serum IgG antibody from homologously primed mice the day before, and 7 days following challenge. Italicized font represents positive reactions with titres greater than 1/512 at 7 days post challenge. Challenge serovar- homologous reactivity is underlined. Values are depicted as pre-challenge/post-challenge.

		C J	H	A	B	L2	F G	K
	C-C	_512/>8196_	32/128	128/128	*128/1024*	32/32	32/128	32/128
	C-A	*512/>8196*	0/128	_128/2048_	*512/1024*	0/128	0/128	32/128
Prime-Boost Serovar	C-K	*512/4096*	*0/512*	0/128	*512/512*	*0/512*	0/128	_32/512_
	C-H	*512/>8196*	_32/512_	*32/512*	*32/512*	0/0	*128/512*	*32/512*
	C-B	*128/2048*	0/32	32/32	_128/1024_	*0/512*	0/32	32/32

increase in antibody titer against serovar C, a 6-fold increase to serovar B and zero to 4 fold increase to serovars A, H, F/G and K. Boosting with heterologous serovars brought about an 8-fold increase in antibody titer against both serovar C and the boosting serovar, accompanied by lesser increases against other serovars. This strong recall of antibody to serovar C by heterologous boosting is the signature of an OAS response. The results of the MIF assay also demonstrated that boosting with serovars more closely related to serovar C increased antibody cross-reactivity more than did boosting with serovar B. This conclusion is supported (p=0.0002) for the differences in post-boost serological reactivity observed between the B and C serogroups (Fisher's exact two-tail test on sera with reactivity to serovars A,H, F/G, and K with reactivity greater than 1:32). The serological reactivity to the chlamydial HSP 60 was also measured as a positive control antigen in an ELISA to ensure that a secondary response had occured. It is known that multiple boosts are required in mice to produce a measurable Hsp60 antibody response (Dr. Rosanna Peeling, Laboratory Centre for Disease Control Canada). Chlamydial Hsp60 IgG antibody O.D. were measured pre- and post-boost and were found to be elevated in all sera at day 187, indicating that the mice had indeed been primed with EBs and were responding in a secondary type response (data not shown).

2. Peptide Epitope Reactivity

To evaluate whether the differences in the MIF patterns could be correlated with differences in responses to the serovar C MOMP VD1 peptide epitope, sera were tested using the pin peptide ELISA (Zhong et al., 1994a). Earlier experiments demonstrated that while naive mice did not develop measurable serovar C MOMP VD1 IgG antibody

responses 7 days after immunization, homologous boosting of primed mice demonstrated a typical secondary antibody response to the serovar C VD1 peptide. We found the same results here with C-VD-1 titers rising approximately 25 fold from 1/200 to 1/5000 within 7 days (figure 9). Heterologous boosting with serovars (A, H and K) also resulted in a recalled antibody response to the serovar C VD1 peptide epitope. As expected, boosting with serovar B did not recall antibodies to the serovar C VD1 peptide since its MOMP differs substantially in amino acid sequence in the VD1 region (figure 2). Our findings that the OAS response is in part directed to the VD1 region of the MOMP and that VD1 specific OAS responses occur among serologically related strains confirmed results independently reported by others (Zhao et al., 1996; Fan and Stephens, 1997).

3.   *Fine specificity of OAS antibody recognition of serovar C VD1 using complete amino acid replacement analysis*

Variations in whole EB reactivity seen in the MIF assay following boosting with *C. trachomatis* suggested qualitative differences in the ability of MOMP antigenic variants to recall antibody from the pool of memory B cells. We speculated that determination of the fine specificity of the C VD1 epitope specific antibody response may further demonstrate qualitative differences in recall at the single epitope level. We, therefore, performed the pin peptide ELISA using serially substituted peptides.
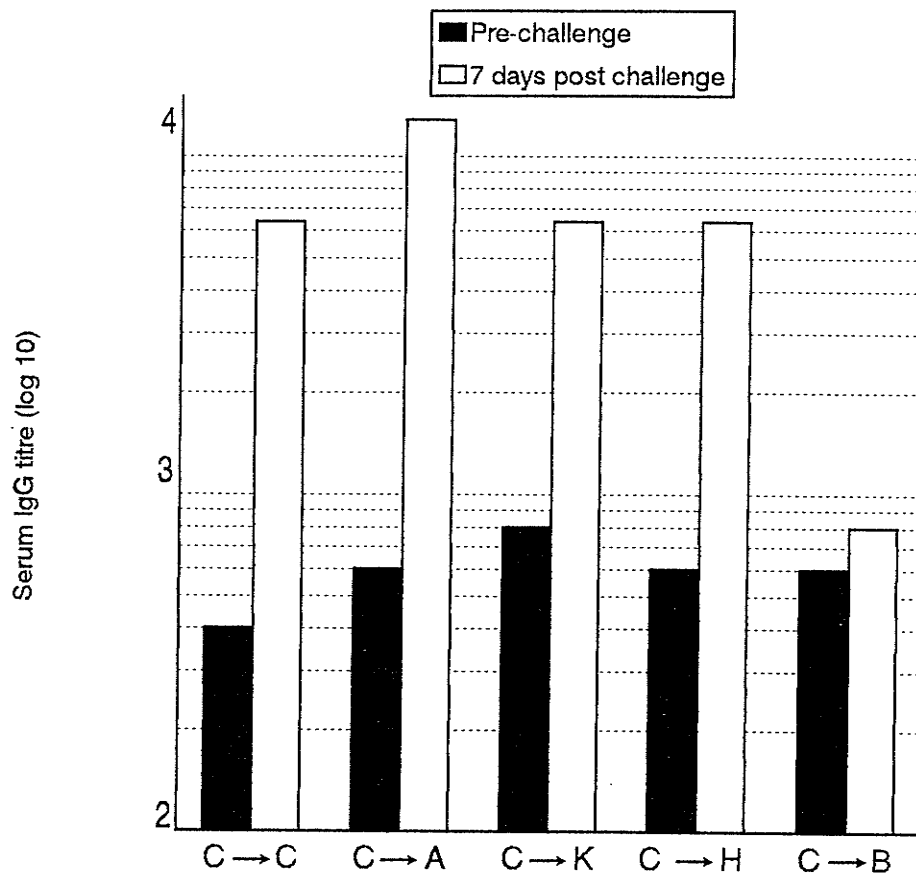
Figure 9:Antigenic sin to the MOMP C-VD1 peptide epitope. Pre and post challenge serum IgG reactivity with the serovar C VD-1 peptide epitope (DVAGLQND). The log of the reciprocal of the highest dilution of sera giving greater than 5-fold over background is shown on the Y-axis. The C complex members (C,A,H, and K) all recall quantitatively similar high titer responses by day 7 in serovar C primed mice. Challenge with serovar B does not recall a C-VD1 antibody response. Results are representative of values obtained in three different priming/challenge experiments.

Among animals primed with serovar C, the pre-boost critical amino acid residue pattern with the serovar C VD1 peptide epitope was -AGLxN- for all groups of mice (Figure 10). Homologous boosting altered the pattern of fine specificity of antibody recognition to -GLQN-. Boosting with heterologous serovars also altered the antibody recognition pattern but in distinctively different ways. Boosting with serovar A altered the antibody recognition pattern to -GLxxD-, serovar K to -GL-, and serovar H to -AGL-. Boosting with serovar B did not alter the pattern of critical residues compared to the prechallenge sera consistent with the absence of recall of serovar C VD1 antibodies.

Differences in fine specificity, as observed in the amino acid replacement analysis of serovar C VD1 peptide, were remarkably concordant with the patterns observed in the MIF assay in that less stringent requirements for specific amino acids within the epitope region were correlated with broader MIF cross-reactivity. More quantitatively, amino acid replacement analysis demonstrated that the total number of intolerant amino acid residue substitutions decreased after boosting with heterologous serovars within the C serogroup. For example, homologous boosting generated antibodies that exhibited a total of 40 intolerant amino acid residue substitutions in the peptide epitope sequence. Heterologous boosting with related serovars consistently produced antibodies with fewer intolerant substitutions. Sera from serovar A boosting had 29 intolerant substitutions, while sera from boosting with serovars K and H each had a total of 23 and 22 intolerant substitutions respectively.

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Figure 10 (facing page): Fine specificity mapping with complete substitution peptide analogs of the C-VD1 epitope sequence VAGLQND. Sera from each prime-boost group was reacted with the individual substitution analogs. Analogs for each amino acid position are in alphabetical order in the single letter code. The values represent the binding of the analog as a percentage of the parental peptide. Substitution analogs with values less than 20% of the parental peptide are termed non-binding and are in bold type face. The critical binding motifs and the total number of intolerant residues are shown on the right. OD values above that of the parent peptide presumably represent heteroclitic antibody binding. The preboost serum IgG reactivity is shown at the top of the figure and is identical for all pooled pre-boost sera. The C-complex drift variants, A, K, and H, recalled quantitatively similar VD-1 antibody responses (see figure 9) with different patterns of fine specificity and had reduced numbers of intolerant substitution (more broadly reactive). X stands for any amino acid.

SERUM

Per Cent Activity when substituted with amino acid residue

Serum	PARENT RESIDUE	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	# OF NON-BINDING PEPTIDES	EPITOPE FINGER PRINT
C pre-boost	V	60	114	33	48	148	99	111	134	128	89	63	36	65	53	123	70	86	100	162	178	0	x
	A	100	170	36	5	83	46	11	53	7	35	2	63	111	124	101	35	36	11	86	79	5	A
	G	8	32	5	2	84	100	6	48	3	4	9	5	10	10	50	11	3	62	111	79	12	G
	L	5	11	3	0	80	82	1	33	35	100	7	17	5	9	52	4	0	15	114	116	12	L
	Q	48	72	13	31	60	32	48	70	44	64	53	40	63	100	96	33	47	55	100	122	1	x
	N	17	137	93	92	219	95	45	98	2	67	13	100	39	3	180	104	92	42	276	128	4	N
	D	103	235	100	101	258	158	174	325	146	241	133	121	69	103	252	130	121	143	238	349	0	x
																						total = 34	
C → C	V	57	65	5	10	133	51	47	122	79	135	58	36	74	34	123	34	63	100	78	118	2	x
	A	100	105	89	46	89	80	51	35	41	40	3	57	90	62	136	113	79	12	94	81	2	x
	G	10	12	3	4	55	100	31	45	5	41	5	7	0	13	65	5	16	2	107	69	12	G
	L	2	10	4	7	67	125	7	47	8	100	10	10	11	15	53	10	12	16	73	61	13	L
	Q	41	1	35	9	45	30	44	41	33	132	44	10	101	100	170	17	16	33	92	73	5	Q
	N	32	36	42	35	93	47	42	34	7	51	9	100	14	48	101	58	19	18	108	66	5	N
	D	49	113	100	87	102	74	91	134	35	124	92	66	10	62	99	60	58	83	112	193	1	x
																						total = 40	
C → A	V	95	174	55	52	140	131	69	152	132	126	74	60	89	50	161	89	126	100	208	235	0	x
	A	100	138	55	9	150	89	34	78	40	61	39	61	114	45	219	83	66	50	170	183	1	x
	G	7	51	2	3	107	100	0	79	0	59	0	1	0	8	67	2	8	77	160	138	11	G
	L	0	58	7	8	92	34	8	43	9	100	10	8	8	9	86	11	7	3	124	151	12	L
	Q	78	53	53	56	108	50	37	114	34	110	72	46	82	100	61	56	105	34	32	86	0	x
	N	102	58	118	105	100	61	49	71	43	128	71	100	165	185	83	111	48	4	124	75	1	x
	D	4	179	100	144	93	262	307	110	489	193	152	120	389	410	3	159	5	12	324	306	4	D
																						total = 29	
C → K	V	63	144	46	43	268	97	96	172	90	165	63	42	66	2	157	45	74	100	150	185	1	x
	A	100	219	105	45	160	77	52	74	95	64	34	86	65	87	227	174	175	54	128	121	0	x
	G	3	63	5	0	157	100	58	101	10	80	9	3	5	5	78	10	37	78	174	138	9	G
	L	1	43	4	0	77	72	1	34	0	100	8	35	2	2	96	4	0	8	120	111	11	L
	Q	37	53	51	59	114	49	53	100	0	86	56	40	41	100	135	45	67	88	167	137	1	x
	N	66	75	90	83	222	80	58	107	3	129	46	100	71	71	172	100	83	91	202	147	1	x
	D	85	169	100	228	203	109	123	209	43	215	156	100	63	97	164	94	55	116	142	295	0	x
																						total = 23	
C → H	V	64	126	36	9	111	76	68	60	82	88	58	48	58	51	98	44	69	100	104	147	1	x
	A	100	197	99	3	187	139	3	159	10	100	11	88	141	92	282	139	128	141	283	184	4	A
	G	4	115	12	8	140	100	79	134	45	109	13	35	3	44	121	9	57	110	290	197	6	G
	L	7	88	0	6	98	56	1	42	2	100	38	47	10	10	99	10	7	10	126	142	10	L
	Q	58	162	96	92	132	86	79	106	38	85	70	80	69	100	145	55	76	87	177	138	0	x
	N	44	167	171	99	208	128	80	91	9	92	30	100	55	57	167	91	86	70	261	173	1	x
	D	83	158	100	85	163	97	96	161	31	134	91	106	47	72	141	74	60	94	142	236	0	x
																						total = 22	
C → B	V	118	340	73	67	300	132	167	153	173	125	82	51	70	62	223	78	158	100	421	400	0	x
	A	100	322	72	11	248	121	12	175	8	82	5	108	132	104	271	65	119	109	459	316	4	A
	G	0	153	0	5	218	100	60	183	13	157	10	10	10	50	100	6	106	201	419	251	8	G
	L	14	111	0	10	149	47	11	57	10	100	37	46	5	10	137	8	0	5	313	312	10	L
	Q	12	95	68	58	144	65	76	115	13	73	63	59	72	100	164	57	90	106	310	253	2	x
	N	14	98	121	125	307	98	84	207	9	144	0	100	16	64	212	127	114	104	519	282	4	N
	D	161	318	100	11	315	113	138	296	16	246	173	188	6	160	288	107	113	182	226	377	3	x
																						total = 31	

Collectively, the data show that OAS antibodies recalled by heterologous boosting are qualitatively different from antibodies recalled by homologous boosting.

(II) Discussion and Conclusions

We evaluated the effect of sequential exposure to *C. trachomatis* antigenic variants on antibody responses. We found that the responding B cell population to both whole chlamydial organisms and to the VD-1 epitope produce phenotypically diverse and distinct binding characteristics. The serum responses were typically of higher reactivity to the priming serovar and demonstrates classical original antigenic sin. We demonstrate that OAS antibodies to serovar C EBs are readily detectable by the MIF assay from sera of mice primed with serovar C and boosted with serovars A, K, H or B. Homologous boosting primarily recalled serovar C specific antibodies whereas heterologous boosting with serovars in the C serogroup recalled antibodies with increased reactivity to multiple serovars including the serovar used for boosting.

The fine specificity of epitope recognition by antibody reflected the nature of the recalled VD1 specific antibody repertoire. Homologous sera (serovar C primed and boosted) exhibited a different VD1 critical amino acid antibody recognition pattern than that found in sera prior to boosting, changing from -AGLxN- prior to boosting to -GLQN- post-boosting. The MOMP C VD1 specific OAS antibody recalled by VD1 sequence variants of serovars A, K, and H had unique patterns of fine specificity that differed from the fine specificity pattern produced by homologous boosting (see figure 10). The qualitative difference in the antibody recognition pattern to the same nominal

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epitope suggests that different variable region genes are selectively used in the OAS antibody response despite quantitatively similar responses to serovar C VD1 as detected in the pin-peptide ELISA. We implicate the existence of memory B cells with a diverse pool of affinity selected variable region genes to serovar C VD1 in order to explain the unique phenotype of OAS antibody generated by heterologous boosting.

Previous experiments provided information relevant to OAS and suggest a partial understanding of the antibody variable region genotypes used in response to serovar C VD1. We previously identified several hybridomas that recognized serovar C VD1 MOMP including a hybridoma (MAb C1.6) that produced antibody capable of neutralizing infectivity of both serovar C and A in cell culture (Zhong et al., 1994a). Remarkably, MAb C1.6 had a critical amino acid binding pattern (-GLxxD-) identical to that observed with sera from serovar C primed, serovar A boosted mice in the present study. Thus, the serovar C primed, serovar A boosted OAS antibody response may preferentially activate B cells with MAb C1.6-like variable region genes. We suggest that similar B cell lineages also exist that are capable of selective activation by serovars K and H.

The serological characteristics observed in the MIF assay were corroborated by the pin-peptide assay. Thus, serogroup C MOMP variants such as A, K and H recalled serovar C VD1 peptide specific antibodies. Recall of these antibodies was dependent on the extent of sequence sharing among the serovars since serovar C VD1 antibodies were not recalled by boosting with serovar B, which shares little sequence identity in the VD1 region with serovar C. This is consistent with the findings of others (Zhao et al., 1996;

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Fan and Stephens, 1997) who initiaited a remarkably similar series of experiments using soluble synthetic VD-1 peptides in solid phase ELISA . They also concluded that the chlamydial OAS antibody response is strongly directed to the variable domain (VD) I of the major outer membrane protein (MOMP) of the priming and challenge *C. trachomatis* serovars, and that the response is correlated with the extent of primary sequence sharing between the MOMP VDs of the two serovars. Significantly, their data shows that the OAS antibody response is associated with the development of cross-reactive neutralizing antibodies to both the priming and challenge strains (Fan and Stephens, 1997).

Broad strain chlamydial immunity may be built upon successive exposure to different serovars. Collectively, these results, along with the protracted period of time required to acquire blanket immunity to *C.trachomatis* (~10 years) (Brunham et al., 1996; Mabey et al., 1996) , led us to us hypothesize a model for the role for OAS in acquired B cell immunity (figure 11). Initial exposure to serovar C drives the expansion of a heterogenous set of serovar C VD1 reactive B cells into the memory compartment. The serovar C VD1 memory B cell pool is then able to undergo selective recall by homologous and heterologous serovars based on the activation fitness of antigenic variants.

Figure 11 (facing page): Model of original antigenic sin (OAS) following sequential exposure to heterologous MOMP variants. (1) Priming stage: Primary exposure to serovar C EBs drives naive B cells with VD-1 specificity to proliferate and differentiate. This establishes the C-VD1 specific B cell memory pool. Primaryresponses plasmablasts produce high titer IgG to the C-VD1 epitope which wanes appreciably by day 144. (2) Recall Stage: Challenge with heterologous or homologous EBs recalls B cells from memory differentially, depending on VD-1 relatedness. The MOMPs of C-complex members (serovars C, A, K & H) differ only by amino acid substitutions in VD-1, and are all able to recapitulate an original high titer IgG response that reacts with the C-VD1 by day 7. However, the response is phenotypically different in each case, as each MOMP variant recalls a unique fine specificity for VD-1. Serovar B does not recall antibody that reacts with C-VD1. The B boosted sera has the same reactivity and fine-specificity as pre-challenge sera. This reveals that the memory B cell pool to a single epitope can be diverse and contain subsets of B cells with fortuitous cross-reactivity which are recalled to antigenic drift variants. This may represent a cumulative host defense mechanism.

1. PRIMING STAGE

2. RECALL STAGE

C-VD1 SPECIFIC MEMORY B CELL POOL

Proliferation and Differentiation

Memory B cell Selective Clonal Expansion:

Germinal Centre Reaction

TYPE SPECIFIC

C-MOMP

C-VD1

C-VD1

C-VD1

C-VD1

C-VD1

C-VD1

C-VD1

C/A-VD1

C/K-VD1

Primary B cell pool

Plasma cells

Increased type specificity -GLQN-

H-MOMP

C/H-VD1

C/H-VD1

C/H-VD1

C/H-VD1

C/H-VD1

C/H-VD1

C-MOMP

MOMP C-VD1 Antibody (negative effect on naive B cells)

CROSS-REACTIVE

Increased Cross-reactivity -AGL-

Differential binding of antigenic variants of the MOMPs with affinity matured membrane IgG activates different memory B cells, thus engendering an broadened OAS response which seems tailored to the antigenic variant. This is supported by the broadened recognition of neutralizing MOMP epitopes following heterologous boost (figure 10; Fan and Stephens, 1997). We speculate that if the target epitope is protective in vivo, OAS antibodies may provide early immunity to re-infection when compared to that generated by naive individuals. Indeed, this model is supported by the ability of *C. trachomatis* OAS antibody to neutralize both a priming and heterologous challenge strain in vitro (Fan and Stephens, 1997), and by the cross-protective immunity engendered between serovars A and C as observed in the monkey eye infection model (Grayston et al., 1970). Given that *C. trachomatis* infections are antigenically variant, it may be that immunity is built on the OAS phenomenon and requires the generation of a diversified memory B cell pool in addition to T cell responses. If so, the order in which antigenic variants prime the host may be important considering the extreme variation in MOMP structure. It has been found, for example, that the order of exposure to haptenated antigens is important in the immunoregulation of subsequent antibody responses to related haptens (Angell-Killie, et al., 1995). *C. trachomatis* serovars such as B and C have been classified as senior serovars because they induce antibodies more broadly cross reactive than do junior serovars (Wang and Grayston, 1970). Thus, priming with a senior serovar and subsequent boosting by a junior serovar may best generate cross protective antibodies.

An identical phenomenon has also been observed with *Plasmodium falciparum* infection (Marsh and Howard, 1986). Children develop extremely strain-specific

antibodies to *P. falciparum* infected erythrocytes whereas adults have broadly cross-reactive antibodies. When antibodies from adults are immuno-affinity purified from *P. falciparum* infected erythrocytes and evaluated for type or cross-reactivity, they are found to exhibit broad cross-reactivity. This demonstration of type 1 crossreactivity (crossreactivity exhibited by the same antibody paratope on different antigens) of the individual homogeneously purified antibodies (Berzofsky and Schechter, 1981) directly shows that the individual antibody molecules from the adults are qualitatively different and more cross-reactive than the individual antibodies of the children. The existence of such multispecific antibodies in adults, who have presumably been exposed a larger number of times to the antigenically variable pathogen, shows that one antibody molecule can be selected to react with several different antigens. Thus the diversity of reactive targets of a sera can be broadened to become much greater than the diversity of the B cell clone. This supports our conclusions, and suggests that the crossreactivity of an individual antibody v-region is a selectable property in secondary responses. The differences in antibody reactivity between children and adults is thought to explain in part the susceptibility of children and resistance (partial) of adults to *P. falciparum* infection. Indeed, Nara (1996) suggested that OAS provides cross-strain protection against antigenic variants of a pathogen by enriching host antibody responses to broadly neutralizing determinants. Thus, the OAS response could also represent an evolved host defense mechanism against antigenic variation.

Current vaccine technologies lack the ability to generate broad immune responses (Nara, 1998). For example, in the case of influenza infection, it is generally recognized

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that antibody provides the major protection against infection with virus of the same strain as the immunizing virus or vaccine (Epstein et al., 1998). Public health strategies have focussed their efforts upon developing individual vaccines for the predominant types of influenza as they arise (Nara and Garrity, 1998). This is despite the fact that heterotypic antibodies were identified early-on in influenza vaccine trials (Richman et al., 1974).

OAS may have important implications for vaccine induced immunity. In fact, the immunologic capacity of variant antigens such as the MOMP to induce an OAS response may be exploitable in a vaccination strategy. Our results suggest that sequential immunization to a limited number of antigenic variants should engender an OAS response that leads to the accumulation of cross-protective antibodies. More quantitatively, Suga et al (1994) determined that all 50 monoclonal antibodies produced by heterologous immunization with two structurally related haptens were crossreactive with both the priming and boosting antigens compared to 5 of 48 weakly crossreactive antibodies produced by homologous boost. Remarkably, 19 of 50 of the heterologous mAbs were catalytic compared to 9 of 48 from the homologous boost which proved the value of the OAS in selecting for crossreactive antibodies. Sequential immunization rather than the simultaneous delivery (Schachter, 1987) of antigenically variant MOMPs can also avoid the problem of antigenic competition that occurs when structurally related antigens are co-administered (Schwartzkoff et al., 1993). Indeed, sequential vaccination with heterologous antigens has been noted to be a largely unexplored area of vaccinology (Ada and Ramsey, 1996). The concept of OAS has broad significance for vaccination not only against *C. trachomatis* but also for other antigenically variant pathogens such as

herpes simplex virus (Ohashi and Ozaki, 1981), rotavirus (Green et al., 1990;Perez-Schael et al., 1997) and HIV (Klinman et al., 1991) for which cocktail type formats are being contemplated.

Heterotypic antibodies can provide protection. Heterotypic immunity provided by prior mucosal influenza A infection conferred resistance to humans during the 1969-70 pandemic of influenza (Gill et al., 1971) despite an antigenic shift in the hemagglutinin molecule (Gill et al., 1987). That heterotypic immunity was responsible for providing protection from an emerging variant was later proven experimentally in animals (Schulman and Kilbourne, 1965; Yetter et al., 1980a; Yetter et al., 1980b). Although heterotypic immunity did not completely prevent infection it dramatically improved recovery and reduced the severity of disease. Studies by Couch et al (1974) suggested that resistance in the Australian population during the 1969 pandemic was provided by preexisting and crossreactive anti-neuraminidase antibodies. These studies suggest that a chlamydial vaccine designed to engender crossreactive antibodies may broaden immunity.

Crossreactive B cells may also reduce disease severity. Although antibody molecules and not memory B cells serve to exclude a pathogen from entering a host (Ahmed and Gray, 1996) memory B cells clearly provide early protection that serves to limit the development of some diseases. In cholera disease, for example, a rapid local memory B cell response is associated with a three fold increase in IgA anti-toxin producing cells in the lamina propria within 16 hours of a re-exposure to cholera toxin

(Lyck and Holmgren, 1987). Because *C. trachomatis* has an incubation time ranging from 48-72 hours it is thus conceivable that memory responses can occur in time to impact upon the initial release of new organisms throughout the mucosa. Thus, mucosal heterotypic protection seen against re-infection with variant strains of *C. trachomatis* (Grayston et al., 1970), or influenza A (Amerding et al., 1982), is likely due in part to the local production and concentration of cross-reactive scIgA antibodies produced in an OAS secondary response. The relatively lower avidity of secondary antibody for the boosting strain (compared to the avidity to the antigens of the priming strain) may be compensated for by high concentration of antibody in the mucosa as a result of the known concentrating effects of the local mucosal immune system on local antibody production. This is consistent with a recent study showing that antibody concentration is more important than avidity in antibody mediated protection (Bachmann et al., 1997). Interestingly, the original notion suggesting that OAS may be a beneficial host response was first proposed for heterotypic influenza A infection (Angelova and Shvartsman, 1982).

The VD-1 epitope is a dominant target and recruits phenotypically diverse antibody responses. Heterologous immunization revealed a striking correlation between whole EB and MOMP VD-1 epitope reactivity. MIF analysis measures both type 1 (crossreactivity exhibited by the same antibody paratope on different antigens) and type 2 specificity (crossreactivity due to other antibodies binding to other conserved antigens) (Berzofsky and Schechter, 1981) and is termed a "mixed" specificity due to the complexity of surface antigens. That is, other antigens besides the MOMP, are shared and

identical on all serovars and are potentially contributing to binding in the MIF. Despite this potential source of crossreactive targets there is only limited reactivity in the MIF following heterologous boost with serovar B which is known only to differ in the MOMP. The VD-1 pin-peptide fine specificity analysis strictly measures type I specificity to a nominal epitope, wherein there is selection for binding from the same pool of antibody paratopes. The fact that both whole EB reactivity and VD1 reactivity are broadened simultaneously in OAS corroborates the presence of a larger B cell pool, from which crossreactive specificites are being selected, to both the whole EB and to VD-1. Moreover, this confirms that the VD-1 epitope is a particularly dominant target in crossreactive recall responses in Balb/C mice. The role of the VD-1 epitopes in recall responses was unknown prior to our initiation of this work. Clearly, the VD-1 epitope is a dominant target in the OAS response between related serovars, albeit by phenotypically distinct populations of antibodies. Thus these data are consistent with the existence of a heterogeneous pool of MOMP reactive B cells in primed mice (Figure 11).

While the data support the notion that genetically distinct populations of antibodies are responding to different challenge immunizations, this analysis falls short of this. We have only measured the polyclonal antibody phenotype without directly identifying the individual immunoglobulin variable region genes that encode them. We recognize that the relationship between phenotypic and genetic diversity in epitope specific antibody responses is not always clear-cut (Day, 1990). Lessons from other immunochemical studies have shown that phenotypic diversity cannot consistently predict genetic diversity, and that somatic hypermutation and alternative usage of V

genes contribute to specific B cell pools. Despite the limitations of this type of serological study, cross-reactive secondary antibody responses have in the past been a valuable guage of epitope specific B cell diversity (Fish et al., 1989; 1991). However, in the case of MOMP antibody responses, direct analysis of V-genes in VD-1 specific hybridomas (Zhong et al., 1994a; and below) supports the suggestion that genetic diversity exists within the host antibody repertoire to VD-1. We have extended these studies as outlined below.

OBJECTIVE 2:   *Characterization of the Balb/c Monoclonal Antibody*
Response to a MOMP Peptide Epitope.

(I) Rationale

In order to test the ability of an immunogenic peptide to elicit expression of antibody genes used to target the native epitope, we had to first identify the anti-native MOMP V gene repertoire. Our previous observation, namely that variant MOMP could recall epitope specific antibody with distinct fine specificities in identically primed mice suggested that V-gene usage to VD-1 is ostensibly diverse (Berry et al., 1999; and above). However, the relationship between phenotypic and genetic diversity in epitope specific antibody responses is not always clear-cut and requires direct analysis (Day, 1987). For instance, while some epitope specific antibody responses are encoded by diverse antibody genes reflecting genetic redundancy (Schilling et al., 1980; Clarke et al., 1990), others appear to be restricted in their diversity, and are encoded by recurring variable region gene assemblages (Kavaler et al., 1990; Kalinke et al., 1996). Thus direct genetic analysis of epitope specific antibodies is necessary to form conclusions regarding epitope specific antibody diversity.

Dominant B cell specificities emerge in an immune response. Despite the complexity of antigens, such as whole bacterium, the host antibody response is focussed to a remarkably small number of surface exposed epitopes. Initially, many B cells may recognize various epitopes epitopes on an incoming pathogen and all are turned on against it (Weiss, 1993). This is supported by the finding that most variable region gene families are turned on early in an immune response (Kelsoe et al., 1989; Kelsoe, 1991).

Over time, however, the complex and cooperative mechanisms of the immune system gradually select for B cells producing antibodies with the best fitness. A subset of these responding cells proliferate more rapidly and some terminally differentiate into plasmablasts, and eventually constitute the bulk of the antibody response. The antigens/epitopes recognized by these antibodies are termed immunodominant. Short of a direct analysis of antigen specific B cells (Jena et al., 1996), hybridoma cell lines have been used to study most epitope specific antibody responses.

Monoclonal antibodies produce a snapshot of the antibody repertoire at the time of fusion. Hybridomas (Kohler and Milstein, 1984) are hybrid cells produced from the fusion of primary tissue derived B cells with a myeloma cell line containing with a selectable marker. It is still unclear whether memory B cells or plasmablasts B cell forms successfully fuse to myeloma in order to create a hybridoma, but activation by antigen is known to be required (Shen, et al., 1986). The selection and subsequent screening for antigen specificity functionally selects for immortalized antigen specific hybridomas, and thus produces a virtually unlimited supply of monoclonal antibodies. Importantly, the selected hybrid B cell or hybridoma, also contains the rearranged V-gene that encodes the antibody making genetic analysis possible. Moreover, the variable region genes of hybridomas are quite stable over time as the somatic mutational apparatus appears to be inactive (Pollock et al., 1988). The use of monoclonal antibodies has become fundamental to immunology. Such studies have revealed details of the mechanisms of genetic diversity and affinity maturation of antibodies, which is relevant to immunization.

Genetic information on several VD-1 mAbs exists. Previously, our lab (Zhong et al., 1994a) created a panel of eight hyperimmune mAbs that mapped to the VD-1 epitope of serovar C MOMP. While the variable region genes of these 8 mAbs were not identical, several of the antibody genes were highly related and differed only in point mutational changes. In particular, 5 clones (C1.1-1.5) all utilized the same V_H-D_H-J_H assemblage in the same reading frame, moreover the light chains of all 8 mAbs were encoded by the same Vk gene. These data suggested that the VD1 repertoire would be encoded by resurring antibody V-genes and may even be restricted to only a few V-gene alleles. However, we cannot derive these conclusions without examing the antibodies used to target VD-1 in independent mice. The clonal relationship of these earlier hybridomas to one another is unclear as the splenocytes from the two mice used to derive these clones were pooled prior to fusion. Thus we set out to generate multiple mAbs from different stages of the immune response. The creation of VD-1 specific hybridomas from different stages of the anti-MOMP response will allow us to directly determine the genetic diversity of antibodies to this epitope via molecular analysis of the antibody genes utilized in individual hybridoma clones. We hypothesize that the comparison of clones from different stages will reveal if: A) The response is restricted to particular variable region genes, or B) There is patterned and recurrent usage of certain variable region gene assemblages, or C) The variable region gene structures are used randomly and there is no recurrence. The study of epitope specific antibody repertoires may also shed light on the fundamental role of pathogens in shaping host antibody repertoires (See Introduction and ; Weiss, 1993)

1.   Results of Site Specific Murine mAb Production

Monoclonal antibodies were selected for VD-1 reactivity. Supernatant from hybridomas produced in immunized mice were screened directly for VD-1 pin-peptide immunoreactivity in a solid phase peptide ELISA. Multiple fusions were necessary to obtain clones from three different stages of "immunity". We identified 14 IgG positive hybridoma clones with VD-1 specificity. Four were from the early stage (day 7), six from the mid-stage (day 21), and four from the late or hyper-immune stage (greater than day 150) (see Table 1). The immunization schedule is detailed for clarity in figure 5 (methods). We specifically screened for IgG in the day 7 mice despite the general knowledge that isotype class switch does not usually begin until around day 8. This was partially due to the fact that the anti-IgM secondary antibody used for ELISA detection was highly reactive with the pin-peptides (which abrogated the specificity of the test) and to be consistent with the class of the clones from later stages. The possible limitations this may have on our study are discussed later (see discussion, this section). Isotype analysis was performed using whole chlamydial EBs as antigen and revealed that all antibodies were IgG-types with kappa light chains (Table 5).

Table 5: Summary of results from early, mid-, and late fusions. Early hybridoma clones AH2, BC5, and HC10 are from a single fusion, and 12B7 is from an independent fusion. All of the mid-clones are from the same fusion. Late-clone 5C6 is from an independent fusion from the other late clones.

FUSION	Number of Spleenocytes Fused	C-VD1 Positive Clones	Cloned Lines	Isotype
EARLY (d7)	8.4×10^{7}	4	AH2	IgG2a/K
			BC5	IgG2a/K
			HC10	IgG1/K
			12B7	IgG1/K
MID (d21)	9.5×10^{7}	6	Nort 2	IgG2a/K
			Nort5	IgG2a/K
			Nort6	IgG2a/K
			Nort8	IgG2a/K
			Nort9	IgG3/K
			Nort10	IgG1/K
LATE (>d150)	3.0×10^{8}	4	5C6	IgG2a/K
			4B6.A	IgG2a/K
			2C9.F	IgG1/K
			2D8.A	IgG3/K

2. *Immunochemical characterization: Confirmation of Native Epitope Reactivity*

Immunochemical analysis was performed in order to verify the reactivity of all 14 mAbs to the whole organism and to the MOMP protein. Using highly purified chlamydial EBs, mAb supernatants were tested for reactivity in a dot-blot assay. All 14 mAbs reacted with the whole organism along with another VD-1 specific mAb, C1.8, as a control (Figure 12, middle). This confirms the reactivity of these antibodies to the whole organism. Ascites from each of the mAbs was prepared and tested for chlamydial protein binding specificity using metabolically labelled chlamydial EBs in an immunoprecipitation experiment (Zhong et al., 1994a). All 14 mAbs precipitated the 40-Kda MOMP protein, confirming their specificity for the MOMP VD-1 epitope (figure 12, bottom). All 14 of the monoclonal antibodies also have MOMP reactivity in western blot analysis performed with antibody prepared from ascites (data not shown) which corroborates the specificity for MOMP. Thus this panel of monoclonal antibodies generated in EB-immunized mice are specific to the chlamydial MOMP and react with peptides corresponding to the VD-1 epitope.

3. *Relative Affinity and in vitro Neutralization of Chlamydial Infectivity*

The relative affinities of the the purified monoclonal antibodies were compared on whole EBs (Table 6). The mAbs have widely varying affinity for whole EBs. However, in general the affinity of the antibodies increases with time post-immunization, and with the number of boosts. The average affinity by group was

Variable Domain-1 Pin-Peptide Screen
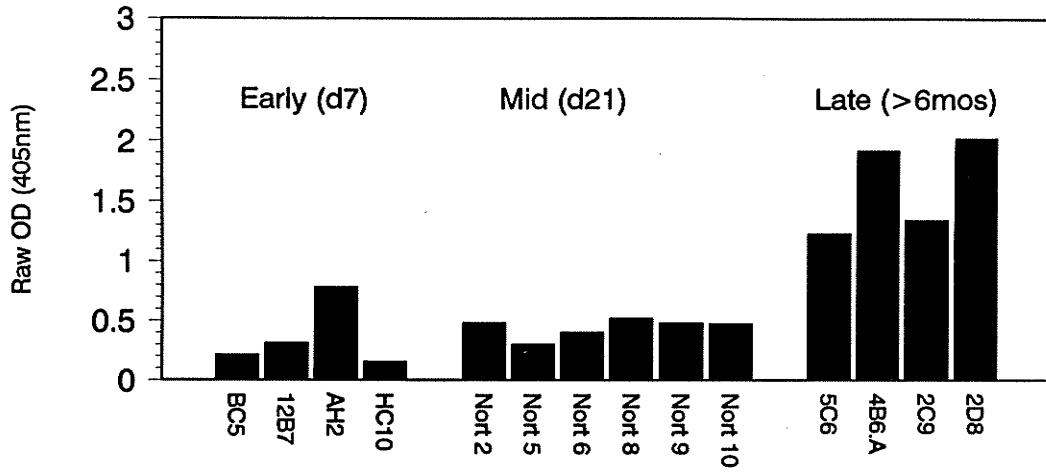
Figure 12 (facing page): Immunochemical analysis of 14 anti-MOMP epitope antibodies. The upper panel shows the raw O.D. of the neat supernatants that initially idenified these hybridoma clones in primary screening. Whole EB reactivity was assesed in immunodot blot and all 14 mAbs react with native EBs. The mAb C1.8, a previously identified anti-chlamydial serovar C mAb that mapped to VD-1 (Zhong et al., 1994a) and is labelled with the synbol +. All 14 of the mAbs react with whole chlamydial EB in dot blot. This is consistent with serovar C EB reactivity observed in whole organism ELISA (data not shown). MOMP protein specificity was evaluated in immunoprecipitation. The arrow shows the location of the MOMP molecule. Raw ascites from each of the 14 mAbs recognizes the MOMP protein specifically. mAb C1.8 was again used as a control antibody and was characterized previously (Zhong et al., 1994a).

Table 6: Relative affinity of mAbs to whole EBs. Monoclonal antibody was serially diluted in 2% BSA and reacted with the EB coated plates in ELISA. The maximum titre giving 5-fold over background is shown in column 1. The corresponding antibody concentration required for each mAb at to yield an optical density of 0.5 is given in column 2. The antibody concentration required to yield neutralization of serovar C EB cell infectivity to 50% of maximum is shown in column 4. The overall group mean for relative affinity and neutralization for early, mid-, and late panels of mAbs is given in column 3 and 5 respectively. Control mAb C1.8 also recognizes the VD-1 epitope of MOMP as shown previoulsy (Zhong et al., 1994a).

	mAb	End-Point Titre	Protein Concentration (ug/ml)	Group Mean (ug/ml)	Neutralization (ND50) ug/ml	Group Mean ND50 (ug/ml)
E	BC5	1/4	5		20	
	12B7	1/4	5	3.13 (+/- 2.17)	15	11.25 (+/- 7.5)
	AH2	1/16	1.25		5	
	HC10	1/16	1.25		5	
M	Nort 2	1/4	5		3.6	
	Nort 5	1/64	0.31		5	
	Nort 6	1/16	1.25	1.25 (+/- 1.88)	17	10.1 (+/- 6.9)
	Nort 8	1/64	0.31		5	
	Nort 9	1/64	0.31		20	
	Nort 10	1/64	0.31		10	
L	2D8	1/1024	0.19		1.25	
	4B6	1/64	0.31		0.25	
	2C9	1/256	0.078	0.150 (+/- 0.15)	5	2.31 (+/- 2.5)
	5C6	1/64	0.31		5	
	C1.8	1/1024	0.019		0.03	

estimated as the concentration of antibody (ug/ml) that would yield an optical density value of 0.5 at 405 nm on whole EB ELISA. There were, however, exceptions to this in all groups. For example early mAbs AH2 and HC10 had relative affinity equivalent to that of mAb Nort6 from the mid-stage group of antibodies. Mid-stage antibody Nort 2 bound with a lower relative affinity and required a concentration of 5 ug/ml to reach O.D. of 0.5. As well, some mid-stage antibodies had a relative affinity equivalent to that seen in the late antibody group. When grouped, however, the average affinity (by group) for early antibodes was 3.13 (+/- 2.17), for mid-antibodies 1.25 (+/-1.88), and for late antibodies 0.150 (+/-0.15). Thus, in general, less antibody was required in late antibodies to produce a binding signal compared to mid antibodies, which required less antibody than early antibodies.

The ability of the VD-1 mAbs to neutralize infectivity of serovar C EB s was evaluated on HaK cell monolayers. The results of neutralization are summarized in table 6. The concentration of a given mAb required to reduce EB infectivity to 50% (neutralizing dose (ND)$_{50}$) tends to decline from early to late stage. Some of the individual mAbs did not follow this trend. For example early mAbs AH2 and HC10 had ND50s or 5 ug/ml, equivalent to that observed for mid mAbs Nort5 and Nort 8. Mid-stage antibody Nort 6 and Nort 9 had ND50s of 17 ug/ml and 20 ug/ml respectively and were comparable in potency to early mAbs BC5 and 12B7. The late mAbs as a group all had ND50s less than or equal to 5 ug/ml. The average mAb concentration (ug/ml) required to produce a ND50 by group was 11.25 (+/-7.5) for early stage, 10.1 (+/-6.9) for mid stage, and 2.31 (+/-2.5) for late stage. These data are generally consistent with an

antigen mediated maturation in antibody affinity.

4.   Immunoglobulin Variable Region Gene Sequencing and Classification

In order to determine the gentic identity of the VD-1 antibody repertoire, and the

basis for affinity maturation to MOMP we sequenced the mAbs variable region genes.

The cDNA for all 14 VH and Vk immunoglobulin region genes were amplified by the

polymerase chain reaction. All of the chains could be amplified with either degenerate

oligonucleotide primers that bind in the framework 1 region, or leader region primers that

prime upstream in the untrans lated region. In some cases it was neccessary to utilize an

initial preamplification of cDNA using a Rapid amplification of cDNA ends (RACE)

homopolymer tailing procedure (see methods; Berry and Brunham, submitted), in

combination with isotype specific downstream oligonucleotides that prime in the N-

terminus of CH1 or 2 (See table 3 and accompanying figure 7). Despite succesful

amplification of cDNAs of the correct size, not all of the PCR products were amenable

to direct cycle sequencing for reasons that are not entirely clear. Likely reasons for this

include internal primer homology and cDNA secondary structures. Additional sequence

information was obtained by cloning PCR products and performing sequencing with

primers that annealed to the vector. The complete and partial nucleotide sequences for

VH and Vk are shown in figures 13 and 14 respectively. The inferred amino acid

sequences were established using PCGENE data analysis program. Variable region gene

classification and homology searches were performed using the Wu and Kabat database,

and the Immunogenetics

CDR1 (H1)

```
                    10        20        30        40        50        60        70        80        90
AH2-H
BC5-H                         TGACCCACCTATCCCCGGCCCTTTATGCAGAGCAGCTGGTGCTTCTGGGACACCATCACC*****TCATTCCTCCG
HC10-H
12B7-H                                                      AAACTCTCCTGTGCAATCCGAGGATACGATTTC*******CCAACCCAT
Nort5-H
Nort6-H
Nort8-H
5C6-H                                         CCCTCAAAGAGCCTGTCCCATCACTTG***CAACTGTCTCGGTTTACA
4B6-H       GCCTTGTCAAACTAGGTTCCTGGCACCTGCAGTCTCAGCCTAAACAGACCTGTGTCCTACACGAGCGAACTT*CCTGCAAGAGCTCTCACA
2C9-H       GAGGTCCAGCTGCAACAGTCTGGACCAGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGCAAGACTTCT*******GGATACACA
2D8-H       AGTGTTGGCTTTATTAGAACAGCTAATGGTAACAGTGTGAGGTGAACTGAGAGCTTGTACAGCTGGGTCCTGAGACCCTGTGCACTCTGGT
```

CDR2 (H2)

```
            91        100       110       120       130       140       150       160       170       180
AH2-H         ATCATCTAACGGATCACTCGGATTGTCTCGACAGGTATGACCGTGTCAGGACCAAGTGACTTGATCGACGGATAACCACACACGTA
BC5-H         GTCCTCTTCATGCCAAAGGCCCAGGAAGACCACTGCACANATCCCAGCTCGGCACATGGCAGCCCACTCAGGGACACCCATCGATGGAGCT
HC10-H        ATTACTAGGCTTCGCTGCGTATCGCGCACGTTACATCCCATGACGACCGATCGCTACCAAACGCTGAATGAGTACCCATGTCCATCAATAC
12B7-H        GACATGTCGTGGGTCCGCAAGACTCCGGAGAAGAGGCTGGAGTTGGTCGCAGCCATT***ACTAGTGATGGTGGTAGCACCTACTATCCAG
Nort5-H
Nort6-H
Nort8-H
5C6-H         ACCAGCTGGGCTGATGTACACCTGAGAGAGCTAAAGTTAGAGTGGATGACCCTGGGAGCTC*AGAGTTCAAGACCAGCATTGAAATATTGTA
4B6-H         GATACACATCACCTCAATACACCATAGACTACAGAGCAGTTGAGCTAGATGGATTGAG*****TATTAATCCTAACAATGGTGGTATTAGCT
2C9-H         TTCACTAAATACACCATGCACTGGGTGAAGCAGAGCCATTAAGAGCCTTGAGTCGGATTGCAGGTATTAATCCTAACAATGGTGGAACTCAG
2D8-H         CACACTGATACTACATGAGCTGGCCTCGCAGCTCCAGAAGCACTGAGTGGTGGTTATAGAACAAGCTAATGGTAACAAACCGGAATACAGTG
```

```
            181       190       200       210       220       230       240       250       260       270
AH2-H       AGTCGCTCCGATGTTATGGCTTCTCGGTTCAAAGCGAAGTCTCACTCGCTTGTCAGACGTGCTTCGATTTGCTGTGTCGGTATATAAT
BC5-H       GGGCAGGCAGCAGCATGACATGCACCCACAGTTTANAAACTGTGAGGAAGCAGGAAGGTCGCCACCACTACTGGTTTACTGAGGATTA
HC10-H      AACCTCTAAGTCAGAATCGTCTAGAACTGAATCAACAGCTCAAAAGTACCATCTCAGAAACATCACTA
12B7-H      ACACCATGAAGAGACGATTCATCATCTCCAGAGACAATACCAAGAAGACCCTGTACCTGCAAATGAGCAGTCTGAGGTCTGAGGACAC
Nort5-H                   AGGCCACATGCAGACTGTAGACAAGTCCTCCAGTCACACGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGATTC
Nort6-H                        GCTGCGTACATCTCAGAGATAGTCCAAACCATCTTCAAATGAACACCCTGAGAGCTGAGGACAG
Nort8-H                  AGGCCACATGCAGACTGTAGACAAGTCCTCCAGTCACACGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGATTC
5C6-H       TGAATCCAGGAATGAAGCTGAAAGAGGTAAA
4B6-H       ACAACCAGAAGTTTAAGGAACACATTGACTGTAGACAAGTCCTCCAGCACAGCCTACATGGAGCTCCGCAGCCTGACACTGAGGATTC
2C9-H       CTACACTACTATCCAGACACCATGAAGAGACGATTCATCATCAAGAAGACCCTGTACCTGCAAATGAGCAGTCTGAGGTCTGTGGACA
2D8-H       CATCTGTGAAGGGTCGGTTCACCATCTCCAGAGATAGTTCCCAAACCATCCTCTATCTTCAAATGAACACCCTGAGAGCTGAGGACAG
```

CDR3 (H3)

```
                        Df116.1   TTTATTACTACGGTAGTAGCTAC
                        DSP2.3    TCTACTATGGTTACGAC
                        DSP2.8    CCTAGTATGGTAACTAC
                        Q52       CAACTGGGAC                              TACTTTGACTACTGGGGC   JH2
            271       280       290
AH2-H       CGACTTCGATTTCCGTGCTCCA       GTGTACTGGACT
BC5-H       CGCCGTCTATTTCTGTGCTCGA           AGTTGGNACG                       TGGTTCGAN            JH2?
HC10-H
12B7-H      AGCCTTGTACTACTGTGCAAGA          CTTACTTATGGCAAACTA                TACTTTGACTACTGGGGC   JH2
Nort5-H     TGCAGTCTATTACTGTGCAAGA  GGTTATTACTACGTTAGTAGCTAC  GGGAGTTAC       TACTTTGACTACTGGGGC   JH2
Nort6-H     TGCCACTTATTACTGTGCAAGA           GATATGAG           GGGTACCGTTTAT  GCTCTGGACTACTGGGGT   JH4
Nort8-H     TGCAGTCTATTACTGTGCAAGA  GGTTATTACTACGTTAGTAGCTAC  GGGAGTTAC       TACTTTGACTACTGGGGC   JH2
5C6-H
4B6-H       TGCAGTCTATTACTGTGCAAGA  GGTTATTACTACGTTAGTAGCTAC  GGAAGTTAC       TACTTTGACTACTGGGGC   JH2
2C9-H       AGCCTTGTATTACTGTGCAAGA          CGGGACAAACTA                      TACTTTGACTACTGGGGC   JH2
2D8-H       TGCCACTTATTACTGTGCAAGA  GATATGAGGGGTACCGTTTATGCTCTGGAC            TACTG               JH1/2
```
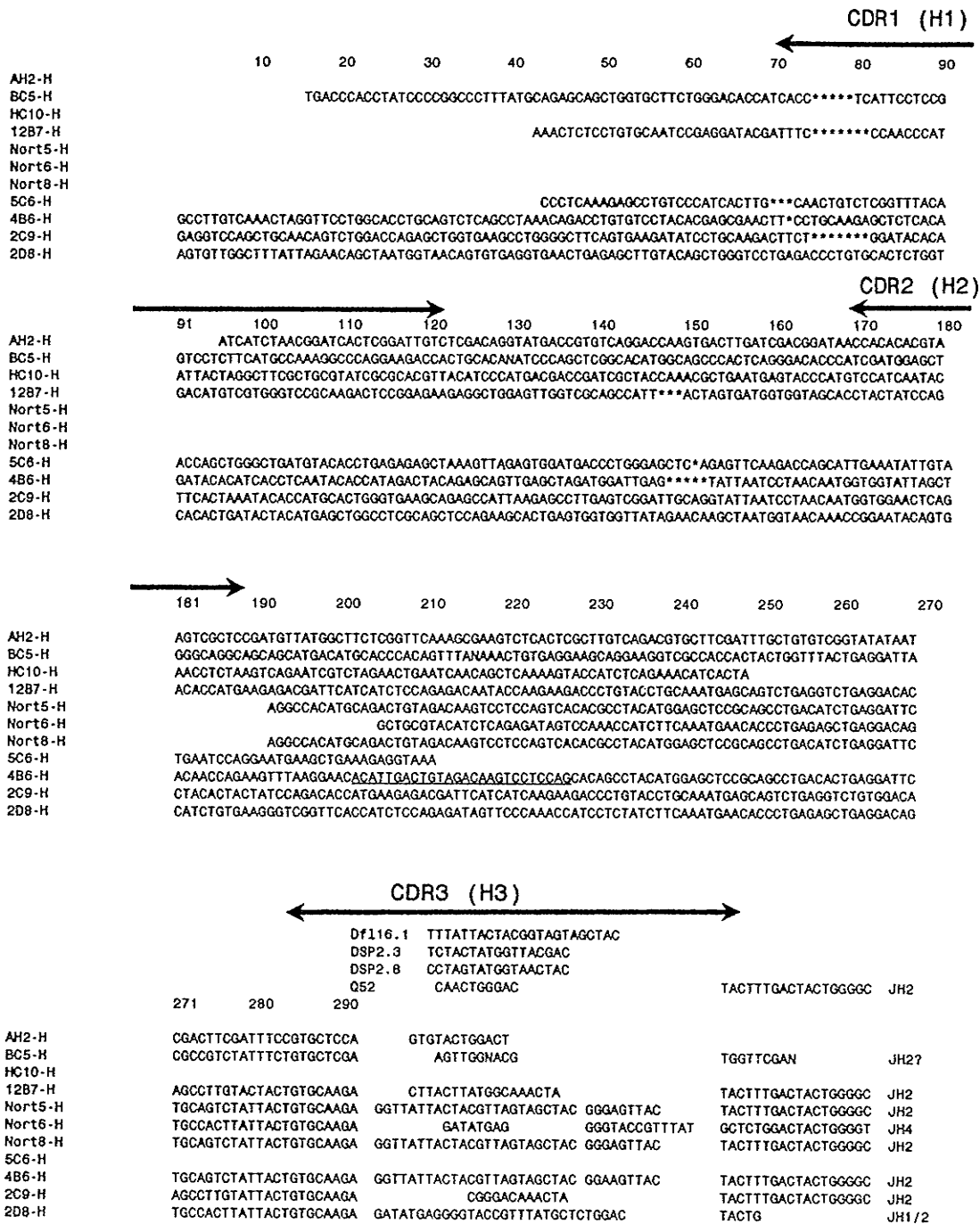
Figure 13: Immunoglobulin heavy chain variable region cDNA sequences. The gamma chain cDNA sequences are shown in alignment according to the numbering of Kabat et al. (1987;1991). -, indicates consensus identity; *, indicates a deletion. Sequence corresponding to oligonucleotide primer designed for VH6-JH2 run-off blot is underlined.

181

```
                                                                              CDR1 (L1)
                                                                        ←──────────────
          10        20        30        40        50        60        70        80        90
consensus GACATTGTGCTGACACAGTCTCCTGCTTCCTTAGCTGT*ATCTCTGGGGCAGAGGGCCACCATCTCATACAGGGCCAGCAAAAGTGT***
AH2-k     ------------------------------------------*---------------------------------------------***
BC5-k     ------------------------------------------*---------------------------------------------***
HC10-k    ------------------------------------------*---------------------------------------------***
12B7-k    ------------------------------------------*---------------------------------------------***
Nort5-k
Nort6-k   C-A-----T--C--C--------A--AAT-*AT-TCTCATCTC---------A--T------GA-C-G---T-----ATC-----***
Nort8-k   ------------------N-----------------*---------------------------------------------***
Nort9-k
5C6-k     ------------------------------------------*---------------------------------------------***
4B6-k     ------------------------------------------*---------------------------------------------***
2C9-k     ------------------------------------------*---------------------------------------------***
2D8-k     ------------------------------------------*---------------------------------------------***

                                                                              CDR2 (L2)
              ──────────────────────────────→                            ←──────────────
          91   100       110       120       130       140       150       160       170       180
consensus *CAGTACATCTGGC*TATAGTTATATGCACTGGAACCAACAGAAACCAGGA****CAGCCACCCAGACTCCTCATCTATCTTGTATCCAA
AH2-k     *------------*-------------------------------------****---------------------------------
BC5-k     *------------*-------------------------------------****---------------------------------
HC10-k    *------------*-------------------------------------****---------------------------------
12B7-k    *------------*-------------------------------------****---------------------------------
Nort5-k              ---A--*----TG------------------------------****---------------------------------
Nort6-k   *-----AG-TC-C****AGC-AC-TGT-----T----G-----G------***T-CT-------A----TGG--T---A-CAC-----
Nort8k    *------------A--*----TG------------------------------****---------------------------------
Nort9-k
5C6-k     *------------*-------------------------------------****---------------------------------
4B6-k     *------------*-------------------------------------****---------------------------------
2C9-k     *------------*-------------------------------------****---------------------------------
2D8-k     *------------*-------------------------------------****---------------------------------


              ────────→
          181  190       200       210       220       230       240       250       260       270
consensus CCTAGAATCTGGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCATCCTCAACATCCATCCTGTGGAGGTGGAGGATG
AH2-k     -------------------------------------------------------------------------------------
BC5-k     -------------------------------------------------------------------------------------
HC10-k    -------------------------------------------------------------------------------------
12B7-k    -------------------------------------------------------------------------------------
Nort5-k   -----------------------------------------------------C-----------------------A-------
Nort6-k   ---G-CT--------------TC-C--------------------G-CTCT-ATTCT----CA---AGCAGA-------CT------
Nort8-k   -----------------------------------------------------C-----------------------A-------
Nort9-k                                                       ---CACAAT--G-GGCC-----TAT--A---A
5C6-k     -------------------------------------------------------------------------------------
4B6-k     -----------------------------------------------------C-----------------------A-------
2C9-k     -------N-----------------------------------------------------------------------A-------
2D8-k     -----------------------------------------------------C-----------------------A-------


                          CDR3 (L3)
                   ←────────────────────────→
                                                N
          271  280       290       300             310       320       330
consensus CTGCAACCTATTACTGTCAGCACATTAGGGA          TACACGTTCGGAGGGGGGACCAAGCTGGAA    JK2
AH2-k     ------------------------------          GCT  ------------------------------
BC5-k     ------------------------------          ...  ------------------------------
HC10-k    ------------------------------          ...  ------------------------------
12B7-k    ------------------------------          ...  ------------------------------
Nort5-k   ------------------------------          ...  ------------------------------
Nort6-k   ----C--T-T------C-----GTAC--T-G          ...  ------------------------------
Nort8-k   ------------------------------          ...  ------------------------------
Nort9-k   A-C--A-T-----TCTAT--GTACG-TGA--          ...  ------N-----------------------
5C6-k     ------------------------------          ...  ------------------------------
4B6-k     ------------------------------          ...  ------------------------------
2C9-k     ------------------------------          ...  ------------------------------
2D8-k     ------------N-----------------          ...  ------------------------------
```
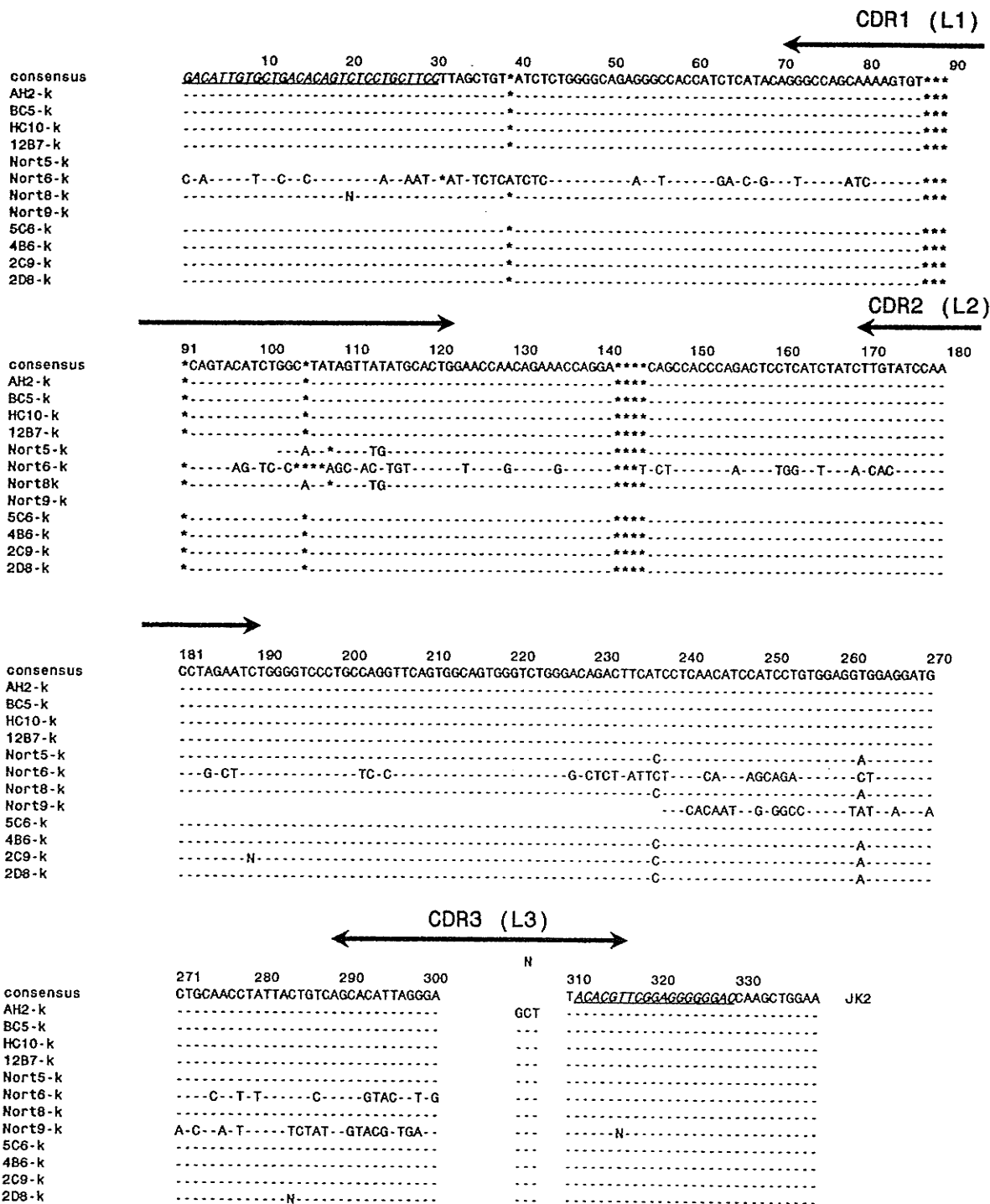
Figure 14: Immunoglobulin kappa chain variable region cDNA sequences. The kappa chain cDNA sequences are shown in alignment according to the numbering of Kabat et al. (1987;1991) to an overall consensus sequence. -, indicates consensus identity; *, indicates a deletion. Sequences corresponding to oligonucleotide primers designed and used for Vk21-JK2 run-off blot are underlined and italicized.

data base (Koln Germany). The sequences were classified according to their best matches from the known V-genes in the repository. The V-genes are classified by family, where possible, and this is summarized in Table 7. It is immediately obvious that the VD-1 antibody repertoire is not dominantly resticted in VH gene usage. However, the same Vk21 and Jk2 segment was used in 10 of 14 mAbs. The sequence of this Vk21 allele has 65% nucleotide identity with the dominant Vk2 gene found to dominantly encode all kappa chains of VD-1 mAbs isolated previously to C-VD1 (Figure 15) (Zhong et al., 1994a).

5.   *Molecular Profile of the Somatic Evolution of VD-1 Antibodies*

An overall analysis revealed that a diverse population of V genes are used to target the VD1 epitope; however upon closer examination a patterned recurrence of certain V genes was obvious. For comparative purposes we included in our analysis eight additional anti-serovar C VD-1 mAbs identified previously in our lab (Zhong et al., 1994a). Table 7 summarizes the Variable gene minicassette usage in all 22 VD-1 mAbs. Te question mark stands for sequences that were illegible and not obtained. Three structural patterns can be found. 1) The Vk21-Jk2 assmblage is found in 10/14 mAbs without significant sequence divergence. 2) The VH5-JH2 assemblage in early mAb 12B7 and several late mAbs. 3) The identical inferred amino acid sequence for H3 (CDR3 of the VH chain) was found in mid-mAbs Nort 5 and 8, and late mAB 4B6.A (figure 16). These meet our definition of recurrence in that they are from different mice to the same epitope. Early mAbs 12B7 and 5 late mAbs (C1.1. C1.2, C1.3, C1.4, C1.5) isolated previously (Zhong et al.,1994a), utilize a VH5 heavy chain

Table 7: Molecular profile of immunoglobulin V-genes used to target the Chlamydial MOMP epitope C-VD1 in BALB/c mice.

	CLONE	ISOTYPE	VH	DH	JH	VK	JK
E	AH2	G2a/k	Misc.	Q52?	?	Vk21	Jk2
	BC5	G2a/k	Misc.	Q52?	JH2?	Vk21	Jk2
	HC10	G1/k	Misc.	?	?	Vk21	Jk2
	12B7	G1/k	VH5	DSP2.8	JH2	VK21	Jk2
M	Nort2	G2a/k	?	?	?	?	?
	Nort5	G2a/k	VH1	DFL16.1	JH2	Vk21	Jk2
	Nort6	G2a/k	VH7	DSP2.10	JH4	Vk4/5	Jk2
	Nort8	G2a/k	VH1	DFL16.1	JH2	Vk21	Jk2
	Nort9	G3/k	?	?	?	Vk9	Jk2
	Nort10	G1/k	?	?	?	?	?
L	C1.1	G3/k	VH5	DFL16.1	JH2	Vk2	Jk1
	C1.2	G3/k	VH5	DFL16.1	JH2	Vk2	Jk1
	C1.3	G1/k	VH5	DFL16.1	JH2	Vk2	Jk1
	C1.4	G2b/k	VH5	DFL16.1	JH2	Vk2	Jk1
	C1.5	G1/k	VH5	DFL16.1	JH2	Vk2	Jk1
	C1.6	G1/k	VH5	DSP2.9	JH2	Vk2	Jk1
	C1.7	G3/k	VH9	DFL16.1e	JH4	Vk2	Jk2
	C1.8	G3/k	VH9	DFL16.2?	JH3	Vk2	Jk2
	5C6	G2a/k	VH2	?	JH2	Vk21	Jk2
	4B6.A	G2a/k	VH6	DFL16.1	JH2	Vk21	Jk2
	2C9.F	G1/k	VH1	DSP2.8?	JH2	Vk21	Jk2
	2D8.A	G3/k	VH8	DSP2.3 (2?)	JH1/2	Vk21	Jk2

CDR1

Vk2-like
ELVMTQSPLSLPVSLGDQVFISC<u>RSSQ T I VHS</u>*NGT* TYLE<u>WY</u>LQKPGQSPK
H-VLTQSPASLAVSLGQRATISY <u>RASKSVSTSSGYSYMH</u>WNQQKPGQPPR
Vk21-like

CDR 2   CDR3
Vk2-like
LLIY<u>KVSNRFS</u>GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYC<u>FQGS</u>
LLIY<u>LVSNLES</u>GVPARFSGSGSGTDF ILNIHPVE VEDAATYYC - <u>QHI</u>
Vk21-like

Vk2-like
<u>HVPWT</u>FGGGTKLE I K
<u>RELTRS</u>EGGPSWNKT
Vk21-like

Figure 15 : Alignment of the inferred amino acid sequence of the two dominant kappa

light chains used to encode VD1 antibodies in BALB/c. A portion of the consensus

nucleotide sequences for cDNAs of each type were translated in PCGENE and aligned.

Amino acid residues that comprise the CDR regions are underlined. These kappa light

chains have 65% nucleotide identity despite being derived from different Vk gene families.

Two sequon consensus sequences (N K/G T) are present. One in Vk2 CDR1 and the other

is inferred from the Vk21 gene and is downstream of CDR3. These have been bolded and

italicized and represent potential sites of N-glycosylation.

mAb	VH	H3	DH	JH
		92 97 115		
		⟵————————————————⟶		
Nort 5	VH1	VYYCARGYYYVSSYGSYYFDYWGQ	FL16.1	JH2
Nort 8	VH1	VYYCARGYYYVSSYGSYYFDYWGQ	FL16.1	JH2
4B6.A	VH6	VYYCARGYYYVSSYGSYYFDYWGQ	FL16.1	JH2
Nort 6	VH7	TYYCARDMRGTVYAL****DYWGQ	SP2.10	JH4
C1.3	VH5	MYYCARHNYGDRL***YYFDYWGQ	FL16.1	JH2

D

Figure 16: Homology in the VH-CDR3 (H3) regions of several monoclonal antibodies to C-VD1. Depicted above is the inferred amino acid sequence in the single letter code. Nort 5 and 8 are identical in both VH and Vk and are from the same mid-fusion suggesting they are clonal. Late mAb 4B6.A can not be clonally related as it is from a different mouse and a different fusion. The homology and the importance of CDR3 as an antigen contact domain suggests that these mAbs have been ligand selected for binding to VD-1. While the mid clones and 4B6.A are sequence identical at the amino acid level in CDR3 of the heavy chain, they have some differences at the nucleotide level further suggesting a functional selection of the expressed molecule. The C-VD-1 specific mAb C1.3 was isolated years earlier and has some homology in CDR3 as well.

V gene recombined with JH2.

(II) Discussion and Conclusions

We have directly examined the genetic basis for the phenotypic diversity of antibodies produced in response to the MOMP VD-1 epitope. The 14 VD1 specific monoclonal antibodies bind to the same nominal pin-peptide epitope and to the native MOMP molecule.

There appears to be a relationship between affinity maturation and the stage of fusion. The VD-1 mAbs have apparently undergone affinity maturation during the response to chlamydial MOMP shown by the selection of antibodies with improved binding affinity. This is consistent with studies on antibody responses to most other antigens, which have been shown to improve in both binding kinetics and affinity to an apparent affinity "ceiling" (Foote and Milstein, 1995). There is overlap in the range of whole EB binding-affinities of antibodies isolated from different stages of the immune response (Table 6). Similar exampls of affinity overlap have been seen in other antibody responses (Berek et al., 1985; Berek and Milstein, 1987). This may be the result of both external variation; such as bias in B cell sampling by hybridoma immortalization, and the potential effects of adjuvants in creating antigen depots capable of repeated boosting (Griffiths et al., 1984), as well as intrinsic differences; due to independent maturation of individual B cell clones relative to their precursor cells (Vora and Manser, 1995), and possible fluctuations in the available antibody repertoire in individual mice at the time of immunization.

Affinity for whole EBs is consistent with a trend in improved neutralization of in vitro infectivity. The relationship of affinity is consistent with improved neutralization of chlamydial infectivity in vitro (Table 6). For example late mAbs neutralized infectivity better than early mAbs at the same concentration. It is not clear however why late mAB 2C9, which has a high affinity for whole EBs and for the C-VD1 pin peptide (data not shown), neutralizes EBs with similar ND50 as early and mid antibodies. Others have similarly observed biological disparity in the comparison of antibodies of the same immunochemical specificity (Barnett et al.,1996; Greenspan and Cooper, 1995). It is possible parameters of antibody binding, such as on-rates (Vijayakrishnan et al., 1997), may correlate better with antibody-mediated neutralization. Although some exceptions exist, the mean affinity of VD1 mAbs increases as they are extracted later on in the immune response, which is consistent with helper T cell dependent affinity maturation. This is relevant to vaccine design. The demonstration of affinity maturation in our panel of VD-1 mAbs supports the use of MOMP as a vaccine candidate to which T cell dependent antibody responses are induced.

The anti-VD-1 antibody response is not dominantly restricted to a few VH genes. Information on epitope specific antibody responses to protein or glycoprotein Ags is surprisingly scarce. Analysis of the genetic basis for these antibody responses is important as protein antigens are constitutively borne on most pathogens, and many are targets of protective helper T cell dependent antibody responses (Ikematsu et al., 1998).

While the repertoire of VH genes appears to shift in its usage, the Vk gene repertoire to VD-1 is highly restricted in their usage. There is limited light chain diversity

in the VD-1 antibody response and 2 light chains are used in 18 of 22 total VD-1 specific hybridoma clones (Table7). Epitope specific restricted Vk usage has been found previously in several other anti-pathogen responses in mice (Akolkar et al., 1987;Patera et al., 1995), and in humans (Lucs et al., 1998). This suggests that the light chain can have an important role in antigen binding specificity. Strikingly, the Vk21 gene has been found to be used in several other murine antibody responses to epitopes on: collagen (Mo et al., 1993); the influenza A hemaglutinin Sb epitope (McKean et al., 1984); human CD4 (Lohman et al, 1992); red blood cells (Casadevall et al.,1994); and trichosanthin (Wang ad Yeh, 1996). It is dominant in response to influenza, predominat to human CD4 and collagen. This supports the theory of multispecificity by demonstrating that individual V-genes can encode antibody molecules that have a spectrum of binding reactivities. As an aside, trichosanthin is a potent cytotoxin related to ricin. Thus trichosanthin, or a homoloque, may represent one such antigen that is capable of exerting stringent and immediate selection for survival that is thought to be neccessary in order for antigens to shape germline genetic basis for host antibody repertoires (Weiss, 1993). The protective capacity of Vk21 may be evaluated by performing toxicity studies with trichosanthin in Vk21 knockout mice.

It is noteworthy that Vk21 has been found to recurringly encode antibodies to two pathogens, influenza and chlamdiae. It raises thee possibility that the amino acid sequence encoded by Vk21-JH2 has some general structural features that make it particularly well adapted for binding protein epitopes on pathogens. This may well be the case as Vk21 encodes a particularly well studied idiotype (Day, 1990) and has a groove

like structure typical of antibodies that bind to peptide epitopes (Bonnycastle et al., 1996). The fact that Vk21 is recurringly used to target the MOMP, as well as other antigens suggests that there may be some conservation between the epitopes borne on these antigens. Given this, it is possible (although unlikely) that there is some functional relationship between proteins used by a plant cytotoxin (with homology to other toxins including ricin and bacterial homologues such as Shigella toxin, Cholera toxin, Diptheria toxin;Zhang and Liu, 1992;), that acts to inactivate eukaryotic ribosomes, and the MOMP. It is particularly provocative when one considers the close proximity of chlamydial EBs to host ribosomes in ther intracellular environment. A quick homology search revealed that some domains on trichosanthin have homology to a 120-KD major structural protein of Rickettsia. The 120 Kd protein on this intracellular bacterial pathogen is organised into an S-layer perhaps suggesting a similar organization for the chlamydial MOMP (Gilmore et al., 1989).

Repertoire drift occurs in the antibody response to MOMP. Thus the genetic basis for affinity maturation to the chlamydial MOMP appears to involve the use of different VH-Vl pairings as the response matures, in addition to somatic mutation, consistent with our previous findings (Zhong et al., 1994a). This represents both repertoire shift and drift, similar to responses that have been seen to the Vesicular stomatitis virus glycoprotein antibody response as it matures in mice (Kalinke et al., 1997). This is also consistent with the results of Stark and Caton (1991) who found that new V-genes were recruited to an antigenically variable epitope of influenza hemagglutinin, rather than somatically mutated version of the same v-gene. The molecular anlaysis of V-genes to C-

VD1 directly corroborates the results of the OAS study which suggested genetic diversity in the antibody response to the native epitope. However, despite this diversity, we found several structural commonalitites between the antibody genes of different VD-1 hybridomas.

There may be a relationship between restricted kappa usage and VH usage. There was an epitope specific recurrence of certain antibody structures to VD-1. Three structural features were redundant. 1) The usage of the identical Kappa light chains. Vk21 was used in all early monoclonals and in four late mAbs. We found the presence of the same VK21 kappa chain structure from different mice at different stages. Epitope specific light-chain recurrence was found previously by Akolkar et al. (1987) in respose to a carbohydrate epitope.It is worth noting that experimentally induced restrictions in light chain diversity has previously been observed to lead to a shift in VH gene utilization in transgenic mice (Bot et al., 1996). Thus biased usage of Vk21 may effect the usage of particular VH-genes in response to MOMP.

(2) The recurrent usage of the VH5-like allele with JH2 in one early and in several late mAbs. The VH5-JH2 assemblage tolerates extreme junctional diversity. The antibodies isolated previously from the late stage (C1.1-5) use the same DH and JH minicassettes as well as the identical Vk assemblage with minor variation. This along with the fact that they were generated in a single fusion (albeit with splenocytes from two mice combined) makes it impossible to rule out a common clonal origin. Nonetheless, the early clone, 12B7, uses the same VH5 with JH2. MAb 12B7 is encoded by a different DH gene, which has been documented in anti-hapten (Gridley et al.,1985) and anti-influenza HA

Cb-site (Kavaler et al., 1990) antibody responses. VH5-JH2 dominance may be explained by preferential stimulation and expansion, perhaps due to higher affinity antigen receptors or expansion by cross-reactive antigens prior to immunization (Kavaler et al., 1990) On the other hand, the high prevalence of VH5-JH2 antibodies to the VD-1 epitope may be a consequence of the ability of this assemblage to tolerate great variability in the junctional regions and still retain specificity (Kavaler et al., 1990).

(3) The recurrent usage of the identical CDR3 region associated with middle stage mAbs Nort 5 and 8, and late mAb 4B6.A. These combinations used VH1 or VH6-like genes. Perhaps the VH6 -like gene is most interesting of these two. This is due to its low homology with any of the known V-gene families. Therefore we conclude that it is either a member of an as of yet unknown V-gene family, or is extremely mutated away from its germline counterpart. The latter is expected of clones such as 4B6.A which were created from B cells of hyperimmunized mice. These B cells are expected to have undegone extensive somatic mutation and selection.

The finding of the same H3 amino acid sequence in two different mAbs to the same epitope is remarkable. The V_H CDR3 (or H3) contains the highest genetic and structural diversity, and it is, structurally, the most important antibody binding domain in terms of antigen contact sites (Mariuzza et al., 1987; Casadevall and Scharff, 1991). However, there are other examples where the identical CDR3 region has been isolated in antibodies to the same epitope or to mimics of the same epitope (Sollazo et al., 1989; Caton et al., 1990; Garcia et al., 1992; Casadevall et al., 1994; Seidl et al., 1997). In some cases different D region nucleotides were found to encode the same inferred amino acid

sequence (Casadevall et al., 1994). Unsusually, there are even examples where individual minigenes are preferentially used (Siekevitz et al., 1983 and references therein). That minigenes can be important in antibody binding has been demonstrated in the anti-arsonate antibody response, where changing the J_H segement drastically reduced affinity (Duchosal et al., 1989). A study of the chemically and structurally complementary epitopes of guanosine and cytidine revealed that an antibody to an anti-cytidine molecule (an anti-idiotype antibody) had the same CDR3 paratope that was also used in an anti-guanosine antibody (Cottet andBordenave, 1994). Most remarkably the molecules used the same DH (D.SP2.2) and JH3 regions but with conserved differences in the actual nucleotide sequence suggesting a stringent functional selection for conservation of this paratope. Other examples of recurrent heavy chain CDR3 structures have been observed in antibodies to the same epitope in idiotype-anti-idiotype studies (Garcia et al., 1992) , and other anti-pathogen studies of antigenicity (Casadevall and Scharff, 1991). Recurrent H-3 structures have been proposed to be generated in a series of homology-directed rearrangements, without N-region diversification (Feeney, 1990; 1992). It cannot be concluded that the N-region additions at the 3' end of the H-3 of 4B6 and Nort 8 are indeed N-diversity because somatic mutation can be in the form of insertions (Wilson et al., 1998).

In summary, several forms of recurrent antibody response were found simultaneously. Few other examples of such a diverse yet patterned antibody response have been found with multiple recurring elements (Casadevall et al., 1995; Ikematsu et al., 1998) Other potential reasons for the recurrence of particular antibody structures will be discussed in more detail in the next chapter. The results are however, consistent with selective forces that act upon B cell during, and not prior to an immune response (Manser et al., 1984). That is some form of a B cell receptor and VD-1 ligand interaction which caused an expansion of clones with higher epitope affinity (Manser et al., 1984). Regardless of the mechanism for recurrence, there are now many additional examples of the usage of recurring antibody V genes in epitope specific responses to many antigens (Table 2).

At present, we do not know whether the mAbs isolated at the early stage (d7) represent early class switched IgG antibodies or if they pre-existed naturally prior to antigenic exposure (Mo et al., 1993). These mAbs are likely the result of T cell-dependent antibody response production. Although truly T-independent antibody responses can be highly restricted (Reininger et al., 1990), they do not undergo somatic hypermutation (Manser,). The anti-VD1 V-genes studied here and previously do contain mutation, and in some cases it may have been extensive as some have very low homology to the known germline copies of mouse V-genes.

While these data are provocative they do not allow us to unequivocally conclude that particular antibody genes are recurrently used for MOMP. In order to rule out other trivial explanations, for example, the potential selective effects of the hybridoma procedure, or the pin-peptide screening process. Thus an independent method of analysis was needed to study the relationship between the chlamydial MOMP VD-1 epitope and expression of particular host variable region genes.

OBJECTIVE 3: *The Relationship Between Chlamydial Antibody Responses and Recurrent Immunoglobulin Gene Expression*

(I) Rationale

Studies on the genetic profile of monoclonal antibodies to the VD-1 epitope of MOMP have suggested that there is recurrent usage of particular V gene assemblages by the host immune system to target an epitope in the MOMP of *Chlamydia trachomatis*. Using this information, we set out to explore whether the recurrent expression of these V-genes can be used as a marker for expression of an epitope specific antibody response (V-marker). Similar studies performed with other antigens and have found recurrent antibody responses to individual pathogenic epitopes (Casadevall and Scharff, 1991;Kofler et al.,1992 Kalinke et al., 1996; Seidl et al., 1997; 1997b; Nayat et al.,1998; table 2).

If a relationship between antibody gene expression and an epitope were to exist it seems most likely that it could be observed in antibody responses to pathogenic microorganisms. The identification of such relationships may be obscured by redundancy in V-gene usage due to massive size of the V-gene repertoire expressed in the available B cell compartment. Methods that select for the study of antibody molecules with a common phenotype, such as idiotype binding (Claflin and Berry, 1988), antibody competition (Roost et al., 1996; Kalinke et al., 1997), and specificity for diminutive epitopes (haptens or peptides; table 2) have in many cases identified antibody molecules that are recurringly encoded by a limited number of V-genes. Clearly, each of these in vitro selection methods introduces a certain degree of bias, and this may in some cases have directly skewed these epitope specific V-genes studies. Similarly, we cannot rule

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out that the hybridoma pin-peptide screening procedure may have introduced a bias in the V-gene structures we identified (above). Thus to further evaluate the relationship between V-gene expression and chlamydial antibody responses we felt the need to use a new approach. By modifying a previously published method for V-gene analysis (DeLassus et al., 1995) we developed the PCR-Run off blot. Indeed, DeLassus et al (1995) demonstrated that polyclonal antibody responses to a hapten, known to elicit a restricted antibody response, could readily be monitored for changes in expression levels of individual immunoglobulin V-gene alleles. Herein we describe the use of this method to compare relative expression levels of specific V-gene mRNA in the responding B cell compartment of immunized mice.

Antibody expression is upregulated through increased transcription. Activated B cells either begin an affinity maturation process, whereby some of its progeny enter the memory pool, or they terminally differentiate into a plasmablast. The plasmablast is a much enlarged cell where the endoplasmic reticulum and the nucleus are greatly increased in volume (Watson et al., 1992). This is in order to facilitate the huge increase in production of antibody molecules for secretion into the extracellular environment. Increased antibody expression is facilitated largely via mechanisms that govern transcriptional control (Max, 1993), and results in increased expression of the antigen specific immunoglobulin mRNA. Indeed, it is a physiological fact that responding plasmablasts increase the expression of their rearranged Ig mRNA 100-1000 times over baseline levels (Yuan and Tucker, 1984; Lefkovits, 1995). Thus the presence of a few antigen specific B cell blasts can contribute significantly to the expression profiles of

individual V-genes and the emerging clones can be detected in induced B cell responses (DeLassus et al., 1995). Similarly, upregulation of antigen specific mRNA in blast cells is believed to be responsible for the successful selection of recombinant mAbs in combinatorial antibody libraries produced from immunized animals as antigen specific mRNA is dominantly represented in the cDNA (Barbas and Burton, 1994a). The Run-off procedure (figure 17) compares relative mRNA levels in polyclonal bulk B cell populations through allele-specific PCR detection of the corresponding cDNAs. Thus we reasoned that recurrence could be evaluated by comparing the relative levels of expression of variable region mRNA in mice immunized with chlamydial vs other non-specific bacterial antigens. Splenocytes were collected from immunized and naive mice, and cDNA was generated from the isolated total RNA. Oligonucleotide primers were designed that anneal to the family specific 5' end of the relatively conserved framework 3 or framework 1 region of the heavy chain and kappa chain variable region alleles. These were used in combination with IgG constant region back primers. The primers were designed to span the V-C intron to eliminate any effects from contaminating DNA that may have persisted despite RNA purification steps. These products were used as template in a linear run-off step with biotinylated J-region back primers. The products of this run-off correspond to the CDR3 region of VH and Vk chains. The biotinylated CDR3 cDNAs were size separated using polyacrylamide gels that are capable of single base pair discrimination and blotted to nylon membranes. The relative levels of specific V-gene were visually detected by staining with an avidin-enzyme conjugate.
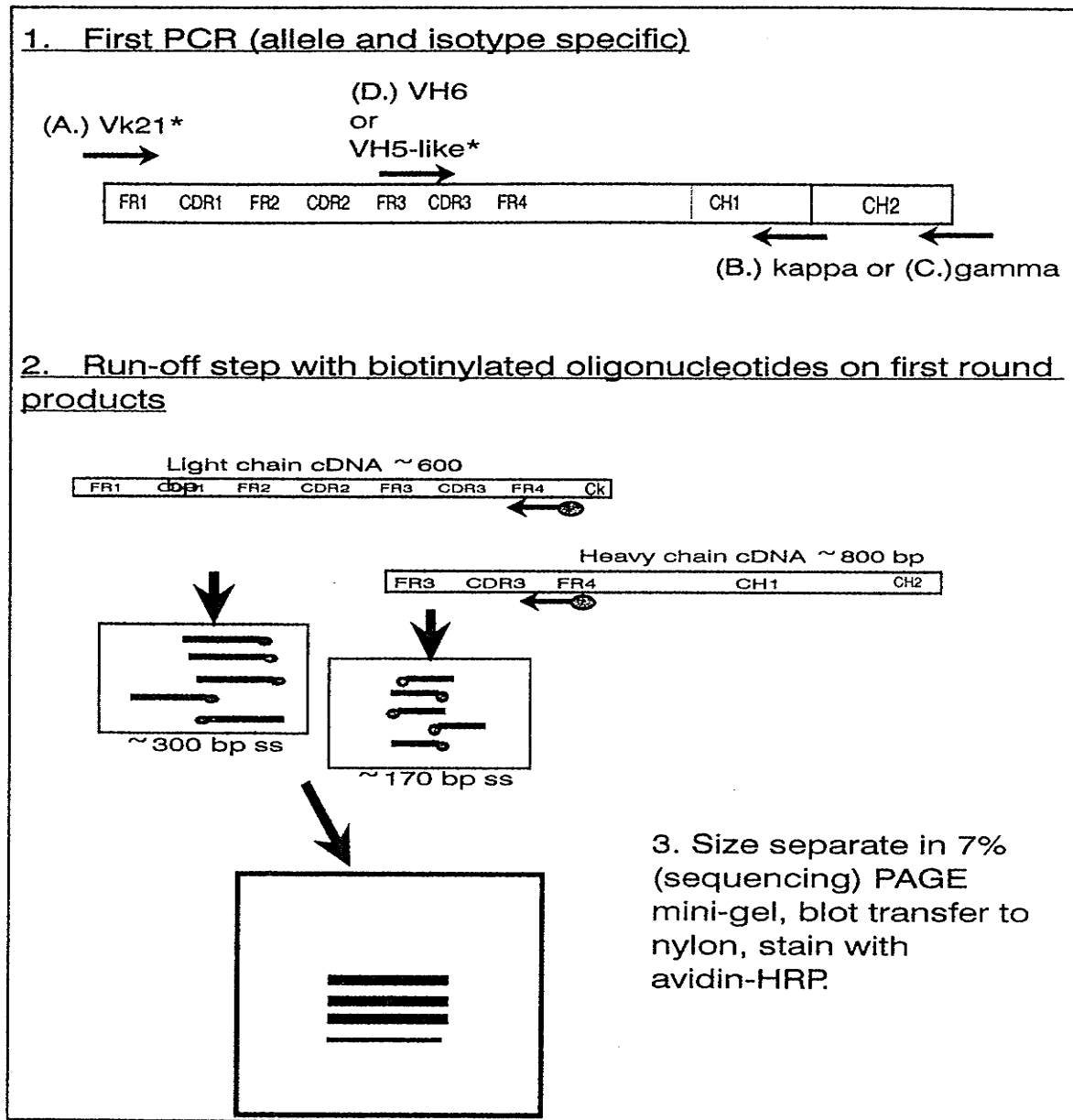
Figure 17: Schematic outline of the run-off blot procedure. Two rounds of enzymatic elongation are performed. Following the first round, the PCR products are passed through a G-50 sephadex column to remove most of the first round primers. In 2, the first round products are subjected to a linear run-off reaction with biotinylated J-region oligonucleotide reverse primers. Biotinylated run-off products are size separated and capillary transferred to membrane. The membrane is stained with commercial avidin-horseradish peroxidase conjugate in 2%-BSA, PBS. This procedure was modified from Delassus et al. (1995). The size separated products differ in size by as little as single base pairs.

Allele specific blots were performed on each of the three "V-marker" V-gene assemblies recurringly identified in CVD1 mAbs: (1) V_{H5}-J_{H2}; (2) VH6-J_{H2}; and V_{k21}-Jk2. The initial round of PCR was specific for the mRNA of V genes rearranged to IgG or Igk constant regions respectively. In this fashion the amplified cDNAs from the spleens of immunized annd naive animals were compared.

1.   *V-Marker Expression in Naive Mice*

In order to evaluate the baseline level of expression of particular V-gene alleles we performed a PCR-Run off blot on Naive mice. Oligonucleotides were designed based upon our monoclonal anti-VD-1 V-gene sequences and conserved constant region primers. The assay was optimized using splenic cDNA from mice immunized with serovar C EBs and has a sensitivity (where a band is visible to the naked eye) of less than 0.1 ng of amplifified cDNA (data not shown). The results of the Run-off assay on the 4 naive mice are shown in figure 18. For all three putative recurrent V-gene alleles a faint band of the correct size was visible indicating the presence and low level expression of rearranged alleles in naive mice.   Because we used purified RNA and our oligonucleotide primers spanned the V-C intron, we can conclude that these V-genes are present in the available repertoire, and can engage antigen.

Figure 18 (facing page): Baseline Variable Region Gene Expression in Naive Mice. (A)

The results of Run-off on Vk21-Jk2 V-gene cDNAs show very light and transient bands

similar in size to biotinylated Run-off products produced with purified Vk21-Jk2 cDNA

(indicated with the symbol +). This cDNA was derived from light chain cDNA of C-

VD1 hybridoma 5C6. (B) Run-off assay performed for the VH6-JH2 and VH5-JH2 V-

markers. Similar to A., very light bands were transiently observed for these cDNAs in

naive mice. This indicates that mRNA expression of these V-gene configurations is

detectable but at very low levels. Numbers represent individual mice.

Baseline Variable Region Gene Expression in Naive Mice

A. V_{K21} + J_{k2}

1   2   3   4   5   +

B. V_{H6} + J_{H2} V_{H5} + J_{H2}

1   2   3   4   5 | # 1   2   3   4   5

2.   V-Marker Expression in Mice Immunized with Bacterial Antigens

We next studied the expression of V_{H5-JH2}, V_{H6-JH2}, and V_{k21}-Jk2 V-gene assemblages of the IgG class in response to *C. trachomatis*. A clinical strain of *Pseudomonas aeruginosa* with a mucoid phenotype (Courtesy of Dr. James Karlowsky, University of Manitoba) and *Mycobacterium tuberculosis* (H37Ra) were used as control antigens because they are antigenically complex and are commonly pathogenic.   The PCR-Run off blots from mice immunized with bacterial antigens were performed as described and are shown in figures 19-21. Upregulation of each of the three V-markers, over baseline levels, was seen in most mice recieving serovar C EBs. Strong bands representing correctly sized biotinlylated CDR3 cDNA products were observed in response to immunization with EBs; For the VH5-JH2 Run-off, 7/8 mice had strong bands (figure 19); For VH6-JH2, 8/8 EB immunized mice (figure 20); and for Vk21-Jk2, 7/8 mice receiving EBs demonstrated increased expression levels. (figure 21). Clearly, there is a day-and-night difference between immunized and naive mice. Remarkably, B cells from mice receiving either *M. tuberculosis* or *P. aeruginosa* showed essentially background levels of the V-markers in the same assay (Figures 19, 20, 21). There was, arguably, an incremental increase in expression of the VH-6-JH2 marker in response to *P. aeruginosa*.

Figure 19 (facing page): Variable region gene upregulation of the VH5-JH2 marker. Strong banding is observed in the lanes containing the size-separated, biotinylated CDR3 cDNAs from 7/8 mice immunized with *Chlamydia trachomatis* serovar C. Predominant dark bands likely represent the effects of clonal expansions and/or plasmablat mRNA "jackpotting" with individual, identically-sized CDR3s. Run-off blots from mice receiving *M. tuberculosis* and or *P. aeruginosa* are shown on the bottom and do not demonstrate change from baseline. A fifth mouse (#5) immunized with *M. tuberculosis* antigens had similar results for this and the other V-markers (data not shown). Numbers indicate individual mice for groups receiving the same antigen as indicated above the lanes for this and the other figures that follow.

Figure 20 (second facing page): Variable region gene upregulation of the VH6-JH2 marker. Strong banding is observed in the lanes containing the size-separated, biotinylated CDR3 cDNAs from 8/8 mice immunized with *Chlamydia trachomatis* serovar C. Run-off blots from mice receiving *M. tuberculosis* and or *P. aeruginosa* are shown on the bottom. All three mice receiving *P. aeruginosa* demonstrate incremental increases in expression levels of this marker. A Run-off blot reaction was performed with 10 ng of heavy chain cDNA of the CVD1-specific hybridoma clone 4B6.A (VH6-JH2 encoded) in the lane marked "mAb". This was an internal control of the assay and blot-transfer procedure.

Figure 21 (third facing page): Variable region gene upregulation of the Vk21-Jk2 marker. Strong banding is observed in the lanes containing the size-separated, biotinylated CDR3 cDNAs from 7/8 mice immunized with *Chlamydia trachomatis* serovar C. Run-off blots from mice receiving *M. tuberculosis* and or *P. aeruginosa* are shown on the bottom and all at at baseline levels for this marker.

Variable Region Gene Upregulation:
VH5-like Heavy Chain Variable region genes recombined with JH2 (IgG)

Serovar C EB Immunized Mice

| #1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
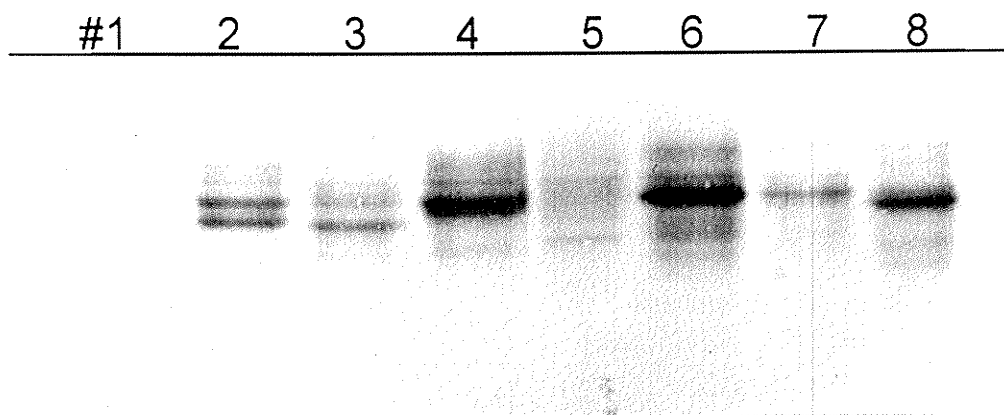
Control Mice:

M. tuberculosis antigens (H37 Ra, CFA)					Pseudomonas aeruginosa (whole organism)		
#1	2	3	4	empty	#1	2	3

Variable Region Gene Upregulation:
VH6-like Heavy Chain Variable region genes recombined with JH2 (IgG)

Serovar C EB Immunized Mice
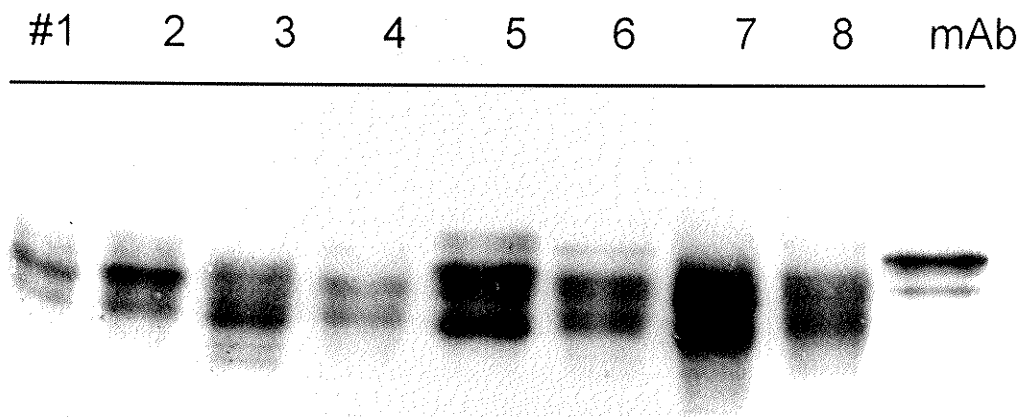
#1	2	3	4	5	6	7	8	mAb

Control Mice:

M. tuberculosis antigens (H37 Ra, CFA)					*Pseudomonas aeruginosa* (whole organism)		

#1	2	3	4	mAb	#1	2	3
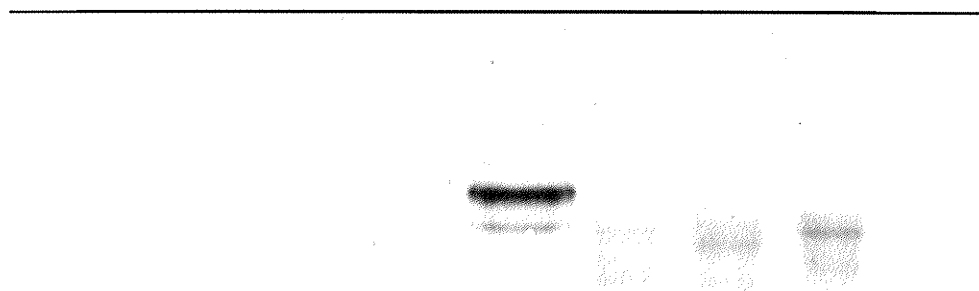
Variable Region Gene Upregulation:
Vk21-like Light Chain Variable region genes recombined with Jk2

Serovar C EB Immunized Mice
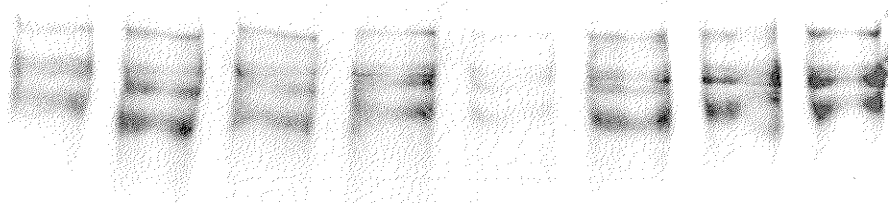
#1	2	3	4	5	6	7	8

Control Mice:

M. tuberculosis antigens (H37 Ra, CFA)					Pseudomonas aeruginosa (whole organism)		
#1	2	3	4	empty	#1	2	3

Next we evaluated the expression of other antibody gene mRNAs. We would expect that all of the immunized mice would demonstrate detectable expression of V-genes. Expression of other V-genes would also indicate that the B cells of these mice had been exposed to antigen, and were responding normally. We used universal consensus primers designed to anneal to most V-gene alleles (Dattamundjar et al., 1996) to measure pan-immunoglobulin gene mRNA expression in the B cell response. These primers anneal in the relatively conserved FR-1 region. Regardless of the antigen, expression of V-gene mRNA was observed in all immunized mice (Figure 22). This shows that antibodies encoded by other alleles are activated to *M. tuberculosis* and *P. aeruginosa* and that the differences in the allele-specific Run-off blots are due to differential expression of V-genes and not an artifact of RNA degradation or failed reverse transcription.

3.   V-Marker Expression in Mice Immunized wth Synthetic Peptides

Synthetic peptides were tested for the ability to induce expression of the V-markers in Run-off blot. In order to challenge our original hypothesis that "... an immunogenic epitope mimic should elicit the same variable region genes or a subset of the same genes that are used to bind the native epitope structure", we evaluated the molecular nature of the in vivo B cell reponse to synthetic peptide vaccine candidates in Run-off blot. The derivation and optimization of these peptides is discussed in Appendix

Figure 22 (facing page): Universal Variable Region Gene Expression in Mice Immunized

with *M. tuberculosis* and *P. aeruginosa*. (A) Universal kappa-Jk2 V-gene expression.

The results of biotinylated Jk2 primer Run-off on cDNAs amplified with universal kappa

primers. The Run-off products produced are similar in size to that produced using

purified Vk21-Jk2 cDNA with the same universal kappa Fr-1 primer (indicated with the

symbol +). This cDNA was derived from light chain cDNA of C-VD1 hybridoma 5C6.

(B) Universal Gamma-JH2 V-gene expression. The results of biotinylated JH2 primer

Run-off on cDNAs amplified with universal Gamma primers. This shows that

expression of rearranged V-genes other than the V-marker alleles can be detected in Run-

off blot. Numbers represent individual mice.

Variable Region Gene Upregulation

A. Universal Kappa + Jκ2 Vgene Expression

M. tuberculosis Ags   P. aeruginosa

\# 1   2   3   4   5   1   2   3   +

B. Universal Gamma + Jh2 Vgene Expression

M. tuberculosis Ags   P. aeruginosa

\# 1   2   3   4   5   1   2   3
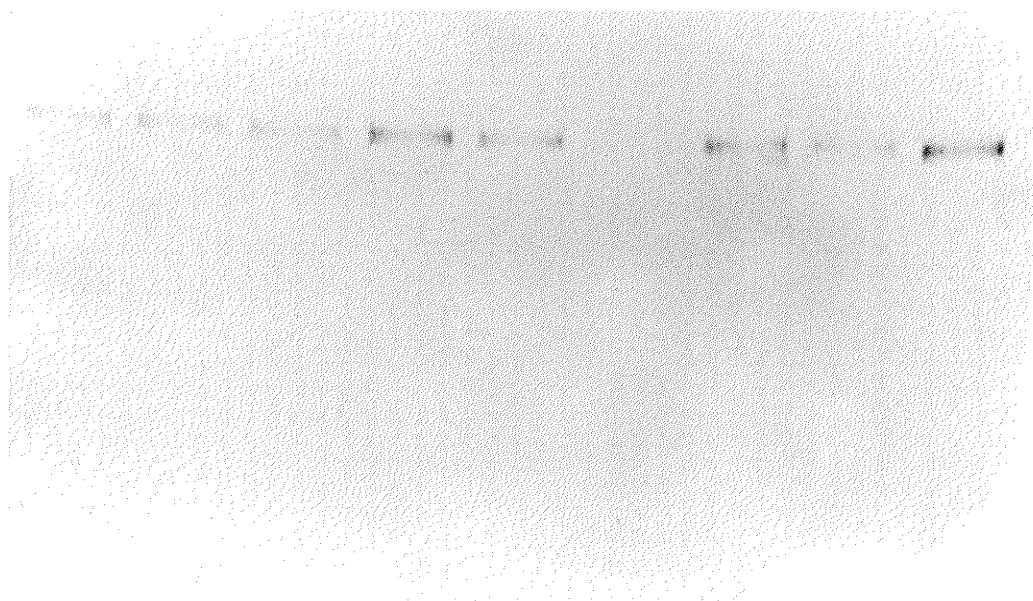
1. Peptides B-3-oxidized and B3-reduced are identical except that the former contains the

corresponding CVD-1 peptide, DVAGLQND, in a disulphide loop, and the latter is

linear. Both contain a promiscuous chlamydial helper T cell epitope. A similarly sized

linear peptide, 0921 (described in Methods, p. 145) with an irrelevant sequence was used

as a control.

The results of V-marker Run-off blot for mice immunized with synthetic peptides

are shown in figures 23-26. Remarkably, whereas complex whole bacterial antigens did

not trigger expression of the V-marker genes (above), the VD-1 epitope containing

synthetic peptides can do so. Indeed, 3 of 8 mice immunized with B3-reduced, and 4 of 7

that received B3-oxidized, showed increases over baseline in expression of the V_{H5}-J_{H3}

immunoglobulin gene marker (Figure 23). More striking, 6 of 8 mice immunized with

B3-reduced, and 3 of 7 that received B3-oxidized had elevated expression of the V_{H6}-J_{H2}

immunoglobulin gene marker (Figure 24). Finally, 5 of 8 mice receiving B3-reduced, and

5 of 7 mice that had been immunized with B3-oxidized showed increased expression of

the Vk21-Jk2 immunoglobulin gene marker (Figure 25). In contrast, mice immunized

with peptide 0921 had only baseline expression of each of these 3 V-markers. This is

despite the fact that peptide 0921 is vigorously antigenic, as shown by a high titre IgG

response in all mice receiving peptide 0921 in peptide ELISA (data not shown). This

strongly suggests that the increased expression of these particular immunoglobulin V-

gene configurations is specific for the presence of the C-VD1 peptide epitope. The results

of the Run-off assays have been summarized for clarity in Table 8.

Figure 23 (facing page): Variable region gene upregulation of the VH5-JH2 marker in response to the B3-peptides. Top: Prominent bands, indicating increased levels of size-separated, biotinylated CDR3 cDNAs, are seen in 3/8 mice immunized with B3-reduced (linear). Bottom: Prominent bands are seen for 4/7 mice receiving B3-oxidized (loop). Numbers indicate individual mice for groups receiving the same antigen as indicated above the lanes for this and the other figures that follow.

Figure 24 (second facing page): Variable region gene upregulation of the VH6-JH2 marker in response to B3 peptides. Top: Strong bands, indicating increased levels of size-separated, biotinylated CDR3 cDNAs, are seen in 6/8 mice immunized with B3-reduced (linear). Bottom: Prominent bands are seen for 3/7 mice receiving B3-oxidized (loop). A Run-off blot reaction was performed with 10 ng (top), or 5 ng (bottom) of heavy chain cDNA of the CVD1-specific hybridoma clone 4B6.A (VH6-JH2 encoded) in the lanes marked "mAb". This was an internal control of the assay and blot-transfer procedure.

Figure 25 (third facing page): Variable region gene upregulation of the Vk21-Jk2 marker in response to B3 peptides.Top: Strong banding, indicating increased levels of size-separated, biotinylated CDR3 cDNAs, are seen in 5/8 mice immunized with B3-reduced (linear). Bottom: Prominent bands are seen for 5/7 mice receiving B3-oxidized (loop).

Figure 26 (fourth facing page): Variable region gene upregulation in response to peptide 0921. Results of Run-off blot performed on cDNA of mice immunized with peptide 0921. Expression of Vk21-Jk2 (top), VH5-JH2 (middle) , VH6-JH2 (bottom) is at baseline (n=8).

Variable Region Gene Upregulation:
VH5-like Heavy Chain Variable region genes recombined with JH2 (IgG)

Synthetic Peptide Vaccine Immunized Mice

linear #1	linear 2	linear 3	linear 4	linear 5	linear 6	linear 7	linear 8

loop #1	loop 2	loop 3	loop 4	loop 5	loop 6	loop 7

Variable Region Gene Upregulation:
VH6-like Heavy Chain Variable region genes
recombined with JH2 (IgG)

Synthetic Peptide Vaccine Immunized Mice

| linear #1 | linear 2 | linear 3 | linear 4 | linear 5 | linear 6 | linear 7 | linear 8 | mAb |

| loop #1 | loop 2 | loop 3 | loop 4 | loop 5 | loop 6 | loop 7 | mAb |
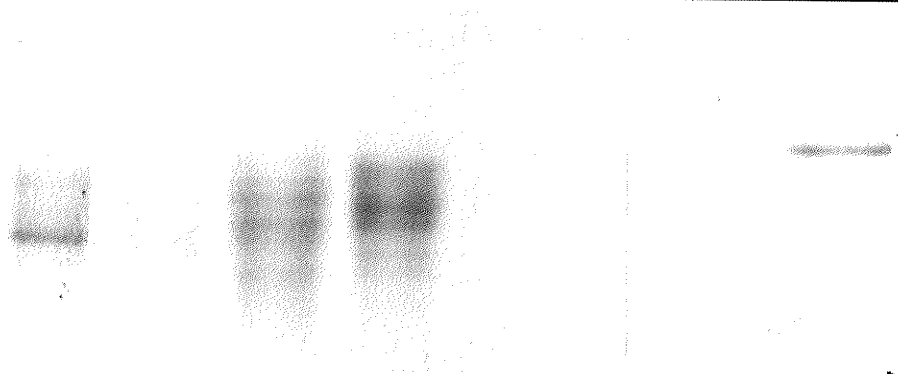
Variable Region Gene Upregulation:
Vk21-like Light Chain Variable region genes
recombined with JK2

Synthetic Peptide Vaccine Immunized Mice

linear #1	linear 2	linear 3	linear 4	linear 5	linear 6	linear 7	linear 8

loop #1	loop 2	loop 3	loop 4	loop 5	loop 6	loop 7
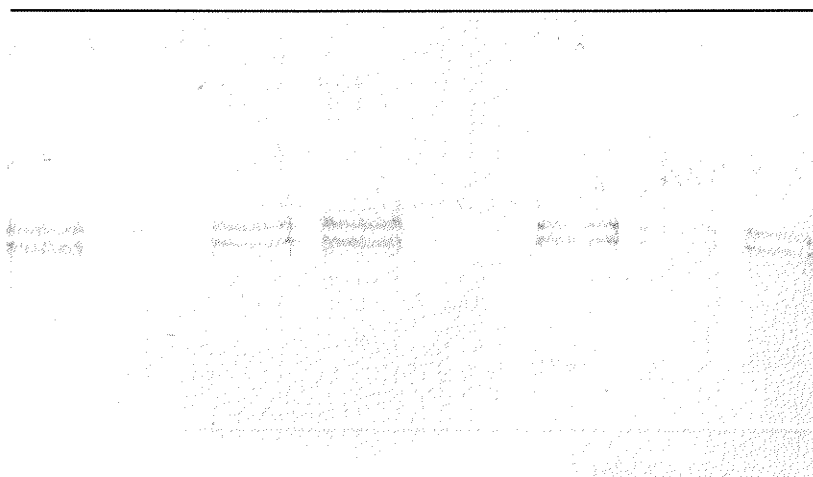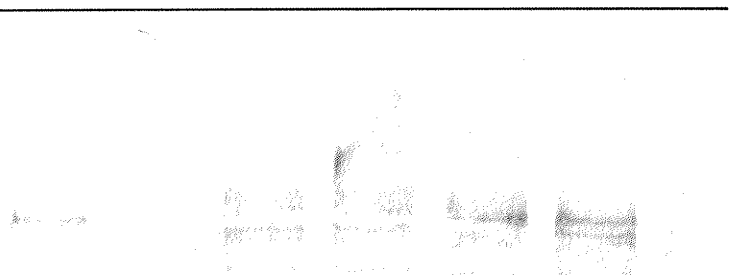
Variable Region Gene Upregulation

Synthetic peptide control 0921

K_{Z1} + J_{K2}

\#   1   2   3   4   5   6   7   8

V_{H5} + J_{H2}
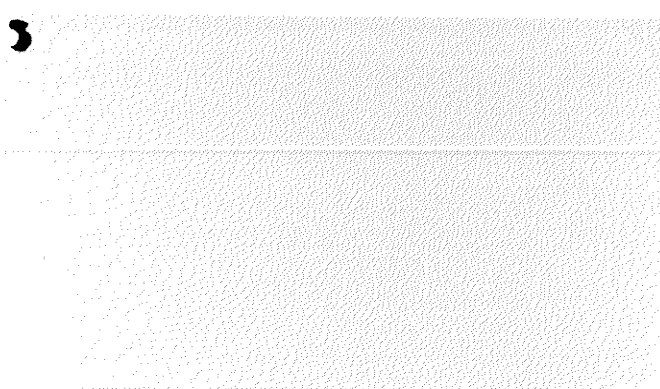
V_{H6} + J_{H2}
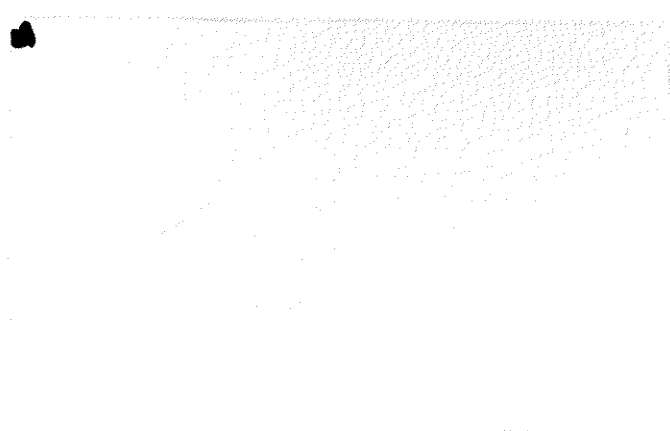
Table 8: Summary of Run-off Blot Assay:

Immunogen	VH5-JH2	VH6-JH2	Vk21-Jk2
Serovar C EBs	7/8	8/8	7/8
CFA (M. tuberculosis H37Ra, Ags)	0/5*	0/5*	0/5
P. aeruginosa (mucoid)	0/3	3/3 (weak)	0/3
Linear VD-1 Peptide	3/8	6/8	5/8
Looped VD-1 Peptide	4/7	3/7	5/7

* Mouse #5 not shown on blot.

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4. Immunochemical characterization of the antibody response

Sera from mice used for Run-off assay were evaluated in pin-peptide and standard ELISA for specific IgG reactivity. Mice immunized with bacteria were positive for homologous serum IgG reactivity in standard ELISA on whole chlamydial EBs, *M. tuberculosis* antigen, or whole *P. aeruginosa* organism (table 9). This corroborates the results of universal Run-off blot for mice immunized with *M. tuberculosis* and *P. aeruginosa* in that it shows that these mice did express antibodies in response to immunization but these were encoded by immunoglobulin V-gene comprised of V-genes other than VH5-JH2, VH6-JH2, and Vk21-Jk2. All mice immunized with serovar C EBs were positive for serum IgG against the C-VD-1 pin-peptides, with titres ranging from 1/200-1/800, compared to background levels in mice immunized with *M. tuberculosis* and *P. aeruginosa* (<1/50) (table 9). Serum IgG reactivity to chlamydial EBs and C VD-1 peptides correlated with upregulation or 2 or more V-markers assessed in individual mice (table 9). Serum IgG reactivity of B3 immunized mice were positive on VD1 pin peptide ELISA, and whole EB ELISA but are low (1/100 for each; data not shown). This was expected as the B3 synthetic peptides, like most synthetic peptides are poor immunogens (Fields and Chanock, 1989), and require multiple immunizations in order to generate high titre antibody. This has been studied in detail for the B3 peptide. The B3 peptides are both capable of inducing high titre pin-peptide and whole EB antibody responses that are neutralizing of i vitro cell infectivity (Appendix 1). A collective summary of the properties of antibody responses to these antigens is given in table 10.

Table 9: Relationship between serologial reactivity and V-gene expression

Antigen	MOUSE	VD-1 pin-peptide Titre	Homologous Titre*	V marker upregulation
C. trachomatis	CEB 1	200	800	2/3
	CEB 2	200	800	3/3
	CEB 3	800	400	3/3
	CEB 4	200	400	3/3
	CEB 5	800	400	2/3
	CEB 6	200	800	3/3
	CEB 7	800	400	3/3
	CEB 8	200	400	3/3
M. tuberculosis	CFA 1	<50	400	0/3
	CFA 2	<50	800	0/3
	CFA 3	<50	100	0/3
	CFA 4	<50	800	0/3
	CFA 5	<50	200	0/3
P. aeruginosa	PA 1	<50	200	1/3
	PA 2	<50	200	1/3
	PA 2	<50	200	1/3

*homologous titres were measured on bacterial antigens corresponding to the homologous bacterial strain/antigen used for immunizattion.

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Table 10: Summary of Properties of Antibody Responses

Immunogen	Antigenicity	Immunogenicity (to EBs)	Protective (in vitro)[1]	Induction of all V-Markers[2]
Chlamydial EBs	+++	+++	Yes	strong
B3-reduced	++	+	Yes	weak
B3-oxidized	++	+	Yes	weak
P. aeruginosa	+++	-	nd	no
M. tuberculosis	+++	-	nd	no

1 Synthetic peptides require multiple boosts to engender neutralizing tires.
2. strong: immunization elicits upregulation of 3/3 V-markers in most individual mice; weak: immunization elicits upregulation of 3/3 V-markers in some mice; no: immunization does not elicit upregulation of all 3 V-markers in any mice.

(II) Discussion and Conclusions

These studies show that several antibody V-gene assemblages are recurrently expressed in mice immunized with serovar C EBs. The V-genes assemblages identified in our study of monoclonal antibodies to C-VD1 (above) enabled us to directly examine the relationship between expression of these V-genes and antibody specificity in immunized mice. All mice immunized with *C. trachomatis* showed an elevated expression of at least 2 of 3 of the antibody gene markers as seen in Run-off blot assay. This corroborates the results of the mAbs study which suggested that although the MOMP epitope specific antiody repertoire was diverse, it shows patterned recurrent usageof particular V-genes. Indeed, 6 of 8 mice showed increased expression of all three markers over baseline in Run-off blot assay. This correlates with serum antibody reactivity to the C VD-1 peptide epitope and to whole EBs from all mice that received chlamydial EBs.

Upregulation of all three V-gene alleles is specific to immunization with *C. trachomatis*. There were no increase over baseline expression of our V-markers in mice immunized with *M. tuberculosis*.Likewise, none of the mice receiving *P. aeruginosa* showed increases in baseline levels of expression of the V_{H5}-J_{H2} and Vk21-Jk2 alleles. Although expression of one of the V- markers , V_{H6}-J_{H2}, is arguably increased over baseline for 3 of 3 mice recieving *P. aeruginosa*. This is consistent with the use of this V-gene to encode mAbs to outer membrane antigens of this similarly, gram-negative porin-containing eubacterium (Emara et al., 1995). In this fashion, the VH6-JH2 served as a positive control in our analysis of V_{H6} expression levels. Moreover, this finding supports the theory that recurrent expression of certain antibody genes occurs in response

to some microbial epitopes. We are not stating that these genes only encode chlamydial antibodies but that the collective expression of all of these markers may be chlamydia-MOMP specific. We expect that other unique patterns of V-gene expression predominate antibody responses to the dominant epitopes of all pathogens including *M. tuberculosis* and *P. aeruginosa,* and that they need to be only characterized. Recently, examples of diverse but patterned V gene usage in human antibody responses have been observed. For example, the immune response to rabies virus glycoprotein G, is composed of diverse lambda light chains, recurring VH3 genes, and restricted Vk gene usage (Ikematsu et al., 1998). We have demonstrated that this knowledge can be used for evaluating vaccine efficacy ex vivo at the molecular level. Similar PCR-based measurements of allele specific V-gene expression have been used for the study of human immunoglobulin (Rassenti et al., 1995) and T cell receptor repertoire diversity (Marguerie et al., 1992) ex vivo. Importantly, for the study of mucosal pathogens, these studies are amenable for usage on mucosal lymphocyte samples (Trentin et al., 1996). Practically, similar protocols are used clinically for the detection of B lymphoid clonality in lymphoproliferative disorders and malignancies of humans (Billadeau, et al., 1991; Deane et al., 1991; Aubin et al., 1994;Thunberg et al., 1997).

Similar and overlapping antibody V-genes are expressed to synthetic MOMP peptides. This supports the results of others who have recently observed that different subunit vaccine formulations elicit distinct but overlapping antibody repertoires (Adderson et al., 1998; Nayak et al., 1998). In our studies, both the linear and looped VD-

1 peptides were able to increase V-marker expression over baseline, and very little difference was seen in the ability of these constructs to do so. However, that both variations of the same peptide epitope can elicit the same antibody genes is consistent with the findings of Nayak et al. (1998). A comparison of the monoclonal antibodies raised to two forms of a synthetic peptide epitope, DPAF, revealed a remarkable homology. A subset of anti-looped peptide and anti-linear peptide mAbs (from different mice) was found to share a common heavy chain variable region.

The VD-1 peptides do not perfectly portray the native epitope. The synthetic peptides do not produce the same pattern of V-gene expression in MOMP specific V-markers. This may be due to differences in epitope valence beween peptides and the EB, adjuvant effects of LPS, or the limited structural information contained within synthetic peptides. Although the numbers are not large it is provocative to note that the synthetic peptides do elicit expression of least one V-gene known to be used against the native MOMP in all mice. This fact along with the structural homology of these peptide to the MOMP VD-1 epitope provides some structural basis to the previously observed biological mimicry shown by these peptides (Zhong et al., 1994b). This indicates that the synthetic peptides bear a resemblance to MOMP, and although this type of biological mimicry is not well understood (Paul, p460), similar host responses are engendered at the molecular level.

There are some important limitations to this analysis. These include: a) We do not know the binding specificity of the CDRs visualized in the blots. b) We do not know the sequence identity of the CDRs visualized in the blots. c) We do not know the frequency of B cells expressing particular V-genes in naive mice. Caveat a) can be tested via recombinant grafting and expression of the CDR regions. Alternatively B cells can be enriched for antigen specificty prior to performing V-gene analysis. Caveat b) requires cloning and sequence determination of JH2 run-off products for comparison with those V-genes used in VD-1 mAbs or in run-off products generated from EB immunized mice. Caveat c) is more difficult to overcome and represents a fundamental shortfall in the knowledge of the immune system. New technology, genome sequencing, functional genomics, and the DNA chip facilitate large scale analysis of gene expression. One day, this may lead to scientists being able to definitively sort out "fingerprints" of V-genes expressed in response to individual epitopes.

Antigen selection alone may not be totally responsible for the recurrent nature of some V-genes. SomeV-gene assemblages have been found at higher frequency in the B cell pre-immune repertoire (Paige and Wu, 1989; Cox et al., 1994; Weber et al., 1994). Indeed, Nadel et al. (1998b) suggested that exposure to environmental antigens had minimal effects on V gene representation for the kappa chain in adult humans, supporting the importance of the initial recombination events i determing the functional antibody repertoire. Moreover, in some cases these frequently made V-genes have been found to be used in some recurrent epitope specific antibody responses (Claflin and Berry, 1988). The high precursor frequency of certain V-genes may be mediated by differential

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efficiency of trancriptional promoters (Fitzsimmons et al., 1998), differences in DNA accessibility prior to recombination (Nadel et al.,1998b), or repertoire skewing by other environmental antigens like the B cell superantigens (Silverman, 1995). Indeed, the more non-germline elements required to generate a particular V-region structure, the lower the probability that such a V-region will be found in the available antibody repertoire (Feeney et al., 1988; Claflin et al., 1989). This is consistent with our sequence data for the Vk21 alleles which are commonly expressed in unmutated form. Limits on heavy chain junctional diversity have been implicated in recurrent anitbody responses (Manser, 1990). Indeed, in addition to antigenic selection, the high probability with which certain VH genes are formed during B cell differentiation may contribute to recurrent expression through non-random assembly of the same structures.

IV. OVERALL SUMMARY OF DISCUSSION AND SPECULATION:

These studies have revealed new and significant insights regarding the nature of host antibody diversity to a variable pathogen protein that are of general interest to the field of immunobiology and of particular interest for the field of chlamydial vaccine development. Both the polyclonal and monoclonal immunoglobulin responses to a neutralizing peptide epitope on the MOMP of *Chlamydia trachomatis* (serovar C) were comprehensively analyzed in Balb/c mice. The most significant conclusions include:

1. Heterologous sequential immunization with sero-related chlamydial strains broadens antibody reactivity both to the whole organism and at the single epitope recognition patterns.

The VD-1 epitope is an important target in secondary homologous and heterologous exposure to *C. trachomatis*. Antigenically drift variant MOMP molecules recall antibodies with enhanced crossreactivity from identically primed mice. The enhancement of antibody reactivity may have general use as a vaccination strategy against antigenically variable pathogens. Importantly, the selective recall of epitope specific antibodies in the OAS responses to VD-1 demonstrates the existence of a phenotypically diverse B cell pool to this epitope. This in turn suggests the existence of genetic diversity encoding these diverse fine specificities.

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2. Structural commonalities exist amoung genetically diverse but functionally

homogeneous antibodies to the identical MOMP VD-1 epitope of C. trachomatis.

To determine the strucutural basis of the anti-C-VD-1 antibody response we have

examined the paratypic and genetic diversity exhibited by a panel of 4 primary, 6

secondary ("mid"), and 12 tertiary ("late") response mAbs specific for this determinant.

Direct analysis of the genotypes of monoclonal antibodies to a neutralizing MOMP

epitope located in VD-1 has revealed that diverse genetic structures can encode antibody

that recognizes the same epitope. These data support the implication of genetic diversity

implied by the previous serological studies, and show that, indeed, diverse antibody

structures are recruited to the MOMP. V-gene sequence analysis of VD-1 monoclonal

antibodies shows that they are encoded by at least 16 different V_H gene segments from 8

V_H gene families, and 6 different V_k gene segments from 4 V_k gene groups. Despite the

apparent redundancy of these antibodies, genetic analysis of the V-gene sequences

expressed to VD-1 shows that there is a patterned usage of particular V-genes

assemblages with structural commonalities. These reccurent structures are in the form of

shared expression of V_H and/or V_k gene assemblages from VD-1 epitope specific

antibodies isolated from independent mice. For example, a V_{H5}-J_H2 assemblage, in

combination with disparate D_H minigenes, encodes an antibody found in both early and in

late clones. In fact, this V-gene pattern dominates the late panel of hybridomas

suggesting that clonal expansion and ligand selection was responsible for the clonal

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dominance of this particular V-gene assemblage. Other structural commonalities include the recurrent usage of the same germline V_k21-J_k2 light chain in all 4 early, 2 mid, and in four of 12 late clones. Remarkably, mid-clones Nort 5 and 8 and late clone 4B6, which utilize V_H genes from V_H1 and V_H6 in combination with a $D_HFL16.1$-J_H2 segment, respectively, have identical inferred amino acid sequence for the CDR3 domain of the heavy chain. The recurent isolation of genetically similar antibodies amongst this functionally homogeneous group of antibodies suggests that antigen-mediated clonal expansion is a powerful selective force upon B cell clones found within the host's antibody repertoire. Furthermore, these data suggest that expression of these genes may be useful as markers of an epitope specific antibody response to MOMP.

3. There is recurrent expression of particular immunoglobulin gene combinations in the host antibody response to C. trachomatis.

The recurrent expression of antibody gene combinations was specific to chlamydial organisms and was correlated with a measurable VD-1 antibody response in the serum. We used the structural commonalities we identified in VD-1 mAbs as molecular markers in a novel PCR-blotting procedure in order to examine the effects of immunization on the baseline mRNA expression of these potentially recurrent VD-1 antibody genes. The results show that indeed these clonotypes are common amongst the available B cell repertoire. Secondly, increased levels of rearranged V_H5-J_H2 , V_k21-J_k2 , and V_H6-J_H2 allele-mRNA were nearly universally observed in mice immunized with chlamydial EBs, but not in response to immunization with other bacterium. The increased

baseline expression of these V gene assemblages correlates with a measurable IgG titres to the serovar C VD1 pin-peptide in the sera. The common availability of B cells expressing V-gene structures that can encode MOMP specificity partially explains the immunodominance of this antigen in the anti-chlamydial antibody response. If a similar relationship is found in the human V-gene locus, we may conclude that there is enormous and inherent selective pressure for antigenic variation of this antigen. These data indicate that BALB/c mice have available a paratypically diverse repertoire of B cell specificities, encoded by distinct V-region structures, that are capable of recognizing a single epitope. However, these specificities are not equally represented in the immune response to the MOMP protein. Although these findings represent only an initial step in the study of chlamydial antibody gene markers, the identification of a relationship between a variable host and variable pathogen protein is provocative with respect to the role of natural selection on individual immunoglobulin genes within the V-gene locus. Knowledge of the specific patterns of host immune system genes expressed in response to invasion by specific pathogens may be useful in diagnosis, particularly with the advent of DNA array chips. These data suggest that an ideal vaccine should be able to elicit the expression of some of the recurrent antibody genes that encode antibodies to the authentic native epitope.

4. VD-1 synthetic peptide vaccines are capable of eliciting the same antibody gene combinations that was evoked by the native epitope.

The immunogenicity of conformationally different VD-1 synthetic pepide vaccines was evaluated in B10 H-2 disparate mice. Antibody in hyperimmunized mice neutralized in vitro infectivity of *C. trachomatis*. Remarkably, the surrogate VD-1 epitopes were capable of eliciting expression of the same antibody gene combinations used to the native VD-1 in some, but not all, of the mice, supporting our hypothesis. The evaluation of the ability of vaccines to trigger expression of specific host defence genes represents a novel method of measuring vaccine immunogenicity.In turn, the ability of the simple VD-1 peptides to elicit antibody structures used to the authentic VD-1 epitope supports the specificity of the V-gene reccurrence to the VD-1 epitope itself. However the presence of only marginal VD-1 peptide and whole EB reactive antibody suggests that the extent of biological portrayal, while not completely understood, is likely poor. This may be due to the selection of these peptide structures with a single monoclonal antibody which may have placed all of our eggs in a single basket. Alternatively, this may simply mean that the short synthetic peptides are not very good at portraying an epitope normally found in the context of an integral membrane protein on the outer surface of a bacterium.

5. Pathogen-Host Coevolution: Revisited

We have identified a relationship between the expression of a variable host and a variable pathogen genes. The genes encode the molecules involved in intimate interactions between host and pathogen. While in some cases, recurrent V-gene expression may be due to clonal expansion and / or structural limitations placed upon dominant B cell specificites, clearly there is selection on the immunoglobulin locus for survival of the whole organism (Smith et al., 1971) . The specific expression of the three V-gene assemblages (VH5-JH2; VH6-JH2; Vk21-Jk2) in response to chlamydial organism shows that B cells expressing these rearranged V-genes are commonly found within the available B cell repertoire of BALB/c. The common presence of these specificities suggests a functional role. Natural selection by common pathogenic structures, and or immunoregulatory network roles may make up this selective influence on the B cell repertoire (Andris et al.,1995). Indeed, more evidence is emerging to support the notion that particular V genes are maintained for rapid and high affinity protection against pathogens (Kalinke et al., 1997). The highly restricted V-gene repertoire of 3-week old rabbits has been postulated to encode antigen specificity to a major pathogen of young rabbits which may provide early protection from infection (Knight, 1992). Consistent with this speculation, some D-proximal V-genes (which are overrepresented in the neonate) have been found to encode some polyreactive antibodies (Holmberg, 1987; Bona, 1988) which may facillitate early protection to multiple pathogens in an underdeveloped immune system. Selection for the emergence of other V-

gene assemblages as the host develops may be less dramatic and influenced by somatic assembly mechanisms and affinity maturation. Many vertebrate antibody repertoires are limited in their usage of allelic V-genes through the biased expression of one or a few V-genes out of their respective total germline repertoires (Berens et al., 1997; Lopez et a.l., 1998; Sun et al., 1998).

The pairing of different VH and Vk in adult and neonatal mice has been shown to occur in a random fashion (Kaushik et al., 1990) and we believe that the recurrent V gene usage we observed is driven by B cell receptor ligand interaction. Bias in the recombination of VH genes in their relation to D proximity (Malynn et al., 1990) does not relate to the C-VD1 specific clones since the members of both VH5 (D-proximal) and VH 6 (D-distall) (see figure 2) were both found to have a recurring relationship with the MOMP epitope. Moreover, a position and age-related preferential gene rearrangement as described for the VH locus does not occur in the Vk locus (Teale and Morris, 1989; Lawler et al., 1989) and thus cannot explain the Vk bias observed. Restricted antibody gene usage has been suggested to be a property of immunodominant epitopes (Noronha et al., 1998).

V.   SUMMARY

In summary we have identified a novel relationship between a variable host and variable pathogen proteins. Through the study of antibody gene usage to a neutralizing epitope on the chlamydial MOMP we have not only defined the molecular basis for a classic serologic phenomenon termed "original antigenic sin" but have also uncovered a novel observation; namely that particular antibody genes are recurrently used in different hosts (of the same species) to respond to identical epitopes. This relationship, between a variable pathogen antigen (the MOMP) and variable host (immunoglobulin) molecules, supports the notion that there may be coevolution between antigenic determinants found on pathogens and the immune recognition molecules encoded within the host genome. This information has important applications in terms of vaccine design (for instance, an ideal vaccine should be able to recall those protective antibody gene combinations that are elicited by the authentic native epitope) and for the possibility to diagnose infectious diseases by the identification of specific antibody gene expression profiles found in host samples. To illustrate this we have compared the ability of MOMP synthetic peptide vaccine candidates to elicit expression of the same V-marker alleles.

Appendix 1

Recombinant and Synthetic MOMP VD-1 Peptides

Early success with whole bacterial cell vaccines has sustained the efforts to develop a chlamydial vaccine although these studies suggested that a subunit vaccine with enhanced immunogenicity would be necessary. In this regard, understanding the molecular basis of immune responses to native chlamydial antigens is fundamental in order to design a vaccine. This is important because some immune responses can be modified through advances in vaccine delivery and design such that an improved immunogenicity can be attained. Importantly, the native immune response can be used to guide the design of vaccines through comparison. In this section we discuss the optimization and immunochemical characterization of phage-borne VD-1 peptides as well as the evaluation of the optimized peptides without the phage carrier.

Immunization with Recombinant Phage-Borne Peptides:

The immunogenicity of several VD-1, conformationally-optimized phage-peptide clones were tested as described previously (Zhong et al., 1994b). Sera from mice hyperimmunized with phage clone #81 (optimized with a VD-1 mAb raised to native EBs) reacted with VD-1 pin-peptides (data not shown), to MOMP protein in western blot and to whole EBs in ELISA (Zhong et al., 1994b). Despite these immunochemical indicators of immunogenicity these sera did not neutralize chlamydial infectivity in *in vitro* cell culture (Figure 27). This paradox however, is consistent with batch variation of EBs observed in repeated ELISAs with the same sera (data not shown).
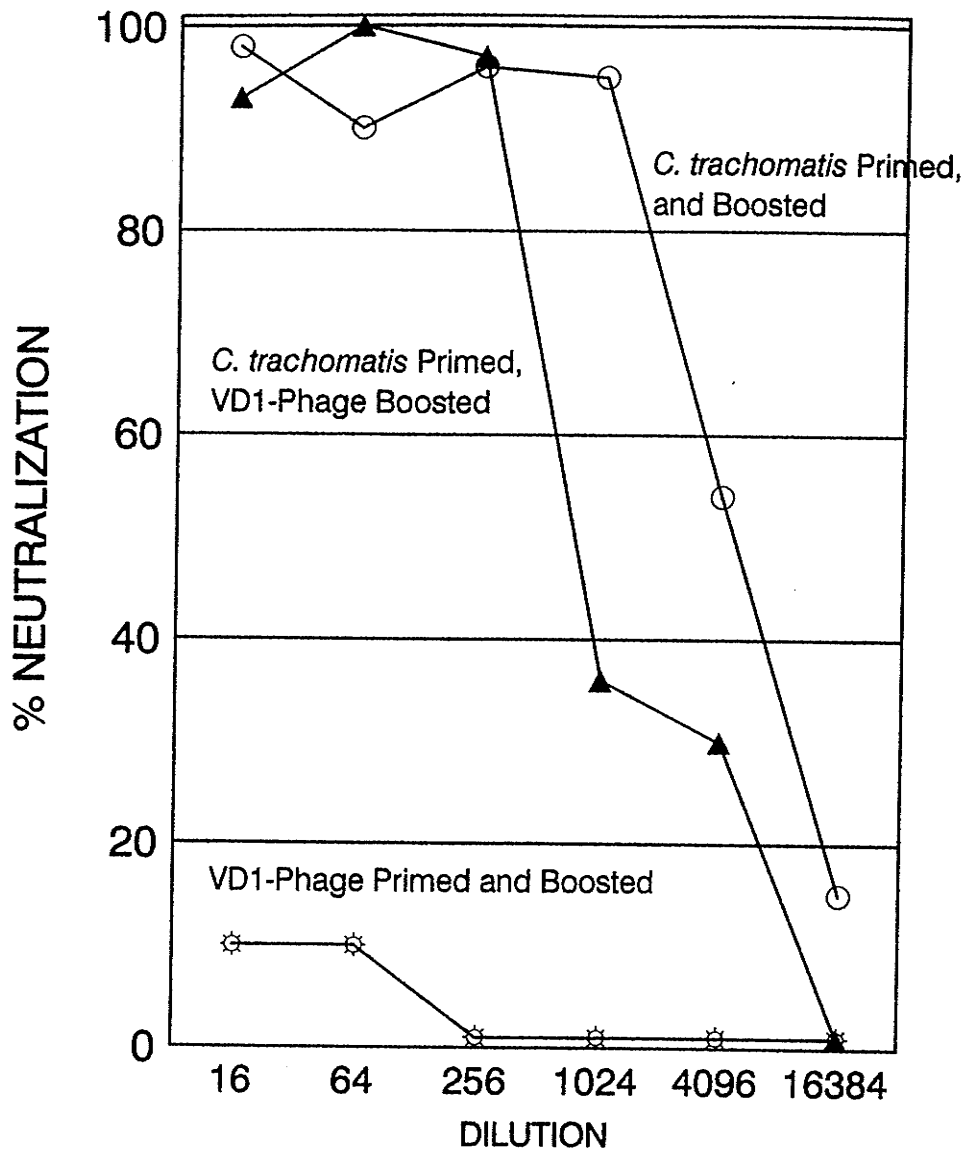
Figure 27: Neutralizing memory response recalled by VD1-Phage and homologous *Chlamydia trachomatis* (Serovar C) EBs in EB primed mice. Pooled sera from groups of mice (n=3-4) were analysed in triplicate in a blinded neutralization assay by Dr. H. Caldwell (NIH Laboratory, Hamilton, Montana). Pooled sera collected from mice that were de novo primed and boosted with C-VD-1 phage was not neutralizing despite having a 1/5000 titre for wild type M13 or recombinant VD-1 phage in ELISA (data not shown). In contrast, the neutralizing sera from EB primed and VD-1 Phage boosted mice (above) reacts minimally with wild-type M13 phage (1/200), but strongly with VD-1 phage (1/4000) in whole phage ELISA (data not shown) indicating that the neutralizing antibodies are specific to the VD-1 peptide borne on recombinant phage.

A possible explanation may be the recessiveness of the MOMP VD-1 epitope in the context of the native bacteriophage capsid proteins. Indeed, upon closer examination of the phage-VD1 sera using western blot, it is obvious that the recombinant pVIII peptides bearing the VD-1 epitope are only a minor component of a much larger immune reaction to native pVIII, pIII and other phage proteins (data not shown). Thus it seems that the population of antibodies raised in a de novo immune response to the phage peptide may be different than those raised against the native MOMP epitope owing both to the lack of MOMP structure or the presence of phage proteins or both (This increased our need to examine the molecular characteristics of anti-MOMP antibodies). Similar disparity between de novo and booster immunogenicity has also been observed in response to a TMV virus decapeptide epitope (Rennick et al., 1980). Notably, another paradoxical seeming reactivity was observed between immunochemical analyses and protective properties of antibodies induced to peptide epitopes of rhinovirus type 2 despite an apparent homogeneity in recognition of a nominal peptide epitope of viral origin (Barnett et al., 1993; 1995; and Barnett et al., 1996). In the case of the VD-1 epitope, some of this disparity may originate from the use of single mAb structures for the optimization of a vaccines. The single specificity of the mAb used for such an affinity selection may place all of our eggs in a single basket. On the other hand this does not preclude the finding that anti-MOMP VD-1 antibodies can bind to phage-borne VD-1. This suggests that the conformation and context of the VD-1 epitope in native and phage settings when tested in vitro creates the perceived one-way reactivity with polyclonal sera. That this is indeed the case is supported by the ability of the phage borne VD-1 construct to recall neutralizing

antibodies from mice primed with the native MOMP (figure 27), that react with the MOMP protein in western blot and boost pin-peptide serum IgG titers (figure 28). This is consistent with the observed ability of a recombinant fusion protein to recall protective antibody against morbillivirus (Obeid et al., 1995).. Thus the context of the phage carrier can impact negatively on the immunogenicity of the recombinant VD-1 peptide. The absolute representation of recombinant pIII proteins is unknown, and may maximally be 10-20% of all copies of the native PVIII phage protein (Dr. Guangming Zhong, University of Manitoba; Greenwood et al., 1991). While secondary immunization with wild type phage shows that the booster effect is peptide specific (Figure 28), we did not compare the ability of looped versus linear phage borne peptides to produce this boosting effect. Thus we do not know if conformational constraints improve the ability of a peptide to recall a secondary MOMP antibody response. Clearly more studies on this would be valuable Other studies have found that polypeptides borne on surrogate carriers are not always highly immunogenic (Schreuder et al., 1996). This contrasts still other studies that have found that phage-borne peptides may be imunodominant on the phage (Greenwood et al., 1991; Willis et al., 1993), although the immunogenicity of the constructs for the native epitopes were not detailed. Thus the properties of individual peptide epitopes are unique, possibly due local topological influences or close proximity of other surface molecules, and therefore precludes generalization.

Figure 28 (facing page): Phage peptide recall of MOMP reactive antibody. Sera was collected and pooled from groupd of BALB/c mice (n=3-4) 7 days following boost with either serovar C EBs, VD1-phage clone 81, or wildtype M13 phage. Sera were tested in VD-1 pin peptide ELISA and whole EB western blot. A representive pre-boost reactivity is shown in the top panel. Notice the minimal MOMP reactive staining in the western blot and a VD-1 pin titre of 1/300. Boost with C-EBs (bottom, left) or VD-1 phage(bottom, middle) recalls high titre VD-1 pin IgG in sera (1/6000 and 1/5000 respectively) and markedly increases the antibody staining of the MOMP. Booster immunization with wild-type M13 phage (bottm, right) does not recall high titer IgG to the VD-1 pin-peptide nor does it change MOMP staining in western blot compared to pre-boost levels.

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EPITOPE SPECIFIC RECALL CAN BE SEEN AT THE WHOLE PROTEIN AND PIN PEPTIDE EPITOPE LEVEL

Rested (pre-boost) Sera

←— MOMP

C. trachomatis Boost

7 DAYS

VD1-Phage Boost

7 DAYS

WT-Phage Boost

7 DAYS
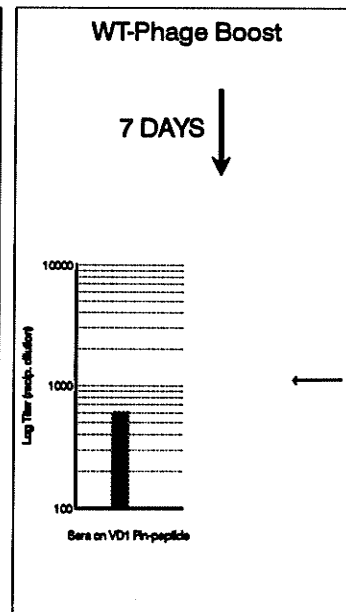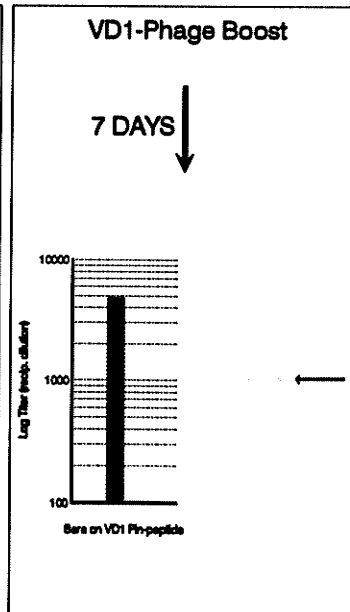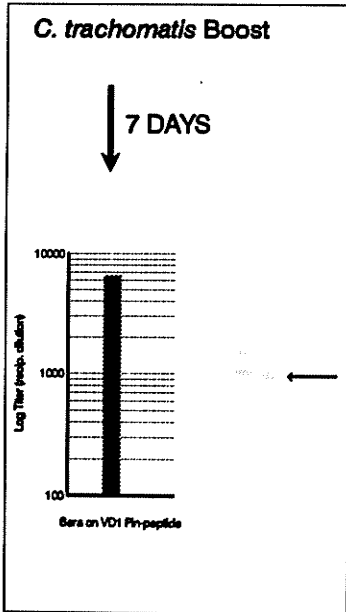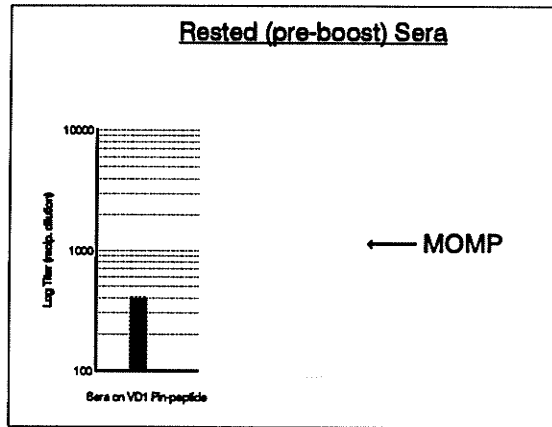
Conformational Optimization of A VD-1 Epitope Functional Mimic Through

Affinity Selection

The structure of the B-cell peptide epitope was optimized by affinity selection, with a conformationally-dependent monoclonal antibody to the native MOMP epitope sequence using randomized epitope display libraries on filamentous phage (Zhong et al., 1994b). The optimal phage-borne epitope was found to be flanked by two cysteine containing oligopeptide sequences:

-D-C-W - MOMP epitope (12-mer) - **W-C-W-** .

The Effects of Conformational Constraints on the Antigenic and Immunogenic

Properties of a Synthetic Peptide Vaccine

Six inbred strains of mice and BALB/c (Table 11) were immunized as briefly described. Groups of four B10A.sgsnj, B10D2/j, B10.BR/sgsnj,C57BL/10snj, B10.AKM/srj (a panel of H-2 disparate congenic mice), and Balb/c mice were pre-bled several days prior to immunization. Each groups received either a peptide (25 ug) , or Serovar C EBs in IFA, in 200ul volume, via intraperitoneal inoculation. Mice were boosted on day 14 and day 35 with 25 ug peptide or EBs and bled via tail vein on day **. Whole blood was incubated at $37^{\circ}C$ for 1 hour and then at $4^{\circ}C$ overnight. The serum was drawn off of the pellet following a brief 10 minute spin at top speed (14,000 X g) in an Eppendorf microcentrifuge at $4^{\circ}C$. Equivalent amounts of sera from each mouse was pooled according to group, and stored at $4^{\circ}C$ prior to analysis.

The oligopeptide vaccines are immunogenic in all B-10 congenic mice suggesting that the A8 TH epitope is indeed capable of engendering help in a variety of H-2 contexts (figure 29a and b).

The oligopeptide vaccines are immunogenic in all B-10 congenic mice suggesting that the A8 TH epitope is indeed capable of engendering help in a variety of H-2 contexts (figure 29a and b).

The looped peptide appeared to be less immunogenic in the B-10 D2 (H-2d) strain than the linear oligopeptide suggesting that the loop structure may affect immunogenicity in this one inbred strain.

Importantly, the looped peptide construct was able to engender antibody responses that were more cross reactive to the native epitope on the surface of serovar C EBs than was the linear oligopeptide. These results can be expressed as an immunogenicity ratio with the EB crossreactivity of the looped peptide as the numerator and that of the linear peptide as the denominator. As shown in figure 30, this ratio for the looped oligopeptide ranged from 1 to 70, and in 4 of 6 strains of mice exceeded unity.

The pooled antisera raised to the looped oligopeptide neutralized chlamydial infectivity two-fold greater than the anti-linear peptide sera (Figure 31). The titer of the anti-looped and anti-linear peptide sera which produced 50% reduction in infection were (IF_{50}) 1/500 and 1/250 respectively. This suggests that the constrained peptide is a better mimic of the native epitope. However this difference between looped and linear sera neutralization was not statistically significant. Moreover the VD-1 peptide titre in polyclonal sera does not correlate with in vitro neutralization. This shows that the peptides are not verbatim mimics of the MOMP VD-1 domain and that only a portion of the anti-peptide antibody is capable of potent neutralization. These or similar peptides may be useful for boosting of antibody responses in an epitope specific fashion.

Table 11: Strains of mice. The strains of mice used to studyB3 peptide immunogenicity are shown.

STRAIN	H-2	Number of mice per immunogen			
		B3-PEPTIDE (OXIDIZED, LOOPED)	B3-PEPTIDE (REDUCED, LINEAR)	B6-PEPTIDE (LINEAR, CYS ALA)	SEROVAR C (EBs)
B10A.sgsnj	a	4	4	4	4
B10D2/j	d	4	4	4	4
B10.BR/sgsnj	k	4	4	4	4
C57BL/10snj	b	4	4	4	4
B10.AKM/srj	m	4	4	4	4
BALB/c	d	4	4	4	4

50ug/mouse/injection, 5X106 EBs/ injection, 3 injections (d0, d14, d28 than bled 14 days later)
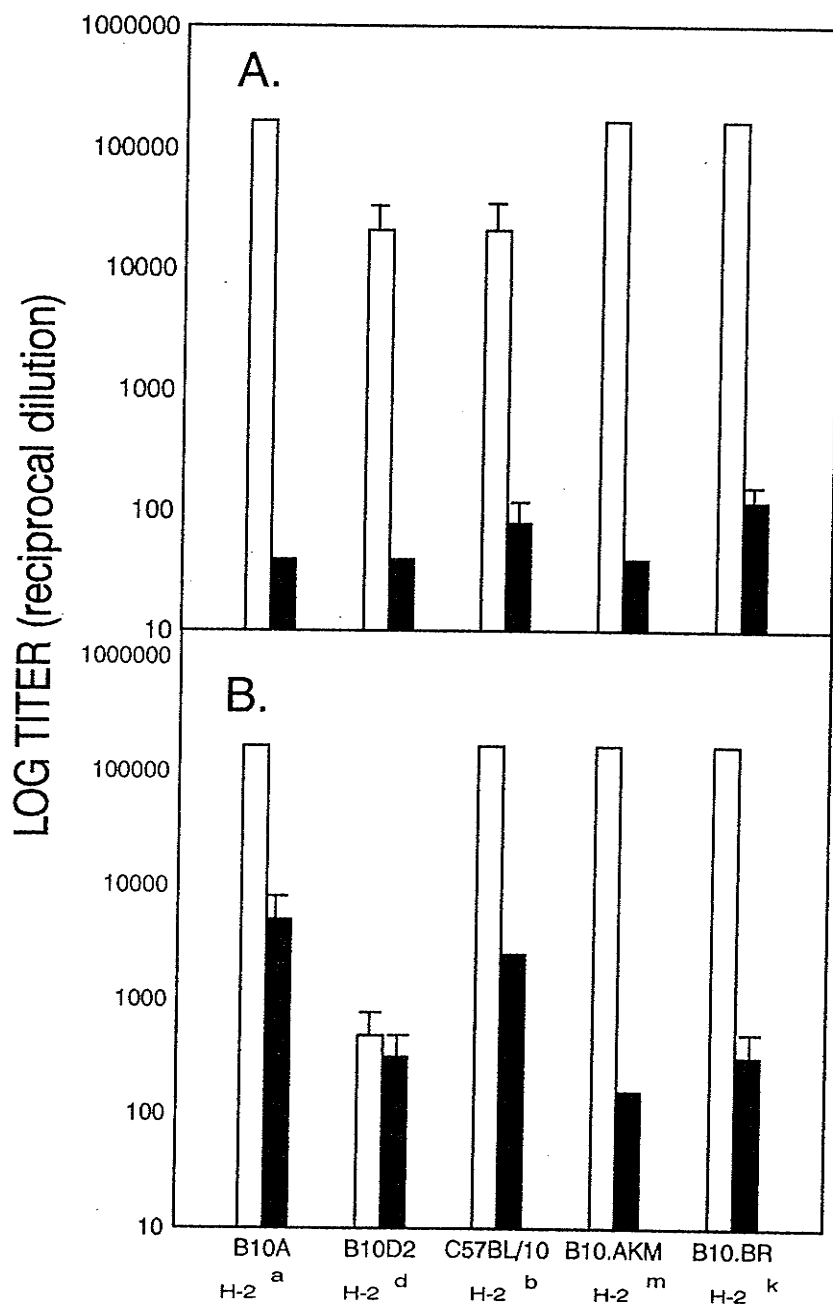
Figure 29: Homologous B3 oligopeptide immunogenicity (white bars) and EB crossreactivity (black bars) in MHC disparate B10 mice. Pooled serum IgG was tested in ELISA on (A.) linear and (B.) looped B3 synthetic peptides. In general the serum raised to the looped B3 construct were more crossreactive with whole chlamydial EBs.
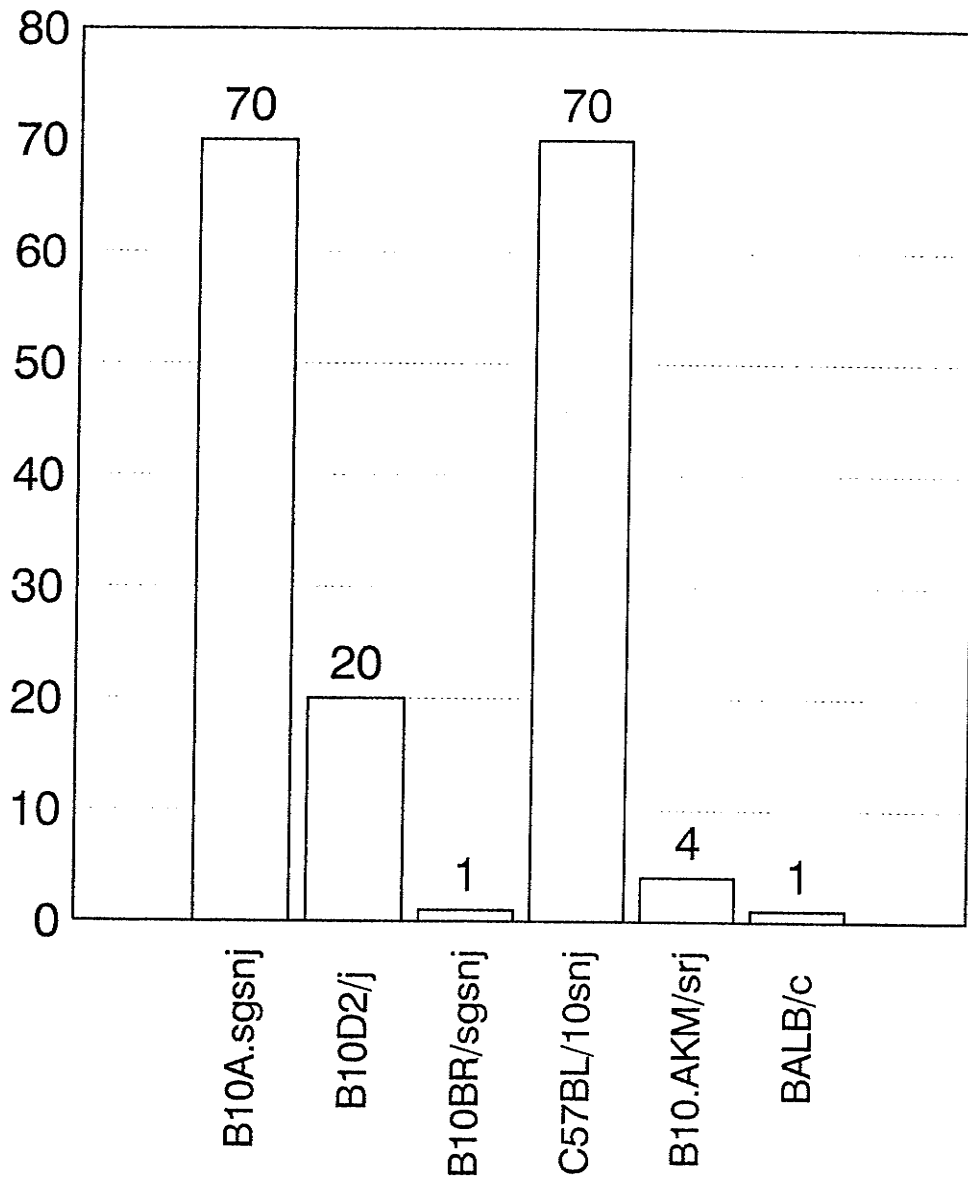
Figure 30:B3 Peptide conformation immunogenicity ratio. Looped and linear peptide immunogenicity was determined by anti-native EB titer raised by peptide divided by anti-native EB titer raised by EB in the same strain.
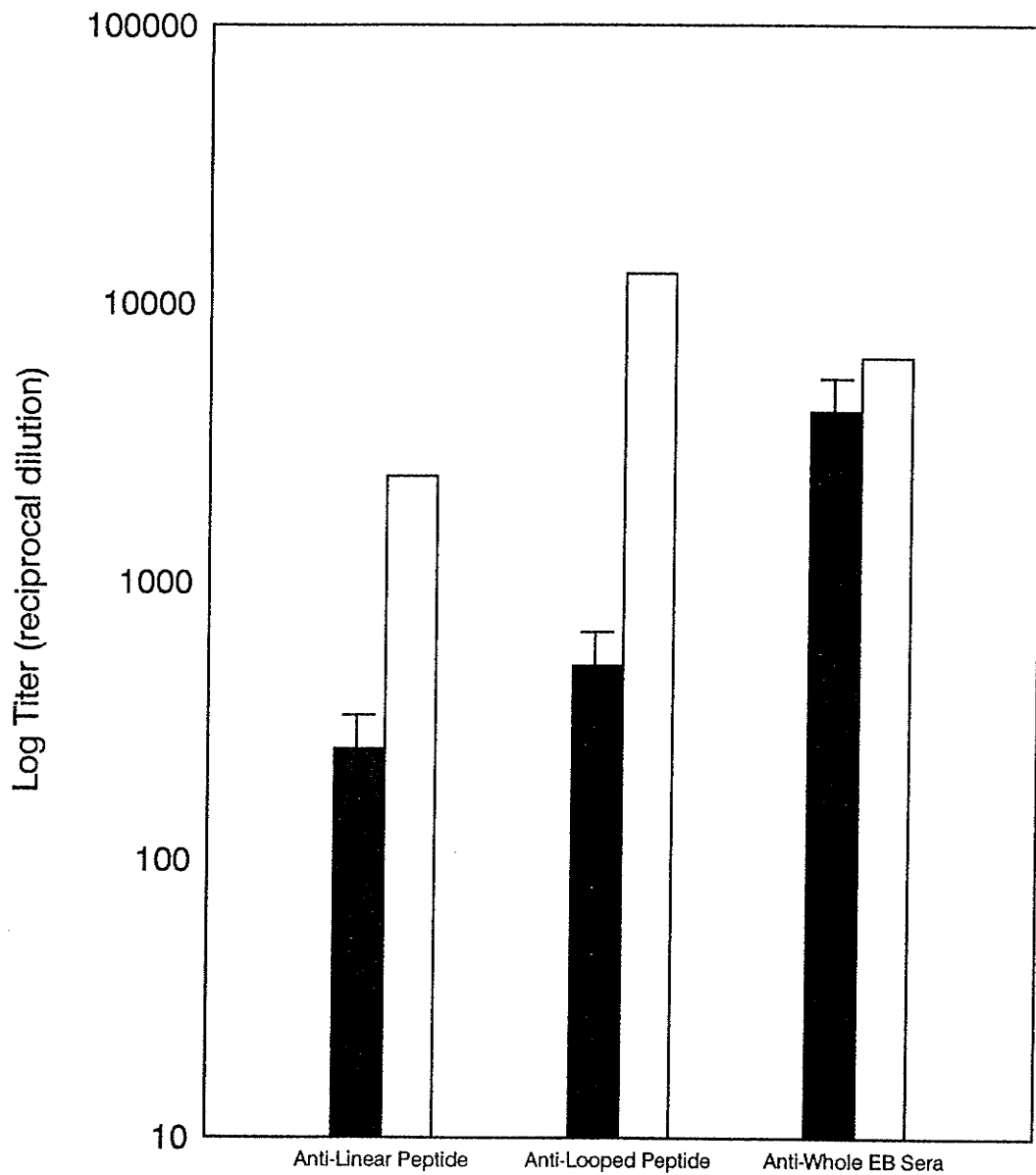
FIGURE 31: B3 peptide anti-sera in neutralization assay. The neutralizing serum IgG titre of pooled anti-serum (equal amounts from all H-2 groups) raised to B3 peptides that produced a 50% reduction in infectivity of *C. trachomatis* in Hak cell assay is shown (black bars; IF$_{50}$). The same sera was tested on C-VD-1 pin-peptides for IgG end-point titres (white bars). The geometric mean whole EB crossreactivity produced in B10 mice immunized with linear vs looped B3 peptides correlates with neutralization and CVD-1 pin titres and are approximately 1/60 and 1/740 respectively. If$_{50}$ titers were determined by triplicate cultures in which preimmune sera was used as control. Pin-peptide ELISA data are reprsentative of three other assays with similar results.

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Appendix 2

Examination of cDNA amplifications produced for substrates in the Run-off Blots.

Contained herein are the EtBr stained agarose gel analytical gels of the products

of the first round of allele specific amplification performed on splenic cDNA isolated

from large numbers of individual mice for Run-off blot assay. All of the PCR reactions

produced bands of the correct size representing Ig V-gene cDNAs (807 bp for the heavy

chain cDNA produced with framework 3 primers and the IgG-3' primer that anneals to

the 3' end of CH2 of all IgG isotypes; 600bp for the light chain cDNA with framework-1

primers and the Ig-kappa-3' primer that anneals to the 3' end of CH1). This confirmed the

success of the RNA isolation and cDNA production for all mice, and that the PCR

products all contained amplified V-gene cDNAs (albeit of unknown assemblage), prior to

J-region run off assay.

Figure 32 : Allele specific PCR in Naive mice.

Figure 33: VH5-IgG amplifications in immunized Mice.

Figure 34: VH6-IgG amplifications in immunized Mice.

Figure 35: Vk21-IgK amplifications in immunized Mice.

VH5 FR3 - IgG - 3` (~ 800bp)

CEB   Tb Ag   P.A.Ag

+ 1 2 3 4 5 6 7 8 1KB 1 2 3 4 5 1 2 3 1KB

VH5 FR3 - IgG - 3` (~ 800bp)
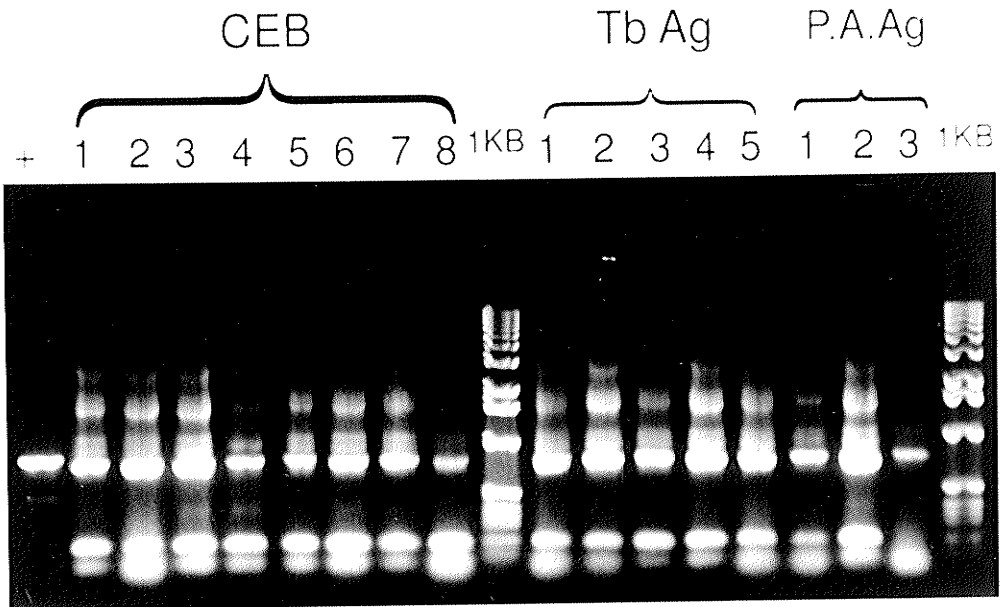
Looped - VD1   Linear - VD1

1KB 1 2 3 4 5 6 7 + 1KB 1 2 3 4 5 6 7 8 +

VH6 FR3 + IgG - 3` (~ 800bp)

C - EB   1KB   Tb Ag   P.A.Ag   1KB

+ 1 2 3 4 5 6 7 8   1 2 3 4 5   1 2 3

VH6 FR3 + IgG - 3` (~ 800bp)

Looped - VD1   Linear - VD1

1KB   1 2 3 4 5 6 7   1KB   1 2 3 4 5 6 7 8 +

VK21 - FR1 + 3`K (~ 650bp)

C - EB   1KB   Tb - Ag   P.A.Ag

1 2 3 4 5 6 7 8   1 2 3 4 5   1 2 3

VK21 - FR1 + 3`K (~ 650bp)

Looped - VD1   Linear - VD1

1KB   1 2 3 4 5 6 7   1KB   1 2 3 4 5 6 7 8
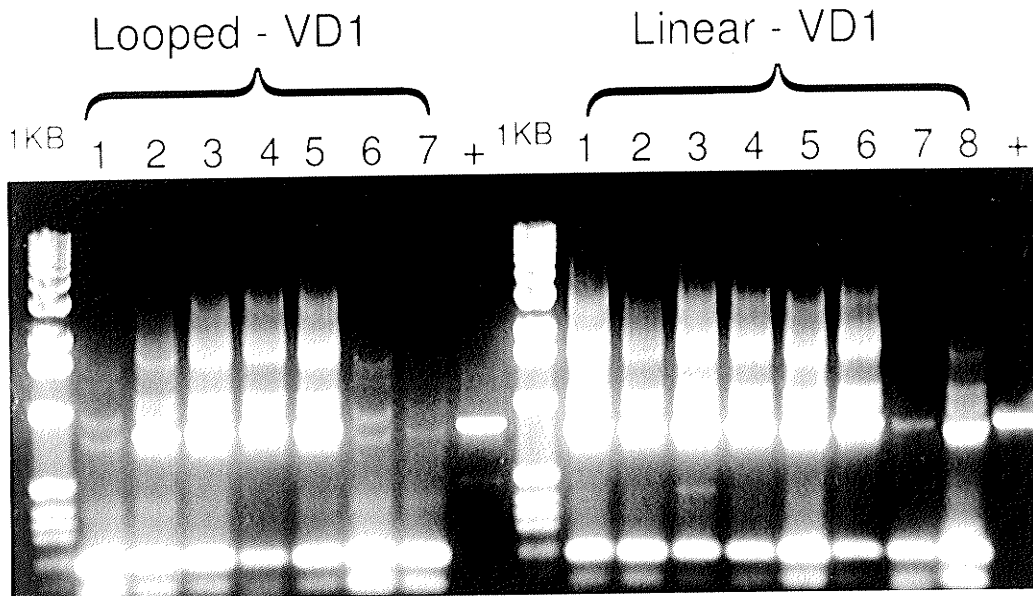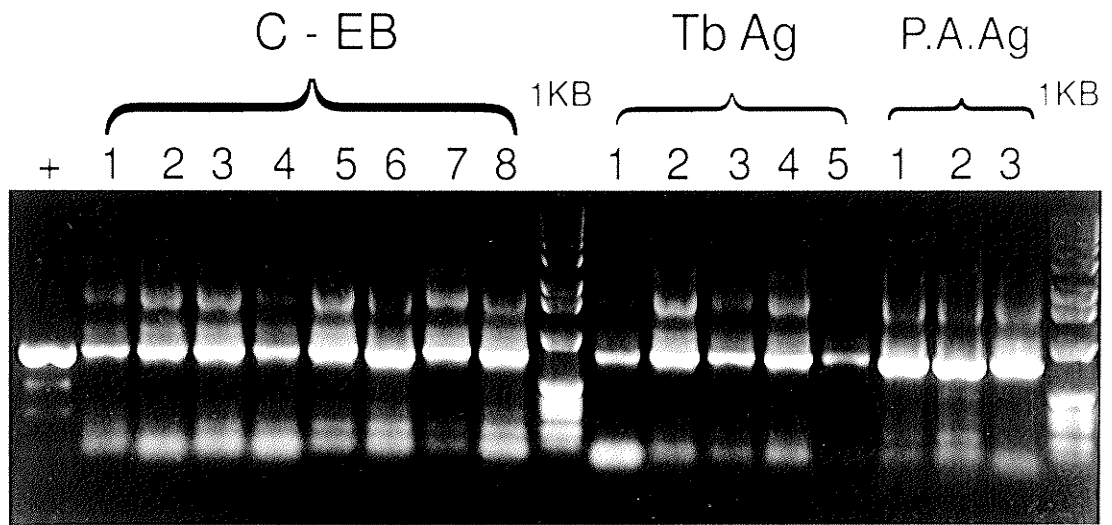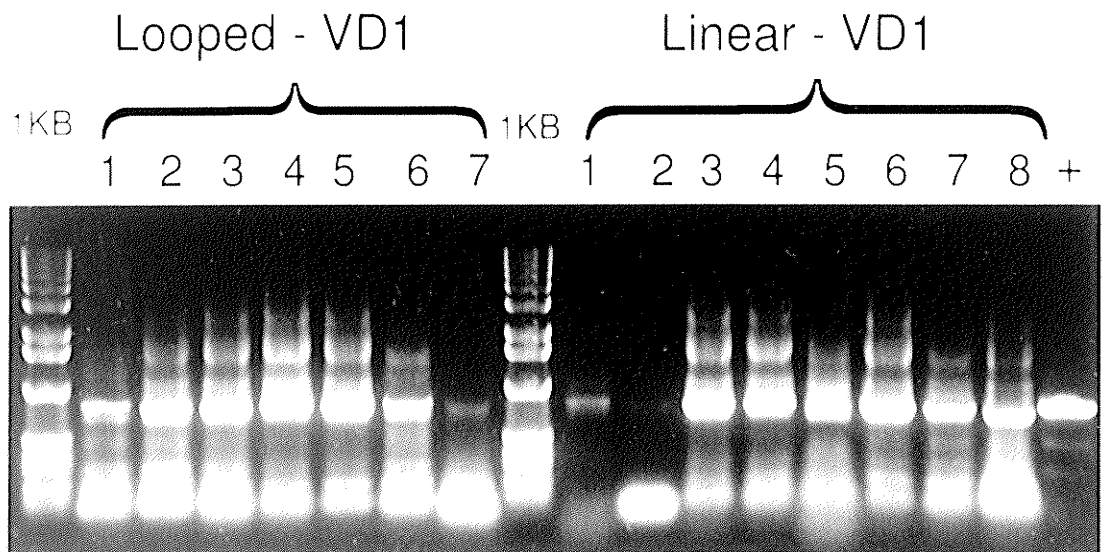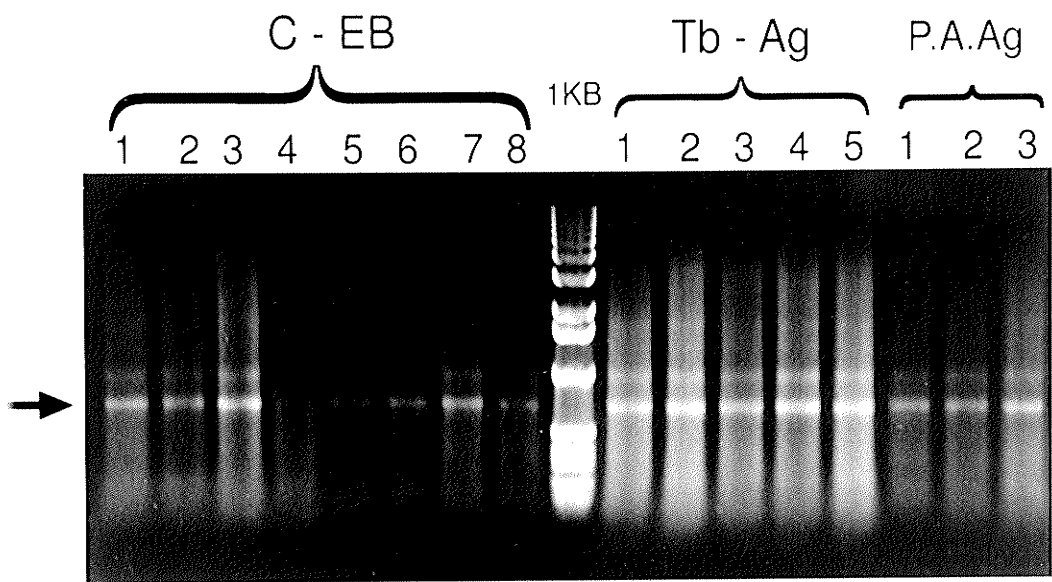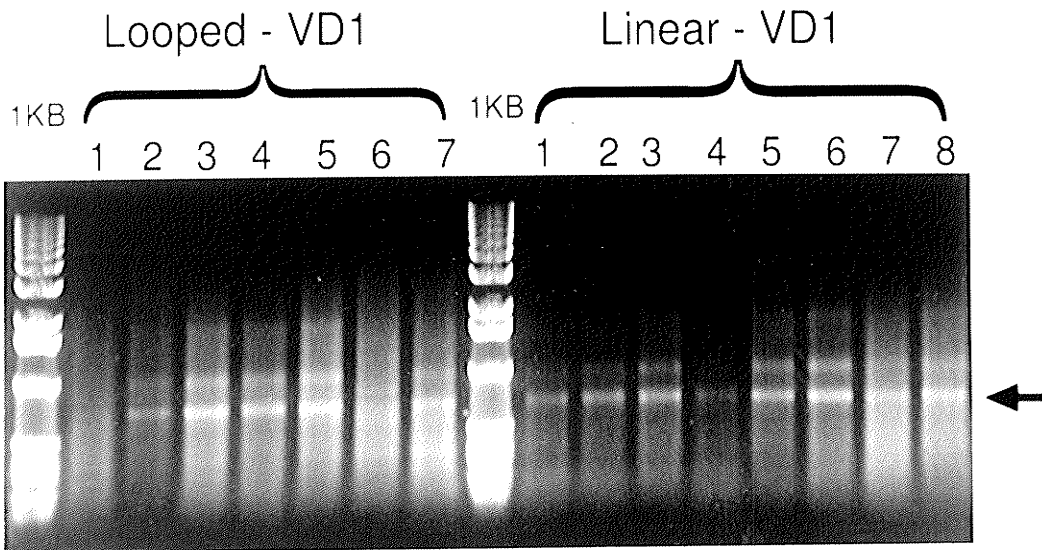
REFERENCES:

Ada, G., and Ramsay. 1996. Vaccines, vaccination and the immune response. Lippincott-Raven, New York.

Adams, J.M., 1980. Immunol. Today 1, 10-17.

Adderson, E.E., Shackelford, P., Quinn, A., and W.L. Carrol. 1991. Restricted IgH chain V gene usage in the human antibody response to *Haemophilus influenza* type b capusular polysaccharide. J. Immunol. 147, 1667-1674.

Adderson, E.E., Azmi, F.H., Wilson, P.M., Shackelford, P.G., and W.L. Carol. 1993. The human VH3b gene subfamily is highly polymorphic. J. Immunol. 151, 800-809.

Adderson, E.E., Wilson, P.M., Cunningham, M., and P. Shackelford. 1998. *Haemophilus influenza* type b polysaccharide-protein conjugate vaccine elicits a more diverse antibody repertoire in infants than in adults. J. Immunol. 161, 4177-4182.

Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: Understanding their relation. *Science* 272. 54-60.

Akolkar, P., Sikder, S., Bhattacharya, S., Liao, J., Gruezo, F., Morrison, S., and E.A. Kabat. 1987. Different Vl and VH germline genes are used to produce similar combining sites with specificity for alpha 1-6) dextrans. J. Immunol. 138, 4472-4479.

Alberti, S., Rodriquez, F., Schirmer, T., Rummel, G., Tomas, J., Rosenbusch, J., and V.J. Benedi. 1995. A porin from Klebsiella pneumoniae: sequence homology, three dimensional model, and complement binding. Infect. Immun. 63, 903-910.

Aldovini, A., and R.A. Young. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature 351, 479-482, 1991.

Alt, F., Yancopolous, T., Blackwell, T., Wood, E., Thomas, M., Boss, R., Coffman, N., Rosenberg, S., Tonegawa, and D. Baltimore. 1984. Ordered rearrangment of immunoglobulin heavy chain V segments. EMBO J. 3, 1209.

Alt, F.W. 1986. New mechanism revealed. Nature 322. 772

Alt, F., Blackwell, K., and G. Yancopolous. 1987. Development of the primary antibody repertoire. Science 238, 1079-1087.

Amerding, D., Rossiter, H., Ghazzouli, I., and E. Liehl. 1982. Evaluation of live and

inactivated influenza A virus vaccines in a mouse model. J. Infect. Dis. 145, 320-330.

Amitt., A., Mariuzza, R., Phillis, S., and R. Poljak . 1986) Three dimensional structure of an antigen-antibody complex to 2.8 A resolution. Science 233, 747-753.

Anderer, F.A., and H. Sclemberger. 1965. Biochim. Biophys. Acta. 97, 503-509.

Anderson, E., Shackelford, P., Insel, R., Quinn, A., Wilson, P., and W. Carroll. 1992. Immunoglobulin light chain variable region gene sequences for human antibodies to *Haemophilus influenza* type b capsular polysaccharide are dominated by a limited number of Vk and Vlambda gene segments and VJ combinations. J. Clin. Invest. 89, 729-738.

Andersson, K., Wrammert, J., and T. Leandersson. 1998. Affinity selection and repertoire shift: paradoxes as a consequence of somatic mutation? Immunol. Rev. 162, 172-182.

Andria, M., Levy, S., ans E. Benjamini. 1990. Diverse VH and Vl genes are used to produce antibodies against a defined protein epitope. J. Immunol. 144, 2614-2619.

Andris, J.S., Ehrlich, P., Ostberg, L., and J.D. Capra. 1992. Probing the human antibody repertoire to exogenous antigen. Characterization of the H and L chains V region gene segments from anti-hepatitis B virus antibodies. J. Immunol. 149, 4053-4059.

Andris, J., Miller, A., Abraham, S., Cunningham, S., Roubinet, F., Blancher, A., and J. Capra. 1997. Variable region gene segment utilization in rhesus monkey hybridomas producing human red blood cell-specific antibodies: Predominance of the VH4 family but not VH4-21 (V4-34). Mol. Immunol. 34, 237-253.

Angell-Killie, J.E., and T.O. Jorgensen. 1995. Immunoregulation in fishII: intermolecular induced suppression of antibody reponses studied by haptenated antigens in atlantic salmon. Dev. Comp. Immunol. 19, 389-404.

Angelova, L.A., and Y.S. Shvartsman. 1982. Original antigenic sin to influenza in rats. *Immunology* 46: 183-188.

Apodaca, G., Katz, L., and K.E. Mostov. 1994. Receptor mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. J. Cell. Biol. 125, 67-86.

Arno, J.N., Ricker, V.A., Batteiger, B.E., Katz, B.P., Caine, V.A., and R.B. Jones. 1990. Interferon-gamma in endocervical secretions of women infected with *Chlamydia trachomatis*. J. Infect. Dis. 162, 1385-1389.

Arnon, R., and M.H.V. Van Regenmortel. Structural basis of antigenicity and design of new vaccines. FASEB J. 6, 3265-3274, 1992.

Arnon, R., Synthetic peptides as the basis for vaccine design. Molec. Immunol. 28, 209-215, 1991.

Atkinson, M., Chang, Y., Celler, J., Huang, C., Paige, C., and G. Wu. 1994. Overusage of mouse DH gene segment, DFL16.1 is strain-dependent and determined by cis-acting elements. Dev. Immunol. 3, 283-295.

Aubin, J., Davi, F., Nguyen-Salomon, F., LeBoeuf, D., Debert, C., Taher, M., Valensi, F., Canioni, D., Brousse, N., Varet, B., Flandrin, G., and E.A. Macintyre. 1995. Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. Leukemia 9, 471-479.

Auvinen, P., Makela, M., Roivainen, M., Kallajoki, M., Vanionpaa, R., and T. Hyypia. 1993. Mapping of antigenic sites of coxsackievirus B3 by synthetic peptides. APMIS 101, 517-528.

Babcook, J.S., Leslie, K.B., Olsen, O., Salmon, R.A., and Schrader, J.W. 1996. A novel strategy for generating monoclonal antibodies from single, isolated lymphocytes producing antibodies of defined specificities. Proc. Natl. Acad. Sci. USA 93, 7843-7848.

Bachmann, M.F, T.M. Kundig, C.P. Kalberer, H. Hengartner, and R.M. Zinkernagel. 1994. How many specific B cells are needed to protect against a virus? *J. Immunol.* 152. 4235-4241.

Bachmann, M.F., Kalinke, U., Althage, A., Freer, G., Brukhart, C., Roost, H-P., Aguet, M., Hengartner, H., and R.M. Zinkernagel. 1997. The role of antibody concentration and avidity in antiviral protection. Science 276, 2024-2027.

Baehr, W., Zhang, Y.X., Joseph, T., Su, H., Nano, F.E., Everett, K.D., and H.D. Caldwell. 1988. Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. Proc. Natl. Acad. Sci. U.S.A. 85, 4000-4004.

Baetteiger, Bes, J.R., Smith, Miller, C.L., and A. Allchin. 1979. Assessment of inactivated influenza A vaccine after three outbreaks of influenza A at Christ's hospital. Lancet i, 33-35.

Baetteiger, B., and L. Pei-Mao. 1998. Human antibodies to linear B cell epitopes in variable sequence regions of the major outer membrane protein of *Chlamydia trachomatis*. In Chlamydial Infections, R. Stephens et al., (eds) Proceedings of the ninth International symposium on human chlamydial infections.

Bailey, R.L., Kajbaf, M., Whittle, H.C., Ward, M.E., and D.C. Mabey. 1993. The

influence of local antichlamydial antibody on the acquisition and persistence of human ocular chlamydial infection: IgG antibodies are not protective. Epidemiol. Infect. 111, 315-324.

Barbas III, C.F., and D. Burton. 1994a. Monoclonal antibodies from combinatorial antibodies; Cold Spring Harbour Laboratories course on making antibody libraries on filamentous phage.

Barbas III, C.F., and D. Burton. 1994b. Human antibodies from combinatorial libraries. Adv. Immunol. 57, 191-280.

Barenfanger, J., and A.B. MacDonald. 1974. The role of immunoglobulin in the neutralization of trachoma infectivity. J. Immunol. 113, 1607-1617.

Barnett, P.V., Rowlands, D.J., and N.R. Parry. 1993. Characterization of monoclonal antibodies raised against a synthetic peptide capable of inducing a neutralizing response to human rhinovirus type 2. J. gen. Virol. 74, 1295-1302.

Barnett, P.V., Rowlands, D.J., Campbell, R., and N.R. Parry. 1995. Monoclonal antibodies to a peptide of human rhinovirus type 2 with different specificities recognize the same minimum sequence. J. Gen. Virol. 76, 1255-1261.

Barnett, P.V., Pullen, L., Staple, R., Lee, L., Butcher, R., Parkinson, D., and T.R. Doel. 1996. A protective anti-peptide antibody against the immunodominant site of the A24 Cruzeiro strain of foot-and-mouth disease virus and its reactivity with other subtype viruses containing the same minimum binding sequence. J gen. Virol. 77, 1011-1018.

Barron, A.,Rank, R., and E. moses. 1984. Immune response in mice infected in the genital tract with mouse pneumonitis agent. Infect. Immun. 44, 82-85.

Batista, F., and M. Neuberger. 1998. Affinity dependence of the B cell response to antigen: A threshold, a cieling , and the importance of off-rate. Immunity 8, 751-759.

Batteiger, B., and R. Rank. 1987. Analysis of the humoral immune response in chlamydial genital infection in guinea pigs. Infect. Immun. 55, 1767-1773.

Bauwens. J.E., Lampe, M.F., Suchland, R.J., Wong, K., and W.E. Stamm. 1995. Infection with *Chlamydia trachomatis* lymphogranuloma venereum serovar L1 in homosexual men with proctitis: molecular analysis of an unusual case cluster. Clin. Infect. Dis. 20, 576-581.

Bavoil, P., Ohlin, A., and J. Schacter. 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. 44, 479-485.

Beare, A.S., Hobson, D., Reed, S.E., and D.A. Tyrell. 1968. A comparison of live and killed influenza virus vaccines. Lancet ii: 418-420.

Beattie, J. 1992. Investigation of the solid- and solution-phase binding reactivities of continuous epitopes recognized by polyclonal guinea-pig anti-recombinant bovine growth hormone antisera. J. Molec. Recog. 5, 99-104.
 in vitro assay effects on binding assays)

Beckers, W., Villa, L., Gonfloni, S., Castagnoli, L., Newton, S., Cesarini, G., and P. Ghiara. 1993. Increasing the immunogenicity of protein antigens through the genetic insertion of VQGEESNDK sequence of human IL-1beta into their sequence. J. Immunol. 151, 1757-1764.

Bemark, M., Liberg, D., and T. Leanderson. 1998. Conserved sequence elements in K promoters from mice and humans: implications for transcriptional regulation and repertoire expression. Immunogenet. 47, 183-195.

Benito, A., Mateau, M.G., and A. Villaverde. 1995. Improved mimicry of a foot and mouth disease virus antigenic site by a viral peptide displayed on beta-galactosidase surface. Bio/Technol. 13, 801-804.

Benjamini, E., M. Andria, C. Estin, F. Norton and C.Y. Leung. 1988. Studies on the clonality of the response to an epitope of a protein antigen. Randomness of activation of epitope-recognizing clones and the development of clonal dominance. *J. Immunol.* 141: 55-63.

Ben-Aissa-Fennira, F., Gaaied, A., Bouguerra, A., and K. Dellagi. 1998. IgM antibodies to P1 cytoadhesin of *Mycoplasma pneumoniae* are part of the natural antibody repertoire expressed early in life. Immunol. Letters 63, 59-62.

Berdoz, J., Monath, T., and Kraehenbuhl, J. 1995. Specific amplification by PCR of rearranged genomic variable regions of immunoglobulin genes from mouse hybridoma cells. PCR Meth. Appl. 4, 256-264.

Berek, C. Griffiths, G., and C. Milstein. 1985. Molecular events during maturation of the immune response to oxalozone. Nature 316, 412-418.

Berek, C., Jarvis, J., and C. Milstein. 1987. Activation of memory and virgin B cell clones in hyperimmune animals. Eur. J. Immunol. 17, 1121-1129.

Berek, C., and C. Milstein. 1987. Mutation drift and repertoire shift in the maturation of the immune response. Immunol. Rev. 96, 23-41.

Berek, C., Berger, A., and M. Apel. 1991. Cell 67, 1121-1129.

Berek, C., Brandl, B., and G. Steinhauser. 1997. Human lambda light chain germline genes: polymorphism in the IGVL2 gene family. Immunogenet.46, 533-534.

Berinstein, A., Piatti, P., Gaggino, O., Schudel, A., and A.M. Sadir. 1991. Enhancement of the immune response elicited with foot-and-mouthdisease virus vaccines by an extract of the *Mycobacterium* sp. wall. Vaccine 9, 883-888.

Berneman, A., Belec, L., Fischetti, V., and J. Bouvet. 1998. The specificity patterns of human immunoglobulin G antibodies in serum differ from those in autologous secretions. Infect. Immun. 66, 4163-4168.

Berry, J.D., Peeling, W., and R.C. Brunham. 1999. Analysis of the original antigenic sin antibody response to the major outer membrane protein of *Chlamydia trachomatis*. J. Infect. Dis. In the press.

Berzofsky, J.A., and A.N. Schechter. 1981. The concepts of crossreactivity and specificity in immunology. Mol. Immunol. 18, 751-763.

Bessen, D., and Fischetti, V.A. 1988. Influence of intranasal immunization with synthetic peptides corresponding to conserved epitopes of M protein on mucosal colonization by group A streptococci. Infect. Immun. 56, 2666-2672.

Bianca, R., and A. Barbour. 1994. Antigen diversity in the bacterium B. hermsii through "somatic" mutations in rearranged vmp gens. Cell 78, 867-876.

Bidart, J., Troalen, F., Ghillani, P., Rouas, N., Razafindrastita, A., Bohoun, C., and D. Bellet. 1990. Peptide immunogen mimicry of a protein-specific structural epitope of human choriogonadotropin. Science 248, 736-739.

Billadeau, D., Blackstadt, M., Greipp, P., Kyle, R., Oken, M., Kay, N., and B. Van Ness. 1991. Analysis of B-lymphoid malignancies using allele-specific polymerase chain reaction: A technique for sequential quantitation of residual disease. Blood, 3021-3029.

Bindon, C.I., Hale, G., and H. Waldmann. 1990. Eur. J. Immunol. 20, 277-281.

Birkelund, S., Johnson, H., and G. Christiansen. 1994. Chlamydia trachomatis serovar L2 induces protein tyrosine phosphorylation during uptake by HeLa cells. Infect. Immun. 62, 4900-4908.

Bittle, J.L., Houghten, R., Alexander, H., Shinnick, T., Sutcliffe, J., and R.A. Lerner.

1982. Protection against foot-and-mouth disease virus by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature 298, 30-33.

Blankenstein, T., and U. Krawinkel. 1987. Immunoglobulin VH region genes of the mouse are organized into overlapping clusters. Eur. J. Immunol 17, 1351-1357.

Bolin, R., and J. Roehrig. 1991. Synthetic peptides of the E2 glycoprotein of venezuelan equine encephalomyelitis virus. II. Antibody to the amino terminus protects animals by limiting viral replication. Virology 185, 281-290, 1991.

Bona, C. 1988. V genes encoding autoantibodies: molecular and phenotypic characteristics. Annu. Rev. Immunol. 6, 327-358.

Bonnycastle, L.L.C., Mehroke, J., Rashed, M., Gong, X., and J.K. Scott. 1996. Probing the basis of antibody reactivity with a panel of constrained peptide libraries by filamentous phage. J. Mol. Biol. 258, 747-762.

Borst, P., and D.R. Greaves. 1987.. Programmed gene rearrangement altering gene expression. Science 235,658-667.

Bot, A., Nangpal, A., Pricop, L., Bogen, B., Kaushik, A., and C. Bona. 1996. Vlambda-light chain genes reconstitute immune responses to defined carbohydrate antigens or haptens by utilizing different VH genes. Mol. Immunol. 33, 1359-1368.

Bottger, V., Bottger, A., Lane, E., and B. Spruce. 1995. Comprehensive analysis of monoclonal anti-proenkephalin antibodies using phage display libraries and synthetic peptides: Revelation of antibody fine specificity caused by somatic mutations in the variable region genes. J. Mol. Biol. 247, 932-946.

Bouvet, J., and G. Dighiero. 1998. From natural polyreactive antibodies to A la carte monoreactive antibodies to infectious agents: Is it a small world after all? Infect. Immun. 66, 1-4.

Brigidio, M., Sabbaga, J., and Brentani, R. 1990. Are synthetic peptides suitable for the detection of continuous epitopes only? Immunol. Lett. 24, 191-198.

Briles, D., Forman, C., Hudak, S., and J.L. Claflin. 1982. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J. Exp. Med. 156, 1177-1185.

Brodeur, P., and R. Riblet. 1984. The immunoglobulin heavy chain variable region locus in the mouse. I. One hundred IgVH genes comprise seven families of homologous genes. Eur. J. Immunol. 14, 922-930.

Brossay, L., Villeneuve, A., Paradis, G., Cote, L., Mourad, W., and J. Hebert. 1994. Mimicry of a neutralizing epitope of the major outer membrane protein of *Chlamydia trachomatis* by anti-idiotypic antibodies. Infect. Immuni. 62, 341-347.

Brown, A.R., C. DeWitt, M. Bosma and A. Nisonoff. 1980. Dominace of an immune response by secondary cells: Quantitation by allotype analysis. *J. Immunol.* 124. 250-254.

Bruck et al., 1986. Proc. Natl. Acad. Sci. USA. 83, 6578.

Brunham, R.C., Kuo, C.C., Cles, L., and K.K. Holmes. 1983. Correlation of host immune response with quantitative recovery of *Chlamydia trachomatis* from the human endocervix. Infect. Immun. 39, 1491-1494.

Brunham, R.C., Kuo, C.C., and W. Chen. 1985. Systemic *Chlamydia trachomatis* infection in mice: A comparison of lymphogranuloma venereum and trachoma biovars. Infect. Immun. 48, 78-82.

Brunham, R., Peeling, R., Maclean, I., McDowell, J., Person, K., and S. Osser. 1987. Postabortal *Chlamydia trachomatis* salpingitis: correlating risk with antigen specific serological responses and with neutralization. J. Infect. Dis. 155, 749-755.

Brunham, R.C., and R.W. Peeling. 1994a. *Chlamydia trachomatis* antigens: role in immunity and pathogenesis. *Infect. Agents Dis.* 3. 218-233.

Brunham, R.C., Yang, C., Maclean, I., Kimani, J., Maitha, G., and F. Plummer. 1994b. *Chlamydia trachomatis* from individuals in a sexually transmitted disease core group exhibit frequent sequence variation in the major outer membrane protein omp1. gene. J. Clin. Invest. 94, 458-463.

Brunham, R.C., Kimani, J., Bwayo, J., Maitha, G., Maclean, I., Yang, C., Shen, C., Roman, S., Nagelkerke, N., Cheang, M., and F. Plummer. 1996. The epidemiology of *Chlamydia trachomatis* within a sexually transmitted disease core. J. Infect. Dis. 73, 950-956.

Brunham, R.C. 1994. Vaccine design for the prevention of *Chlamydia trachomatis* infection. Presented at the eighth international symposium on human chlamydial infection, Chantilly, France, June.

Buchanan, K., Smith, E., Dou, S., Corcoran, L., and C. Webb. 1997. Family specific differences in transcription efficiency of Ig heavy chain promoters. J. Immunol 159, 1247-1254.

Burnet, F.M. 1959. The clonal selection theory of acquired immunity. The University Press, Cambridge.

Burns, C.A., Ebersole, J.L., and M.R. Allansmith. 1982. Immunoglobulin A antibody levels in human tears, saliva, and serum. Infect. Immun. 36, 1019-1022.

Burns, J., Siadat-Pajouh, M., Krishnaney, A., and H. Greenberg. 1996. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing ability. Science 272, 104-106.

Butler, M. 1987. Animal cell technology: Principles and products. Open university Press, Philadelphia.·

Byrne, G.I., Stephens, R.S., Ada, G., Caldwell, H.D., Su, H., Morrison, R.P., Van Der Pol, B., Bavoil, P., Bobo, L., Everson, S., Ho, Y., Hsia, R.C., Kennedy, K., Kuo, C-C., Montgomery, P.C., Peterson, E., Swanson, A., Whitaker, C., Whittum-Hudson, J., Yang, C.L., Zhang, Y.X., and G. M. Zhong. 1993. Workshop on in vitro neutralization of *Chlamydia trachomatis*: Summary of proceedings. J. Infect. Dis. 168, 415-420.

Caldwell, H.D., Stewart, S., Johnson, S., and H. Taylor. 1987. Tear and serum antibody response to *Chlamydia trachomatis* antigens during acute chlamydial conjunctivitis in monkeys as determined by immunoblotting. Infect. Immun. 55, 93-98. Campbell, M.J., Zelenetz, A., Levy, S., and Levy, R. 1992. Use of family specific leader region primers for PCR amplification of the human heavy chain variable region gene repertoire. Mol. Immunol. 29, 193-203.

Campos, M., Pal, S., O'Brien, T.P., Taylor, H.R., Prendergast, R., and J.A. Whittum-Hudson. 1995. A chlamydial major outer membrane protein extract as a trachoma vaccine candidate. Invest. Opthalmol. Vis. Sci. 36, 1477-1491.

Carmarero, J.A., Andreu, D., Cairo, J., Mateu, M., Domingo, E., and E. Giralt. Cyclic disulfide model of the major antigenic site of serotype C foot and mouth disease virus: Synthetic, conformational, and immunochemical studies. FEBS lett. 328, 159-164, 1993.

Carroll, W., Adderson, E., Quinn, A., and P. Shackelford. 1992. Immunoglobulin variable region gene expression in response to *Haemophilus influenzae* type b polysaccharide.J. Infect. Dis. 165 (suppl 1), S57-S61.

Carroll, W., Shackelford, P., and E. Adderson. 1995. The immunoglobulin gene repertoire to *Haemophilus influenzae* type b. Ann. N.Y. Acad. Sci. 764, 374-277.

Carter, R., and D.Fearon. 1992. CD19: Lowering the threshold for antigen receptor stimulation of B cells. Science 256, 105-107.

Casadevall, A. 1998. Antibody mediated protection against intracellular pathogens. Trends in Microbiol. 3, 102-107.

Casadevall, A., and M. Scharff. 1991. The mouse antibody response to infection with Cryptococcus neoformans: VH and VL usage in polysaccharide binding antibodies. J. Exp. Med. 174, 151-160.

Dasadevall, A., DeShaw, M., Fan, M., Dromer, F., Kozel, S., and L. Profski. 1994. Molecular and idiotypic analysis of antibodies to Cryptococcus neoformans glucuronoxylomannan. Infect. Immuni. 62, 3864-3872.
Cascalho, M., Ma, A., Lee, S., Masat, L., and M. Wabl. 1996. A quasi-monoclonal mouse. Science 272, 1649-1652.

Cascalho, M., Wong, J., Steinberg, C., and M. Wabl. 1998. Mismatch repair co-opted by hypermutation. Science 279, 1207-1210.

Casson., L.P., and T. Manser. 1995a. Evaluation of loss and change of specificity resulting from random mutagenesis of an antibody VH region. J. Immunol. 155, 5647-5654.

Casson., L.P., and T. Manser. 1995b. Random mutagenesis of two complementarity determining region amino acids yields an unexpected high frequency of antibodies with increased affinity for both cognate antigen and autoantigen. J. Exp. Med.

Castrignano, S.B., Carlsson, B., Carneiro, MS., Soderstrom, T., and L.A. Hanson. 1993. IgA and IgG subclass deficiency in a poor population in a developing country. Scand J. Immunol. 37, 509-514.

Caton, A. and H. Koprowski. 1991. Influenza virus hemagglutinin-specific antibodies isolated from a combinatorial expression library are closely related to the immune response of the donor.. Proc..Natl. Acad. Sci. U S A 87, 6450-6454.

Caton, A.J., Brownlee, G., Staudt, L., and W. Gerhard. 1986. Structural and functional implications of a restricted antibody response to a defined antigenic region on the influenza virus hemagglutinin. EMBO J. 5, 1577-1587.

Caton, A., Herlyn, D., Ross, A., and H. Koprowski. 1990. Identical D regions expressed by murine monoclonal antibodies to a human tumor antigen. J. Immunol. 144, 1965-1968.

Cebra, J.J., Gearhart, P.J., Kamat, R., Roberson, S.M., and J. Tseng. 1976. Origin and differentiation of lymphocytes involved in the secretory IgA response. Cold Spring Harb.

Symp. Quant. Biol. 41 , 201-215.

Chang, J. 1982. Structural studies of the outer envelope of *Chlamydia trachomatis* by electron microscopy. J. Mol. Biol. 161, 579-590.

Chang, B., and P. Casali. 1995. A sequence analysis of human germline Ig VH and Vl genes. Ann. N.Y. Acad. Sci. 764, 170-179.

Charbit, A., Molla, A., Saurin, W., and M. Hofnung. 1988. Versatility of a vector for expressing foreign polypeptides at the surface of gram negative bacteria. Gene 70, 181-189.

Chaudhary, V.K., Batra, J., Gallo, M., Willingham, M., Fitzgerald,D.J., and Pastan, I. 1990. A rapid method of cloning functional variable region antibody genes in *Escherichia coli* as single chain immunotoxins. Proc. Natl. Acad. Sci. USA 87, 1066-1070.

Chenchick, A., Diachenko, L., Moqadam, F., Tarabykin, V., Lukyanov, S., and Siebert, P.D. 1996. Full-length cDNA cloning and determination of mRNA 5' and 3' ends by amplification of adaptor-ligated cDNA. Biotechniques 21, 526-534.

Chiang, Y.L., Sheng-Dong, R., Brow, M., and Larrick, J.W. 1989. Direct cDNA cloning of the rearranged immunoglobulin variable region. Biotechniques 7, 360-366.

Chothia, C., Lesk, A.M., Tramontano, A., Levitt, M., Smith-Gill, S.J., Air, G., Sheriff, S., Padlan, E.A., Davies, D., Tulip, W.R., Colman, P.M., Spinelli, S., Alzari, P.M. and R.J. Poljak. 1989. Conformations of immunoglobulin hypervariable regions. Nature 342, 877-883.

Chothia, C. 1997 Protein-protein and protein-carbohydrate recognition. In, Molecular aspects of host-pathogen interactions, McCrae, M.A. Saunders, J., Smyth, C., and N. Stow (eds.) Cambridge University Press, New York.

Claflin, J.L., Berry, J., Flaherty, D., and W. Dunnick. 1987. Somatic evolution of diversity among anti-phosphocholine antibodies induced with *Proteus morganii*. J. Immunol. 138, 3060-3068.

Claflin, J.L., and J. Berry. 1988. Genetics of the phosphocholine-specific antibody response to *Streptococcus pneumoniae*. Germ line but not mutated antibodies are dominantly selected. J.Immunol. 141, 4012-4019.

Clarke, S.H., Huppi, K., Ruczinsky, D., Staudt, L., Gerhard, W., and M. Weigert. 1985. Intra- and interclonal diversity in the antibody response to influenza hemagglutinin. J Exp. Med. 161. 687-704.

Clarke, S.H., Staudt, L., Kavaler, J., Schwartz, D., Gerhard, W., and M. Weigert. 1990. V region gene usage and somatic mutation in the primary and secondary responses to influenza virus hemagglutinin. J Immunol. 144, 2795-2801.

Coleclough, C., Perry, R., Karjalainen, K., and M. Weigert. 1981. Aberrant rearrangements contribute significantly to the allelic exclusion of immunoglobulin expression. Nature 290, 372.

Coloma, M.J., Larrick, J.W., Ayala, M., and Gavilondo-Cowley, J.V. 1991. Primer design for the cloning of immunoglobulin heavy-chain leader-variable regions from mouse hybridoma cells using the PCR. BioTechniques 11, 152-154.

Coloma, M., Hastings, A., Wims, L., and Morrison, S.L. 1992. Novel vectors for the expression of antibody molecules using variable regions generated by polymerase chain reaction. J. Immunol. Meth. 152, 89-104.

Conlan, J.W., Clarke, I.N., and M.E. Ward. 1988. Epitope mapping with solid-phase peptides: identification of type-, subspecies-, species- and genus-reactive antibody binding domains on the major outer membrane protein of *Chlamydia trachomatis*. Mol. Microbiol. 2, 673-9.

Conlan, J.W., Ferris, S., Clarke, I.N., and M.E. Ward. 1989. Surface-exposed epitopes on the major outer membrane protein of *Chlamydia trachomatis* defined with anti-peptide anti-sera. J. Gen. Microbiol. 135, 3219-3228.

Cornelis. G.R. 1997.Cross-talk between Yersina and eukaryotic cells. In, Molecular aspects of host-pathogen interactions. McCrae, M., Saunders, J., Smyth, C., and N. Stow eds., Cambridge University Press, New York.

Cory, T.R., Kuo, C.C., and S.P. Wang. 1975. Comparative susceptibility of eleven mammalian cell lines to infection with trachoma organisms. J. Clin. Microbiol. 1, 434-439.

Cotter, T.W., andG.I. Byrne. 1996. Immunity to chlamydia:comparison of human infectionsand murine models. Res. Immunol. 147, 587-595.

Cotter T.W., Meng, Q., Shen, Z., Zhang, Y., Su, H., and H.D. Caldwell. 1995. Protective efficacy of major outer membrane protein-specific immunoglobulin A IgA. and IgG monoclonal antibodies in a murine model of *Chlamydia trachomatis* genital tract infection. Infect. Immun. 63, 4704-4714.

Cottet, M., and G. Bordenave. 1994. Epitope complementarity and idiotypic interactions:

A study of idiotypic like interactions between anti-cytidine and anti-guanosine A/J mouse monoclonal antibodies-II. Analysis of the gene repertoire used to encode these antibodies. Mol. Immunol. 31, 75-76.

Couch, R.B., Kasel, J.A., Gerin, J.L., Schulman, J.L., and E.D. Kilbourne. 1974. Induction of partial immunity to influenza by a neuraminidase-specific vaccine. J. Infect. Dis. 129, 411-420.

Couch, R.B., and J.A. Kasel. 1983. Immunity to influenza in man. Ann. Rev. Microbiol. 37, 529-549.

Couch, R.B., Kasel, J.A., Glezen, W.P., Cate, T.R., Six, H., Taber, L., Frank, A., Greenberg, S.B., Zahradnik, J.M., and W.A. Keitel. 1986. Influenza: Its control in persons and populations. J. Infect. Dis. 153, 431-440.

Coutinho, A. 1993. Lymphocyte survival and V-region repertoire selection. Immunol. Today 1, 38-40.

Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R., Jansonius, J., and J.P. Rosenbusch. 1992. Crystal structures explain functional properties of two *E. coli* porins. Nature 358, 727-733.

Cox, J., Ivanyi, J., Young, D., Lamb, J., Syred, A., and M.J. Francis. Orientation of epitopes influences the immunogenicity of synthetic peptide dimers. Eur. J. Immunol. 18, 2015-2019, 1988.

Cox, J., Tomlinson, I., and G. Winter. 1994. A directory of human germ-line Vk segments reveals a strong bias in their usage. Eur. J. Immunol. 24, 827-836.

Crowley-Nowick, P.A., Bell, M., Edwards, R.P., McCallister, D., Gore, H., Kanbour-Shakir, A., Mestecky, J., and E.E. Partridge. 1995. Normal uterine cervix: characterization of isolated lymphocyte phenotypes and immunoglobulin secretion. Am. J. Reprod. Immunol. 34, 241-247.

Crowley-Nowick, P.A., Bell, M.C., Brockwell, R., Edwards, R.P., Chen, S., Partridge, E.E., and J. Mestecky. 1997. Rectal immunization for induction of specific antibody in the genital tract of women. J. Clin. Immunol. 17, 370-379.

Cui, Z.D., LaScolea, L.J., Fisher, J., and P.L. Ogra. 1989. Immunoprophylaxis of *Chlamydia trachomatis* lymphogranuloma venereum pneumonitis in mice by oral immunization. Infect. Immun. 57, 739-44.

Cui, Z.D., Tristram, D., LaScolea, L.J., Kwiatkowski, T. Jr., Kopti, S., and P.L. Ogra.

1991. Induction of antibody response to *Chlamydia trachomatis* in the genital tract by oral immunization. Infect. Immun. 59, 1465-9.

Dale, J.B., Chiang, E.Y., and J.W. Lederer. Recombinant tetravalent group A streptococcal M protein vaccine. J. Immunol. 151, 2188-2194, 1993.

Dascher, C., Roll, D., and P. Bavoil. 1993. Expression and translocation of the chlamydial MOMP in E. coli. Microb. Path. 15, 455-467.

Dattamajumdar, A.K., Jacobson, D., Hood, L.E., and Osman, G. 1996. Rapid cloning of any rearranged mouse immunoglobulin variable genes. Immunogenetics 43, 141-151.

Davenport, F.M., A.V. Hennesy, and T. Francis. 1953. Epidemiological and immunological significance of age distribution of antibody to antigenic variants of influenza virus. *J. Exp. Med.* 98. 641-647.

David, D., Demaison, C., Bani, L., and J. Theze. 1996. Progressive decrease in VH3 gene family expression in plasma cells of HIV infected patients. Int. Immunol. 8, 1329-1333.

David, V., and N. Maizels. 1989. Templated, Targetted sequence diversification displays a similar choreography in diffeent organisms. Cold Spring Harb.Symp. Quant. Biol. 54, 227-231.

David, V., Folk, N., and N. Maizels. 1992. Germline variable regions that match hypermutated sequences in genes encoding murine anti-hapten antibodies. Genetics 132, 799-811.

Davis, M.M., Kim, S.K., and L. E. Hood. 1980. DNA sequences mediating class switching in alpha-immunoglobulins. Science 209, 1360-1365.

Day, E. 1990. Advanced Immunochemistry. Wiley-Liss Inc. New York.

Dean, D., Schacter, J., Dawson, C.R., and R.S. Stephens. 1992. Comparison of the major outer membrane protein variant sequence sequence regions of B/Ba isolate: A molecular epidemiologic approach to *Chlamydia trachomatis* infections. J. Infect. Dis. 166, 383-392.

Dean., D., and K. Millman. 1997. Molecular and mutation trends analysis of omp1 alleles for serovar E of Chlamydia trachomatis. Implications for the immunopathogenesis of disease. J. Clin. Invest. 99, 475-483.

Deane, M., McCarthy, K., Wiedeman, L., and J.D. Norton. 1991. An improved method

for detection of B lymphoid clonality by polymerse chain reaction. Leukemia 5, 726-730.

Decker, D., Kline, G., Hayden, T., Zaharevitz, S., and N. Klinman. 1995. Heavy chain V gene specific elimination of B cells during the pre-B cell to B cell transition. J. Immunol. 154, 4924-4935.

DeFranco, M. 1993. B lymphocyte activation. In Fundamental Immunology, Paul, W.E. (ed.), Raven Press, New York.

Delassus, S., Gey, A., Darche, S., Cumano, A., Roth, C., and P. Kourilsky. 1995. PCR-based analysis of the murine immunoglobulin heavy-chain repertoire. J. Immunol. Meth. 184, 219-229.

Derfoot, J., Nardelli, B., Huang, W., Ho, D., and Tam, J.P. 1992. Macromolecular assemblage in the design of a synthetic AIDS vaccine. Proc. Natl. Acad. Sci. USA 89, 3879-3883.

Diamond, B., and M.D. Scharff. 1984. Somatic mutation of the T15 heavy chain gives rise to an antibody with autoantibody specificity. Proc. Natl. Acad. Sci. USA. 81. 5841-

Dilosa, R., Maeda, K., Masuda, A., Szakal, A., and J.G. Tew. 1991. Germinal center B cells and antibody production in the bone marrow. J. Immunol. 146. 4071-4077.

Dorman, C. 1995. DNA topology and the global control of bacterial gene expression:implications for the regulation of virulence gene expression. Microbiology 141, 1271-1280.

Duchosal, M., Kofler, R., Balderas, R., Agaudo, T., Dixon, F., and A. Theofilooulous. 1989. Genetic diversity of murine rheumatoid factors. J. Immunol. 142, 1737.
du Pasquier, L. 1993. Evolution of the immune system. In Fundamental Immunology, Paul, W.E. (ed.), Raven Press, New York.

East, I.J., P.E.E. Todd. and S.J. Leach. 1980.. Original antigenic sin: Experiments with a defined antigen. *Mol. Immunol.* 17. 1539-1544.

Eisen, H.N., and G. Siskind. 1964. Variations in affinity of antibodies during the iutinhou. 1986. Lymphocyte population kinetics in the mouse. Immunol. Rev. 91, 5-19.

Frohman, M.A., Dush, M., and Martin, G.R. 1988.. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85, 8998-9002.

Frohman, M. 1994. Cloning PCR products, In The Polymerase Chain Reaction, Mullis,

251

K. ed., 30-31.

Froyen, G., Hendrix, D., Ronsse, I., Fiten, P., Martens, E., and Billiau, A. 1995. Effect of VH and VL ntigenic specificity. *J. Immunol.* 151. 5272-5281.

Emara, M., Tout, N., Kaushik, A., and J. Lam. 1995. Diverse VH and Vk gene encode antibodies to Pseudomonas aeruginosa LPS. J. Immunol. 155, 3912-3921.

Emini, E., Jameson, B., and E. Wimmer. 1983. Priming for and induction of anti-poliovirus neutralizing antibodies by synthetic peptides. Nature 304, 699-703, 1983.

Emini, E., Jameson, B., and E. Wimmer. 1984. Identification of a new neutralization antigenic site on poliovirus coat protein VP2. J. Virol. 52, 719-721, 1984.

Epstein, S.L., Lo, C., Misplon, J., and J.R. Bennick. 1998. Mechanism of protective immunity against influenza virus infection in mice without antibodies. J. Immunol. 160, 322-327.

Ertl, H.C., Dietschold, B., Gore, M., Otvos, L., Larson, J., Wunner, W., and H. Koprowski. Induction of rabies virus specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. J. Virol. 63, 2885-2892, 1989.

Fan, J., and R.S. Stephens. 1997. Antigen conformation dependence of *Chlamydia trachomatis* infectivity neutralization. *J. Infect. Dis.* 176. 713-721.

Fan, T., Lu, H., Hu, L., Shi, G., McClarty, G., Nance, D., Greenberg, A., and G. Zhong. 1998. Inhibition of apaoptosis in chlamydia-infected cells: blockage of mitochondrial cytochrome c release and caspase activation. J. exp. Med. 187, 487.

Fang, W., Weintraub, B., Dunlap, B., Garside, P., Pape, K., Jenkins, M., Goodnow, C., Mueller, D., and T. Behrens. 1998. Self-reactive B cells overexpressing Bcl-xL escape negative selection and are tolerized by clonal anergy and receptor editing. Immunity 9, 35-45.

Fanning, L., Bertrand, F., Steinberg, C., and G.E. Wu. 1998. Molecular mechanisms involved in receptor editing at the Ig heavy chain locus. Int. Immunol. 10, 241-246.

Fayette, J., Dubois, B., Vandenabeele, S., Bridon, J., Vanbervliet, B., Durand, I., Banchereau, J., Caux, C., and F. Briere. 1997. Human dendritic cells skew isotype switching of CD-40 activated naive B cells towards IgA1 and IgA2. J. Exp. Med 185, 1909-1918.

Fazekas de St. Groth, S., and R.G. Webster. 1966. Disquisitions on original antigenic sin I. Evidence in man. *J. Exp. Med.* 124: 331-345.

Fearon, D.T., and R.M. Locksley. 1996. The instructive role of innate immunity in the acquired immune response. Science 272, 50-53.

Feeney, A.J. 1990. Lack of N-regions in fetal and neonatal mouse immunoglobulin V-D-J junction sequences. J. Exp. Med. 172, 1377-1390.

Feeney, A.J. 1992. Predominance of VHDHJH junctions occuring at sites of short sequence homology results in limited junctional diversity in neonatal antibodies. J. Immunol. 149, 222-229.

Feeney, A.J., Atkinson, M., Cowan, M., Escuro, G., and G. Lugo. 1996. A defective Vk A2 allele in Navajos which may play a role in increased susceptibility to *Haemophilus influenza* type b disease. J. Clin. Invest. 97, 2277-2282.

Ferguson, M., Reed, S.E., and P.D. Minor. Reactivity of Anti-peptide and Anti-poliovirus type 3 monoclonal antibodies with synthetic peptides. J. gen. Virol. 67, 2527-2531. 1986.

Fernandez, C. 1992. Genetic mechanisms for dominant VH gene expression. The VHB512 gene. J. Immunol. 149, 2328-2336.

Fields, B., Goldbaum, F., Ysern, X., Poljak, R., and R. Mariuzza. 1995. Molecular basis of antigen mimicry by an anti-idiotope. Nature374, 739-742.

Fish, S., and T. Manser. 1987. Influence of the macromolecular form of a B cell epitope on the expression of antibody variable and constant region structure. J. Exp. Med. 166, 711-724.

Fish, S., E. Zenowich, M. Fleming, and T. Manser. 1989. Molecular analysis of original antigenic sin I. Clonal selection, somatic mutation, and isotype switching during a memory B cell response. *J. Exp. Med.* 170. 1191-1209.

Fish, S., M. Fleming, J. Sharon, and T. Manser. 1991. Different epitope structures select distinct forms of an antibody variable region for expression during the immune response. *J. Exp. Med.* 173. 665-672.

Fitzsimmons, S.P., Rotz, B.T., and M.A. Shapiro. 1998. Asymmetric contribution to Ig repertoire diversity by Vk exons: Differences in the utilization of Vk10 exons. J. Immunol. 161, 2290-2300.

Foote, J., and Winter, G. 1992. Antibody framework residues affecting the conformation

of the hypervariable loops. J. Mol. Biol. 224, 487-499.

Foote, J., and C. Milstein. 1995. Kinetic and affinity limits on antibodies produced during immune responses. Proc. Natl. Acad. Sc. USA. 92, 1254-1256.

Fowke, K.R., et al. 1996. Resistance to HIV-1 infection amoung persistantly seronegative prostitutes in Nairobi, Kenya. Lancet 348, 1347-1351.

Friede, M., Muller, S., Briand, J., Plaue, S., Fernandes, I., Frisch, B., Schuber, F., and M.H.V. Van Regenmortel. Selective induction of protection against influenza virus infection in mice by a lipid-peptide conjugate delivered in liposomes. Vaccine 12, 791-797, 1994.

Freitas, A., Rocha, B., and A. Coutinhou. 1986. Lymphocyte population kinetics in the mouse. Immunol. Rev. 91, 5-19.

Frohman, M.A., Dush, M., and Martin, G.R. 1988.. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85, 8998-9002.

Frohman, M. 1994. Cloning PCR products, In The Polymerase Chain Reaction, Mullis, K. ed., 30-31.

Froyen, G., Hendrix, D., Ronsse, I., Fiten, P., Martens, E., and Billiau, A. 1995. Effect of VH and VL consensus sequence-specific primers on the binding and neutralizing potential of a single-chain FV directed towards HUIFN-gamma. Mol. Immunol. 32, 515-521.

Fukushi, H., and K. Hirai. 1993. *Chlamydia pecorum*: the 4th species of the genus *Chlamydia*. Microbiol. Immunol. 37, 515-522.

Garcia, K.C., Desiderio, S., Ronco, P., Verroust, P., and M. Amsel. 1992. Recognition of angiotensis II: Antibodies at different levels of an idiotypic netwrok are superimposable. Sciece 257, 528-531.

Gavilondo-Cowley, J., Coloma, M.J., Vazquez, J., Ayala, M., Macias, A., Fry, K., and Larrick, J.W. 1990. Specific amplification of rearranged immunoglobulin variable region genes from mouse hybridoma cells. Hybridoma 9, 407-417.

Gearhart, P.J., and D. Bogenhagen. 1983. Clusters of point mutations are found exclusively around rearranged antibody V genes. Proc. Natl. Acad. Sci. USA. 80. 3439-

Geysen, M.N., J. Rodda, J.T. Mason, G. Tribbick, and G.P. Schoofs. 1987. Strategies for

epitope analysis using peptide synthesis. *J. Immunol. Methods* 102. 259-274.

Geysen, H., Tainer, J., Rodda, S., Mason, T., Alexander, H., Getzoff, E., and R. Lerner. 1987. Chemistry of antibody binding to a protein. Science 235, 1184.

Getzoff, E.D., Tainer, J.A., and R.A. Lerner. 1988. The chemistry and mechanism of antibody binding to protein antigens. Adv. Immunol. 43, 1-98.

Ghaem-Maghami, S., Bailey, R., Mabey, D., Hay, P., Mahdi, O., Joof, H., Whittle, H., Ward, M., and D. Lewis. 1997. Characterization of B-cell responses to *Chlamydia trachomatis* antigens in humans with trachoma. Infewct. Immunt. 65, 4958-4964.

Gilmore, R., Joste, N., and G.A. McDonald. 1989. Molec. Microbiol. 3, 1579-1586.

Gill, P.W., Babbage, N.F., Gunton, P.E., Flower, W., and D.A. Garret. 1971. Did the asian virus protect us from hong kong influenza? Med. J. Aust. July 3, 54-53.

Gill, P.W., Cunningham, A.L., and A.M. Murphy. 1987. Should healthy children be vaccinated against influenza? Lancet, 1440-1441.

Gojobori, T., and M. Nei, 1986. Relative contributions of germline gene variation and somatic mutation to immunoglobulin diversity in the mouse. Mol. Biol. Evol. 2, 156-67.

Golvano, J., Lasarte, J., Sarobe, P., Gullon, A., Prieto, J., and F. Borras-Cuesta. Polarity of immunogens: Implications for vaccine design. Eur. J. Immunol. 20, 2363-2366, 1990.

Gough, N., and O. Bernard. 1981. Sequences of the joining region genes for immunoglobulin heavy chains and their role in generation of antibody diversity. Proc. Natl. Acad. Sci.USA 78, 509-513.

Goyenechea, B., and C. Milstein. 1996. Modifying the sequence of an immunoglobulin V-gene alters the resulting pattern of hypermutation. Proc. Natl. Acad. Sci. USA. 93, 13979-13984.

Giorgetti, C.A., and J.L. Press. 1994. A peptide sequence mimics the epitope on the multideterminant antigen Tyr,Glu.-Ala-Lys that induces the dominant H10/Vk1+ primary antibody response. J. Immunol. 152, 136-145.

Grant, F.J., Levin, S., Gilbert, T., and Kindsvogel, W. 1987. Improved RNA sequencing method to determine immunoglobulin mRNA sequence. Nucl. Acids. Res. 15, 5496.

Gras-Masse, H., Jolivet, M., Drobeco, H., Aubert, J., Beachey, E., Adibert, F., Chedid, L., and Tartar, A. 1988. Influence of helical organization on immunogenicity and

antigenicity of synthetic peptides. Mol. Immunol. 25, 673-678.

Grayston, J.T., K.S. Kim, E.R. Alexander, and S.P. Wang. 1970. Protective studies in monkeys with trivalent and monovalent trachoma vaccines. *In* Trachoma and Related Disorders Caused By Chlamydial Agents, *Excerpta Medica Int. Congress Series 223.* 377-385.

Grayston, J., and S. Wang. 1978. The potential for vaccine against infection of the genital tract with *Chlamydia trachomatis*. Sex. Trans. Dis. 5, 73-77.

Grayston, J.T., Kuo, C., Campbell., L., and S. Wang. 1989. *Chlamydia pneumoniae* sp. nov. for Chlamydia species strain TWOT. Int J. Syst. Bacteriol. 39, 88-90.

Greenspan, N.J., and L.J.N. Cooper. 1995. Complementarity, specificity and the nature of epitopes and paratopes in multivalent interactions. Immunol. Today 16, 226-230.

Gridley, T., Margolies, M., and M. Gefter. 1985. The association of various D elements with a single immunoglobulin VH gene segment: Influence on the expression of a major cross-reactive idiotype. J. Immunol. 134, 1236-1244.

Griffiths, G.M., Berek, C., Kaartinen, M., and C. Milstein. 1984. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. Nature 312, 271-275.

Gu, H., Tarlinton, D., Muller, W., Rajewsky, K., and I. Forster. 1991. Most peripheral B cells in mice are ligand selected. J. Exp. Med. 173, 1357-1371.

Hackstadt, T., Todd, W.J., and H.D. Caldwell. 1985. Disulfide-mediated interactions of the chlamydial major outer membrane protein: role in the differentiation of chlamydiae? J. Bacteriol. 161, 25-31.

Hanson, L.A., Bjorkander, J., Carlsson, B., Roberton, D., and T Soderstrom. 1988. The heterogeneity of IgA deficiency. J. Clin. Immunol. 8, 159-162.

Hartman, A., and S. Rudikoff. 1984. VH genes encoding the immune response to beta - 1,6. galactan: somatic mutation in IgM molecules. EMBO 3, 3023-3030.

Hatch, T., Miceli, M., and J. Sublett. 1986. Synthesis of disulfide bonded outer membrane proteins during the developmental cycle of *Chlamydia psittaci* and *Chlamydia trachomatis*. J. Bacteriol. 165, 379-385.

Hayashi, K., and Y. Kumamoto. 1991. The clinical significance of specific IgA antibodies in local secretions in cases with chlamydial urogenital infections - comparison with serum

antibodies and analysis of antigen specificity by immunoblotting assay. Kansenshogaku-Zasshi 65, 1430-1445.

Hayes, L.J., Conlan, J., Everson, J.S., Ward, M.E., and I.N. Clarke. 1991. *Chlamydia trachomatis* major outer membrane protein epitopes expressed as fusions with LamB in an attenuated aro A strain of *Salmonella typhimurium*; their application as potential immunogens. J. Gen. Microbiol. 137, 157-1564.

Hayes, L.J., Yearsley, P., Treharne, J.D., Ballard, R.A., Fehler, G.H., and W.E. Ward. 1994. Evidence for naturally occuring recombination in the gene encoding the major outer membrane protein of lymphogranuloma venereum isolates of *Chlamydia trachomatis*. Infect. Immun. 62, 5659-5663.

Hayes, L.J., Pecharatana, S., Bailey, R.L., Hampton, T., Pickett, M., Mabey, D., Watt, and M.E. Ward. 1995. Extent and kinetics of genetic change in the omp1 gene of n on *Chlamydia trachomatis. Infect. Immun.* 62. 1576-1583

Holgren, J., Czerkinsky, C, and A. Svennerhol,. 1992. Mucosal Immunity: implications for vaccine development. immunobiology 184, 157-179.

Hood, L., Campbell, J., and S. Elgin. 1975. The organization, expression, and evolution of antibody genes and other multigene families. Ann. Rev. Genetics 9, 305.

Hsai, R., Pannekoek, Y., Ingerowkski, E., and P.M. Bavoil. 1996. Type III seceretion in Chlamydia psittaci GPIC. In Stary, A. ed. Proceedings of the third meeting of the European Society for Chlamydia Research., p. 51. Bologna.

Huse, W.D., Sastry, L., Iverson, S., Kang, A., Alting-Mees, M., Burton, D.R., Benkovic, S.J., and Lerner, R.A. 1989. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science 246, 1275-1281.

Hutchins, W., Adkins, A., Emmons, T., and M.A.J. Westerlink. 1996. Molecular characterization of a monoclonal antibody produced in response to a group C meningococcal polysaccharide peptide mimic. Mol. Immunol. 33, 503-510.

Igietsieme, J., Uriri, I., Kumar, S., Ananaba, G., Ojior, O.,Momodu, I., Candai, D., and C. Black. 1998. Route of infection that induces a high intensity of gamma interferon secreting T cell in the genital tract produces optimal protection against *Chlamydia trachomatis* infection in mice. Infect. Immun. 66, 4030-4035.

Ikematsu, W., Kobarg, J., Ikematsu, H., Ichiyoshi, Y., and P. Casali. 1998. Clonal analysis of a human antibody response. III. Nucleotide sequences of monoclonal IgM, IgG, and IgA to rabies virus reveal restricted Vk gene utilization, junctional VkJk and VlambdaJlambda diversity, and somatic hypermutation. J. Immunol.161, 2895-2905.

Insel, R., Varade, W., and E. Marin. 1994. Human splenic IgM transcripts are mutated at high frequency. Mol. Immunol. 31. 383-392.

Iverson, J.O., Hanson, R.P., and J. Spalatin. 1976. Experimental chlamydiosis in wild and domestic lagomorphs. J. Wildl. Dis. 12, 215-220.

Jacob, J., Kelsoe, G., Rajewsky, K., and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centers. Nature 354, 389-392.

Janeway, C.A., and P. Travers. 1996. Immunobiology. 2nd ed. Garland Pub. Ltd., N.Y. 616 p.p.

Jantos, C.A., Heack, S., Roggendorf, R., Sen, M., and J.H. Hegemann. 1997. Antigenic and molecular analyses of different *Chlamydia pneumoniae* strains. J. Clin. Microbiol. 35, 620-623.

Jawetz, E., Rose, L., Hanna, L., and P. Thygeson. 1965. Experimental inclusion conjunctivitis in man. Measurements of infectivity and resistance. JAMA 194, 620-632.

Jeanteur, D., Lakey, J., and F. Pattus. 1994. The porin superfamily: diversity and common features. *In* Bacterial Cell Wall, J. Ghuysen and R. Hakenbeck, eds. Elsevier Science, Berlin.

Jena, P.K., Liu, A.H., Smith, D., and Wysocki, L.J. 1996. Amplification of genes, single transcripts, and cDNA libraries from one cell and direct sequence analysis of amplified products derived from one molecule. J. Immunol. Meth. 190, 199-213.

Jemmerson, R., Antigenicity and native structure of globular proteins: Low frequency of peptide reactive antibodies. Proc. Natl. Acad. Sci. 84, 9180-9184, 1987.

Jemmerson, R., and Hutchinson, R.M. 1990. Fine manipulation of antibody affinity for synthetic epitopes by altering peptide structure: antibody binding to looped peptides. Eur. J. Immunol. 20, 579-585.

Jeong, H., and J. Teale. 1989. VH gene family repertoire of resting B cells: Preferential use of D-proximal families early in development may be due to distinct B cell subsets. J. Immunol. 143, 2752.

Jerne, N. 1974. A network theory of the immune system. Ann Inst. Pasteur Paris 125C, 373.

Johansson, B.E.,and E.D. Kilbourne. 1990. Comparative long-term effects in a mouse model system of influenza whole virus and purified neuraminidase vaccines followed by sequential infections. J. Infect. Dis. 162, 800-809

Johansson, B.E.,and E.D. Kilbourne. 1994. Immunization with purified N1 and N2 influenza virus neuraminidase demonstrates cross-reactivity without antigenic competition. Proc. Natl. Acad. Sci. U.S.A. 91, 2358-2361.

Jones, H.M., Schachter, J., and R.S. Stephens. 1992. Evaluation of the humoral immune response in trachoma to *Chlamydia trachomatis* major outer membrane proteins by sequence-defined immunoassay. J. Infect. Dis. 166, 915-919.

Jones, S.T., and Bendig, M. 1991. Rapid PCR-cloning of full-length mouse immunoglobulin variable regions. Bio/Technology 9, 88-89.

Jovelin, F., Gilbert, D., Brard, F., Bernardi, T., Marche, P., Frebourg, T., and Tron, F. C., McClarty, G., and Brunham, R.C. 1997. DNA vaccination with the MOMP gene protects against *Chlamydia trachomatis* infection. J. Infect. Dis. 176, 1035-1040.

Juul., et al. 1998. A new apparently functional IgVk gene present in some individuals only. Immunogenet. 48, 40-46.

Kaartinen, M., Griffiths, G., Markham, A., and C. Milstein. 1983. mRNA sequences define an unusualy restricted IgG response to 2-phenyloxalozone and its early diversification. Nature 304, 320-324.

Kaartinen, M., Solin, L., and O. Makela. 1991. V genes of oxazolone antibodies in 10 strains of mice. Eur. J. Immunol. 21, 2863-2869.

Kabat, E.A., Wu, T.T., Reid-Miller, M., Perry, H.M., Gottesman, K.S and C. Foeller. 1987. Sequences of proteins of immunological interest, 4th edn. United States Department of Health and Human Services, Washington, D.C.

Kabat, E.A., Wu, T.T., Reid-Miller, M., Perry, H.M., Gottesman, K.S and C. Foeller. 1991. Sequences of proteins of immunological interest, 4th edn. United States Department of Health and Human Services, Washington, D.C.

Kabat, E.A., and T. Wu. 1991. Identical V-region amino acid sequences and segments of sequences in antibodies of different specificties. J. Immunol. 147, 1709-1719.

Kalinke, U., E.M. Bucher, B. Ernst, A. Oxenius, H. Roost, S. Geley, R. Kofler, R.M. Zinkernagel, and H. Hengartner. 1996. The role of somatic mutation in the generation of the protective humoral immune response against vesicular stomatitis virus. *Immunity* 5. 639-652.

Kaluza, B., Betzl, G., Shao, H., Diamantstein, T., and Weidle, U.H. 1992. A general method for chimerization of monoclonal antibodies by inverse polymerase chain reaction which conserves authentic N-terminal sequences. Gene 122, 321-328.

Kaplan, M., Keller, M., Hsu, D., Ch'ng, L., Miller, A., and E. Sercarz. 1988. A predominant idiotype independent of specificity or Ig and H-2 allotypes is found in the primary but not the secondary murine antibody response to lysosyme. Eur. J. Immunol. 18, 1567-1574.

Kaul, R., Trabattoni, D., Bwayo, J.J., Arienti, D., Zagliani, A., Mwangi, F., Kariuki, C., Ngugi, E., McDonald, K., Ball, T., Clerici, M., and F. Plummer. 1998. HIV specific mucosal IgA in a cohort of HIV-1 resistant kenyan sex workers. AIDS, in the press.

Kaumaya, P.T.P., VanBuskirk, A.M., Goldberg, E., and Pierce, S.K. 1992. Design and immunological properties of topographical .,

Kaumaya, P.T.P., Kobs-Conrad, S., Seo, Y., Lee, H., VanBuskirk, A., Feng, N., Sheridan, J., and V. Stevens. 1993. Peptide vaccines incorporating a "promiscuous" T cell epitope bypass certain haplotype restricted immune responses andprovide broad spectrum immunogenicity. J. Mol. Recog. 6, 81-94.

Kavaler, J., Caton, A., Staudt, L., Schwartz, D., and W. Gerhard. 1990. A set of closely related antibodies dominates the primary antibody response to the antigenic site CB of the A/PR/8/34 influenza virus hemagglutinin. J. Immunol. 145, 2312-2321.

Kelsoe, G., Miceli, R., Cerny, J., and D.H. Schulze. 1989. Mapping of antibody specificities to VH gene families. Immunogenetics 29, 288-296.

Kelsoe, G. 1991. The primary antibody Repertoire: The somatic genotype and paratopic phenotype of B cell populations. Immunol. Ser. 55, 81-92.

Kettleborough, C., Saldanha, J., Ansell, K., and Bendig, M.M. 1993. Optimization of primers for cloning libraries of mouse immunoglobulin genes using the polymerase chain reaction. Eur. J. Immunol. 23, 206-211.

Khan, C., Ramos, B., Pierce, R., Hormaeche, R., McNeil, H., Ali, T., Chatfield, S., Capron, A., Dougan, G., and C.E. Hormaeche. Construction, expression, and immunogenicity of multiple tandem copies of the *Schistosoma mansoni* peptide 115-131

of the P28 glutathione *S*-transferase expressed as C-terminal fusions to tetanus toxin fragment C in a live aro-attenuated vaccine strain of *Salmonella*. J. Immunol. 153, 5634-5642, 1994.

Kilbourne, E.D., Johansson, B.E., and B. Grajower. 1990. Independent and disparate evolution in nature of influenza A virus hemagglutinin and neuraminidase glycoproteins. Proc. Natl. Acad. Sci. U.S.A. 87, 786-790.

Kim, S., Davis, M., Sinn, E., Patten, P., and L. Hood. 1981. Antibody diversity: Somatic hypermutation of rearranged VH genes. Cell 27. 573.

Kirkham, P.M., Mortari, F., Newton, J.A., and H.W. Schroeder Jr. 1992. Immunoglobulin VH clan and family identity identity predicts variable domain structure and may influence antigen binding. EMBO J. 11, 603-609.

Kirschbaum, T., Jaenichen, R., and H. Zachau. 1996. The mouse immunoglobulin kappa locus contains about 140 variable gene segments. Eur. J. Immunol. 26, 1613-1620.

Kleinfeld, R., Hardy, R., Tarlington, D., Dangl, J., Herzenberg, L., and M. Weigert. 1986. Recombination between an expressed immunoglobulin heavy chain gene and a germline variable gene segment in ly+ B cell lymphoma. Nature 322, 843-

Klenerman, P., Hengartner, H., and R.M. Zinkernagel. 1997. A non-retroviral RNA virus persists in DNA form. Nature 390, 298-301.

Klinman, N.R., Sigal, N.H., Metcalf, S.K., and P.J. Gearhart. 1976. The interplay of evolution and environement in B cell diversification. Cold Spring Harb. Symp. Quant. Biol. 41, 165-173.

Klinman, N.R., and P. Linton. 1988. The clonotype repertoire of B cell subpopulations. Adv. Immunol. 42. 1-93.

Klinman, N.R., and Gingeras, T.R. 1994. Specific immunoglobulin cDNA clones produced from hybridoma cell lines and murine spleen fragment cultures by 3SR amplification. PCR Meth. Appl. 3, 320-331.

Knight, K. 1992. Restricted VH gene usage and generation of antibody diversity in rabbit. Annu. Rev. Immunol. 10, 593-616.

Kobs-Conrad, S., Lee, H., DiGeorge, A., and Kaumaya, P. 1993. Engineered topographic determinants with alpha/beta, beta/alpha/beta, and beta/alpha/beta/alpha topologies show high affinity binding to native protein antigen lactate dehydrogenase-C4.. J. Biol. Chem. 268, 25285-25295.

Koehler, J., Birkelund, S., and R. Stephens. 1992. Overexpression and surface localization of the *Chlamydia trachomatis* major outer membrane protein in E. coli. Mol. Microbiol. 6, 1087-1094.

Kofler, R., Duchosal, M., and F. Dixon. 1989. Complexity, polymorphism, and connectivity of mouse Vk gene families. Immunogenet. 29, 65-74.

Kofler, R., Geley, S., Kofler, H., and A. Helmberg. 1992. Mouse variable region gene families: Complexity, polymorphism and use in non-autoimmune responses. Immunol. Rev. 128, 6-21.

Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-497.

Kohsaka, H., Carson, D.A., Rassenti, L.Z., Ollier, W.E., Chen, P.P., Kipps, T.J., and N. Miyasaka. 1996. The human immunoglobulin V H. gene repertoire is genetically controlled and unaltered by chronic autoimmune stimulation. J. Clin. Invest. 98, 2794-2800.

Kraj, P., Rao, S., Gla, A., Hardy, R., Milner, E., and L. Silberstein. 1997. The human heavy chain Ig V region gene repertoire is biased at all stages of B cell ontogeny, including early pre-B cells. J. Immunol. 158, 5824-5832.

Krause, R.M., Dimmock, N. J., and D. Morens. 1997. Summary of antibody workshop: The role of humoral immunity in the treatment and prevention of emerging and extant diseases. J. Infect. Dis. 176, 549-559.

Kuby, J., 1992. Immunology. W.H. Freeman and Co. New York.

Kuo, CC. 1988. Host response. In, Barron, A. (ed.) Microbiology of Chlamydia. CRC Press, Boca Raton, 193-208.

Kurosawa, Y., and S. Tonegawa. 1982. Organization, structure, and assembly of immunoglobulin heavy chain diversity DNA segments. J. Exp. Med. 155, 201-218.

Kussie, P.H., Seren, B., Wysocki, L., and M. Margolies. 1994. A single engineered amino acid substitution changes antibody fine specificity. J. Immunol. 152, 146-152.

Kutteh, W.H., and J. Mestecky. 1994. Secretory immunity in the female reproductive tract. Am. J. Reprod. Immunol. 31, 40-46.

Lagervist, A.C.S., Furebring, C., and Borrebaeck, C.A.K. 1995. Single, antigen specific B cells used to generate Fab fragments using CD40-mediated amplification or direct PCR cloning. BioTechniques 18, 862-869

Lamm, M.E. 1997. Interaction of antigens and antibodies at mucosal surfaces. Ann. Rev. Microbiol. 51, 311-340.

Lampe, M.F., Suchland, R., and W.E. Stamm. 1993. Nucleotide sequence of the variable domains within the major outer membrane protein gene from serovariants of *Chlamydia trachomatis*. Infect. Immun. 61, 213-219.

Lampe, M.F., and W. E. Stamm. 1994. Purification of *Chlamydia trachomatis* strains in mixed infection by monoclonal antibody neutralization. J. Clin. Microbiol. 32, 533-535.

Lampe, M.F., Wong, K.G., Kuehl, L.M., and W.E. Stamm. 1997. Chlamydia trachomatis major outer membrane protein variants escape neutralization by both monoclonal antibodies and human immune sera. Infect. Immun. 65, 317-319.

Lampe, M., Wilson, C., Bevan, M., and M. Starnbach. 1998. Gamma interferon production by CTLs i required for resolution of Chlamydia trachomatis infection. Infect. Immun. 66, 5457.

Lan, F., Umeda, M., and K. Inoue. 1996. Extended fitness of variable region primers by a novel PCR protocol. J. Immunol. Meth. 195, 27-32.

Lane, R.D. 1985. A short-duration polyethylene glycol fusion technique for increasing production of monoclonal antibody-secreting hybridomas. J. Immunol. Meth. 81, 223-228.

Langman, R.F., and M. Cohn. 1993. What is the selective pressure that maintains the gene loci encoding the antigen receptors of T and B cells? Immunol. Cell Biol. 70, 397-401.

Langeveld, J., Casal, J.I., Osterhaus, A., Cortes, E., de stuart, R., Vela, C., Dalsgaard, K., Puijk, W.C. Schaaper, W.M.M., and Meloen, R. 1994. First peptide vaccine providing protection against viral infection against viral infection in the target animal: Studies of canine parvovirus in dogs. J. Virol. 68, 4506-4513.

Lanzavechia, A., 1985. Antigen-specific interaction between T and B cells. Nature 314, 537-539.

Larrick, J.W., Danielsson, L., Brenner, C., Wallace, E., Abrahamson, M., Fry, K.E., and Borrebaeck, C.A.K. 1989a. Polymerase chain reaction using mixed primers: Cloning of

human monoclonal antibody variable region genes from single hybridoma cells. Bio/Technology 7, 934-938.

Larrick, J.W., Danielsson, L., Brenner, C., Abrahamson, M., Fry, K.E., and Borrebaeck, C.A.K. 1989b. Rapid cloning of rearranged immunoglobulin genes from human hybridoma cells using mixed primers and the polymerase chain reaction. Biochem. Biophys. Res. Com. 160, 1250-1256.

Larrick, J.W., Coloma, M., Del Valle, J., Fernandez, M., Fry, K., and Gavilondo-Cowley, J. 1990. Immunoglobulin V regions of a bactericidal anti-Neisseria meningitidis outer membrane protein monoclonal antibody. Scand. J. Immunol. 32, 121-128.

Laskin, J., Gray, A., Nisonoff, A., Klinman, N., and P. Gotleib. 1977. Proc. Natl. Acad. Sci. USA 74, 4600.

Lavoie, T.B., Drohan, W.N., and S.J. Smith-Gill, 1992, Experimental analysis by site directed mutagenesis of somatic mutation effects on affinity and fine specificity in antibodies specific for lysosyme. J. Immunol. 148, 503-513.

Lawler, A.M., Kearney, J., Kuehl, M., and P.J. Gearhart. 1989. Proc. Natl. Acad. Sci. USA. 86, 6744.

LeBoeuf, R.D., Galin, F., Hollinger, S., Peiper, S., and Blalock, J.E. 1989. Cloning and sequencing of immunoglobulin variable region genes using degenrate oligodeoxyribonucleotides and polymerase chain reaction. Gene 82, 371-377.

Lebecque, S., Bouteiller, O., Arpin, C., Banchereau, and Y. Liu. 1997. Germinal center founder cells display propensity for apoptosis before onset of somatic mutation. J. Exp. med. 185, 563-571.

Lee, K., Sastry, P., Paranchych, W., and R. Hodges. 1989. Immunological studies of the disulfide bridge region of *Pseudomonas aeruginosa* PAK and PAO pilins, Using anti-PAK pilus and Antipeptide antibodies. Infect. Immun. 57, 520-526.

Lee, K., Panelli, M., Kim, C., Riker, A., Bettinotti, M., Roden, M., Fetsch, P., Abati, A., Rosenberg, S., and F. Marincola. 1998. Functional dissociation between local and systemic immune response during anti-melanoma peptide vaccination. J. Immunol. 161, 4183-4194.

Lefkovits, I. 1995. ...and such are little lymphocytes made of. Res. Immunol. 146, 5-10.

Leonetti, M., Cotton, J., Leroy, S., Mourier, G., and A. Menez. Immunogenicity of T epitope-containing cyclic peptides. Increasing neutralizing antibody responses by

introducing fine chemical changes. J. Immunol. 155, 210-218, 1995.

Letvin, N., Montefiori, D., Yasutomi, Y., Perry, H., Davies, M., Lekutis, C., Alroy, M., Freed, D., Lord, C., Handt, L., Liu, M., and J. Shiver. 1997. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. Proc. Natl. Acad. Sci7-883.

Liew, F.Y., Russell, S.M., Appleyard, G., Brand, C.M., and J. Beale. 1984. Cross-protection in mice infected with influeza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur. J. Immunol. 14, 350-356.

Loh, E.Y. Elliot, J., Cwirla, S., Lanier, L., Davis, M. 1989. Polymerase chain reaction with single-sided specificity: Analysis of T cell receptor delta chain. Science 243, 217-220.

Lohman, K., Attanasio, R., Buck, D., Carrillo, M., Allan, J., and R. Kennedy. 1992. Characteristics of murine monoclonal anti-CD4. J. Immunol. 149, 3247-3253.

Lu, Y., Hall, T., Gay, L., and J.E. Donelson. 1993. Point mutations are associated with a gene duplication leading to the bloodstream reexpression of a trypanosome metacyclic VSG. Cell 72, 397-406.

Lucas, A., Moulton, K., and D. Reason. 1998. Role of kII-A2 light chain CDR3 junctional residues in human antibody binding to the H. influenza type B polysaccharide. J. Immunol. 161, 3776-3780.

Lundkvist, A., Horling, J., Athlin, L., Rosen, A., and B. Niklasson. Neutralizing human monoclonal antibodies against Puumala virus, causative agent of nephropathia epidemica: a novel method using antigen coated magnetic beads for specific B cell isolation. J. gen. Virol. 74, 1303-1310, 1993.

Lundkvist A., et al., 1996. Virus Research 45, 29-44.

Lyaschenko, K., Colangeli, R., Houde, M., Jahdall, H., Menzies, D., and M. Gennaro. 1998. Heterogenous antibody responses in tuberculosis. Infect. Immun. 66, 3936-3940.

Lycke, N., and J. Holmgren. 1987. Long-term cholera antitoxin memory in the gut can be triggered to antibody formation associated with protection within hours of an oral challenge immunization. Scand. J. Immunol. 25, 407-412.

McClarty, G. 1994. Chlamydiae and the biochemistry of intracellular parasitism. Trends in Microbiology, 2, 157-164.

McDermott, M., and J. Bienenstock. 1979. Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory, and genital tissues. J. Immunol. 122, 1892-1898.

McGee, J.R., and H. Kiyono. 1993. New perspectives in vaccine development: mucosal immunity to infections. Infect. Agents. Dis. 2, 55-73.

McHeyzer-Williams, M., McLean, M., Lalor, P., and G. Nossal. 1993. Antigen-driven B cell differentiation in vivo. J. Exp. Med. 178, 295-307.

McKean, D., Huppi, K., Bell, M., Staudt, L., Gerhard, W., and M. Weigert. 1984. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. Proc. Natl. Acad. Sci. USA. 81, 3180-3184.

Mclean, I., Peeling, R., and R. Brunham. 1987. Characterization of *Chlamydia trachomatis* antigens with monoclonal and polyclonal antibodies. Can. J. Microbiol. 34, 141-147.

de la Maza., L.M., Pal, S., Khamesipour, A., and E.M. Peterson. 1994. Intravaginal inoculation of mice with the Chlamydia trachomatis mouse pneumonitis biovar results in infertility. Infect. Immun.62, 2094-7.

Ma, M., Wu, S., Howard, M., and Borkovec, A.B. 1984. Enhanced production of mouse hybridomas to picomoles of antigen using EL-4 conditioned media with an in vitro immunization protocol. In Vitro 20, 739-742.

Mahale, S.D., Pereira, J., Natraj, U., and K.S.N. Iyer. Comparison of antibodies raised against the peptide 10-24 of chicken riboflavin carrier protein cRCP. by classical and multiple antigen peptide MAP. approaches. J. Immunol. Meth. 190, 215-219, 1996.

Mainville, C.A., Sheehan, K., Klaman, L., Giorgetti, C., Press, J., and P.H. Brodeur. 1996. Deletional mapping of fifteen mouse VH gene families reveals a common organization for three IgH haplotypes. J. Immunol. 156. 1038-1046.

Maizels, N., and A. Bothwell. 1985. The T-cell-independent immune response to the hapten NP uses a large repertoire of heavy chain genes. Cell 43, 715-720.

Makela, O., and K. Karjalainen. 1988. Genetic control of early antibody responses. Immunol. Rev. 105, 85.

Malynn, B., Yancopoulos, G., Barth, J., Bona, C., and F.W. Alt. 1990. Biased expression of JH proximal Vh genes occurs in the newly generated repertoires of neonatal and adult

mice. J. Exp. Med. 171, 843.

Manca, F. Kunkl, A., Fenoglio, D., Fowler, A., Sercarz, E., and F. Celada. 1985. Constraints in T-B cooperation related to epitope topology on E. coli Beta-galactosidase. I. The fine specificity of T cells dictates the fine specificity of antibodies directed to conformation-dependent determinants. Eur. J. Immunol. 15, 345-350.

Manning, D., and S. Stewart. 1993. Expression of the major outer membrane protein of Chlamydia trachomatis in E. coli. Infect. Immun. 61, 4093-4098.

Manser, T. 1987. Mitogen driven B cell proliferation and differentiation are not accompanied by hypermutation of immunoglobulin variable region genes. J. Immunol. 139, 234-238.

Manser, T. 1990. Limits on heavy chain junctional diversity contribute to the recurrence of an antibody variable region. Mol. Immunol. 27, 503-511.

Manser, T., Huang, S., and M. Gefter. 1984. Influence of clonal selection on the expression of immunoglobulin variable region genes. Science 226, 1283-1288.

Manser, T., Wysocki, L., Gridley, R., Near, R., and M. Gefter. 1985. The molecular evolution of the immune response. Immunol. Today 6. 94-

Marguerie, C., Lunardi, C., and So, A. 1992. PCR-based analysis of the TCR repertoire in human autoimmune diseases. Immunol. Today 13, 336-338.

Mariuzza, R.A., Phillips, S.E.V., and R.J. Poljak. 1987. The structural basis of antigen antibody recognition. Ann. Rev. Biophys. Chem. 16, 139-159.

Marks, J., Tristrem, M., Karpas, A, and Winter, G. 1989. Oligonucleotide primers for polymerase chain reaction amplification of human immunoglobulin variable region genes and design of family specific oligonucleotide probes. Eur. J. Immunol. 21, 985-991.

Marrack, P., and J. Kappler. 1994. Subversion of the immune system by pathogens. Cell76,323-332.

Marshall, A., Wu, G., and C. Paige. 1996. Frequencyof the VH81x usage duing B cell develoment: initial decline in usage is independent of Ig heavy chain surface expression. J. Immunol. 156, 2077.

Mattila, P.S., Schugk, J., Wu, H., and O. Makela. 1995. Extensive allelic sequence variation in the J region of the human immunoglobulin heavy chaingene locus. Eur. J. Immunol. 25, 2578-2582.

Matsumoto, A. 1982. J. Bacteriol. 151, 1040-1042.

Max, E.1993. Immunoglobulins, structure and function. In Fundamental Immunology, Paul, W.E. (ed.), Raven Press, New York.

Mazanec, M., Kaetzel, C., Lamm, M.E., Fletcher, D., Peterra, J., and J.G. Nedrud. 1995. Intracellular neutralization of sendai and influenza viruses by IgA monoclonal antibodies. Advances in Mucosal Immunology. Mestecky, J., et al., eds. Plenum Press, New York.

Mazzoli, S., Trabattoni, D., Lo Caputo, S., Piconi, S., Ble, C., Meacci, F., Ruzzante, S., Salvi, A., Semplici, F., Longhi, R., Fusi, M., Tofani, N., Biasin, M., Luisa Villa, M., Mazzotta, F., and M. Clerici. 1997. HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals. Nature Med. 3, 1250-1257.

Mestecky, J., Michalek, S.M., Moldoveanu, Z., and M.W. Russel. 1997. Routes of immunization and antigen delivery systems for optimal mucosal immune responses in humans. Behring Inst. Mitt. Feb 98., 33-43.

Miller, M.D., Gould-Fogerite, S., Shen, L., Woods, R., Koenig, S., Mannino, J., and Letvin, N.L. 1992. Vaccination of rhesus monkeys with synthetic peptide in a fusogenic proteoliposome elicits simian immunodeficiency virus specific CD8+ cytotoxic T lymphocytes. J. Exp. Med. 176, 1739-1744.

Milner, E.C.B., Hufnagle, W.O., Glas, A., Suzuki, I., and C. Alexander. (1995) Polymorphism and utilization of human VH genes. Ann. N.Y. Acad. Sci. 764, 50-61.

Minoprio, P., Andrade, L., Lembezat, M., Sasser, D., Travis, W., Arnold, W., and G. Haughton. 1989. Indiscriminate representation of VH-gene families in the murine B lymphocyte response toTrypanosoma cruzi. J. Immunol. 142, 4017.

Mo, J., Bona, C., and R. Holmdahl. 1993. Variable region gene selection of immunoglobulin G- expressing B cell with specificity for a defined epitope on type II collagen. Eur. J. Immunol. 23, 2503-2510.

Mo, J., Scheynius, A., Nilsson, S., and R. Holmdahl. 1994. Germline encoded IgG antibodies bind mouse cartilage in vivo. Sacnd. J. Immunol. 39, 122-130.encode

Moldeveanu, Z., Clements, M.L., Prince, S.J., Murphy, B.R., and J. Mestecky. 1995. Human immun responses to influenza virus vaccines administered by systemic or mucosal routes. Vaccine 13, 1006-1012.

Morrison, R., Manning, S., and H. Caldwell.1992. Immunology of Chlamydia

trachomatis infections, in "Sexually transmitted Diseases", Quin, T., (ed.) Raven Press, New York.

Moss, B., Vaccinia virus: A tool for research and vaccine development. Science 252, 1662-1667, 1991.

Mostov, K.E. 1994 Transepithelial transport of immunoglobulins. Ann. Rev. Immunol. 12, 63-84.

Moulder, J., Hatch, T., Kuo, C., Schacter, J., and J. Storz. 1984. Genus Chlamydia In, Krieg, N., and J. Holt, eds. Bergey's manual of systematic bacteriology, 8th ed. Williams and Wilkins, Baltimore.

Moulder, J.W. 1991. Interaction of Chlamydiae and host cells in vitro. Microbiological Rev. 55, 141-190.

Muller, S., Plaue, S., Valette, M., Briand, J.P., and Van Regenmortel, M.H.V. 1990. Antigenic properties and protective capacity of a cyclic peptide corresponding to site A of influenza virus influenza. Vaccine ALtered fine conformation of the same looped peptide effect immunogenicity of the same nominal peptide-perhaps they effected the processing, if protection by a looped peptide then it suggests that optimal conformation of that peptide was fortuitous if not intentionally designed.

Murdin, A.D., Su, H., Manning, D.S., Klein, M.H., Parnell, M.J., and H.D. Caldwell. 1993. A poliovirus hybrid expressing a neutralization epitope from the major outer membrane protein of Chlamydia trachomatis is highly immunogenic. Infect . Immun. 61, 4406-4414.

Murdin, A.D., Su, H., Klein, M.H., and H.D. Caldwell. 1995. Poliovirus hybrids expressing neutralization epitopes from variable domains I and IV of the major outer membrane protein of Chlamydia trachomatis elicit broadly cross-reactive C. trachomatis -neutralizing antibodies. Infect. Immun. 63, 1116-1121.

Murphy, B.R., Kasel, J.A., and R.M. Chanock. 1972. Association of serum anti-neuraminidase antibody with resistance to influenza in man. New Eng. J. Med. 286, 1329-1332.

Murphy, B.R., Nelson, D.L., Wright, P.F., Tierney E.T., Phelan, M.A., and R.M. Chanock. 1982. Secretory and systemic immunological response in children infected with live attenuated influenza A vaccines. Infect. Immun. 36, 1102-1108.

Murray, E.S., Charbonnet, L.T., and A.B. MacDonald. 1973. Immunity to chlamydial infections of the eye. I. The role of circulatory and secretory antibodies in resistance to

reinfection with guinea pig inclusion conjunctivitis. J. Immunol. 110, 1518-1525.

Mystecky, J. 1987. The common mucosal immue system and current strategies for induction of immune responses in external secretions. J. Clin. Immunol. 7, 265-276.

Nadel, B., Tnag, A., and A. Feeney. 1998. VH replacement is unlikely to contribute significantly to receptor editing due to an ineffectual embedded recombination signal sequence. Mol. Immunol. 35, 227-232.

Nadel, B., Tnag, A., Escuro, G., Lugo, G., and A. Feeney. 1998b. Sequence of the spacer in the recombination signal sequence affects VDJ rearrangement frequency and correlates with nonrandom Vk usage in vivo. J. Exp. Med. 187, 1495-1503.

Nara, P.L., 1996. Humoral immunity to HIV-1: Lethal force or trojan horse? *In* Immunology of HIV infection. S. Gupta, editor. Plenum Pub. Co., N.Y._p.p.

Nara, P.L. 1998. An ounce of prevention. Vaccine 16, 1779.

Nara, P.L., and R. Garrity. 1998. Deceptive imprinting: a cosmopolitan strategy for complicating vaccination. Vaccine 16, 1780-1787.

Nayak, B., Tuteja, R., Manivel, V., Roy, R., Vishwakarma, R., and K. Rao. 1998. B cell responses to a peptide epitope. V. Kinetic regulation of repertoire discrimination and antibody optimization for epitope. J. Immunol. 161, 3510-3519.

Newhall, W.J., 5th. 1987. Biosynthesis and disulfide cross-linking of outer membrane components during the growth cycle of *Chlamydia trachomatis*. Infect. Immun. 55, 162-168.

Newkirk, M., and J.D. Rioux. 1995 Immunogobulin gene usage in the human anti-pathogen response. Infect. Agents Dis. 4, 153-160.

Newton, S.M.C., Jacob, C.O., and B.A.D. Stocker. 1989. Immune response to cholera toxin epitope inserted in Salmonella flagellin. Science 244, 70-72.

Newton, S.M.C., Joys, T.M., Anderson, S.A., Kennedy, R.C., Hovi, M.E., and B.A.D. Stocker. 1995. Expression and immunogenicity of an 18-residue epitope of HIV-1 gp41 inserted in the flagellar protein of a Salmonella live vaccine. Res. Microbiol. 146, 203-216.

Nichols, R.L., Oertley, R.E., Fraser, E.C., MacDonald, A.B.,and D.E. McComb. 1973. Immunity to chlamydial infections of the eye. VI. Homologous neutralization of trachoma infectivity for the owl monkey conjuctivae by eye secretions from humans with trachoma.

J. Infect. Dis. 127, 429-432.

Nicoletti, C., Borhesi-Nicoletti, C., Yang, X., Schulze, D., and J. Cerny. 1991. Repertoire diversity of antibody response to bacterial antigens in aged mice. II phosphorylcholine antibody repertoire in young and aged mice differ in both VH/Vl gene repertoire and specificity. J. Immunol. 147, 2750-2755.

Noronha, E., Wang, X., Desai, S., Kageshita, T., and S. Ferrone. 1998. Limited diversity of human scFv fragments isolated by panning a syntheitc phage display scFv Library with cultured human melanoma cells. J. Immunol. 161, 2968-2976.

Nossal, G., 1994. Negative selection of lymphocytes. Cell 76, 229-239.

Novottny, J., Handschumacher, M., and R.E. Bruccoleri. Protein antigenicity: a static surface property. Immunol. Today 8, 26-31, 1987.

Obeid, O.E., Partidos, C.D., Howard, C.R., and Steward, M.W. 1995. Protection against morbillivirus induced encephalitis by immunization with a rationally designed synthetic peptide vaccine containing B- and T-cell epitopes from the fusion protein of measles virus. J. Virol. 69,1420-1428.

Oettinger, M,. Schatz, D., Gorka, C., and D. Baltimore. 1990. Science 248, 1517-1523.

Ogasawara, K., Naruse, H., Itoh, Y., Gotohda, T., Arikawa, J., Kida, H., Good, R.A., and K. Onoe. A strategy for making synthetic peptide vaccines. Proc. Natl. Acad. Sci. 89, 8995-8999, 1992.

Ogra, P.L., Karzon, D.T., Righthand, F., and M. McGillivray. 1968. Immunoglobulin response in serum and secretions after immunization with live and inactivated polio vaccine and natural infection. N. Engl. J. Med. 279, 893-900.

Ohara, O., Dorit, R., and Gilbert, W. 1989. One-sided polymerase chain reaction: The amplification of cDNA. Proc. Natl. Acad. Sci. USA 86, 5673-5677.

Ohashi, M., and Y. Ozaki. 1981. Studies on the neutralizing antibody to Herpes simplex virus. I Effect of heterotypic antigenic stimulus on the type specificity of neutralizing antibody in rabbits. Arch. Virol. 67, 57-69.

Ohlin, M., Owman, H., Mach, M., and C. Borrebaeck. 1996. Light chain shuffling of a high affinity antibody results in a drift in epitope recognition. Mol. Immunol. 33, 47-56.

Ohno, S. 1970. Evolution by gene duplication. Springer-Verlag, Berlin.

Ojcius, D., Souque, P., Perfetinni, J., and A. Dautry-Varsat. 1998. Apoptosis of epithelial cells and macrophages due to infection with the obligate intracellular pathogen Chlamydia psittaci. J. Immunol. 161, 4220-4226.

Onorato, I.M., Modlin, J.F., McBean, A.M., Thomas, M.L., Losonsky, G.A., and G.H. Bernier. 1991. Mucosal immunity induced03-1809.

Pederson, L., Birkelund, S., Holm, A., Ostergaard, S., and G. Christiansen. 1996. The 18 kd Chlamydia trachomatis histone H1-like protein (Hc1) contains a potential N -terminal dimerization site and a C-terminal nucleic acid binding domain. J. Bacteriol. 178, 994-1002.

Peeling, R.W., and R.C. Brunham. 1996a. Treatment of chlamydial infections. Rec. Res. Dev. Antimicrob. Agents Chemother. 1, 455-469.

Peeling, R.W., and R.C. Brunham. 1996b. Chlamydiae as Pathogens: New species and new issues. Rudersorf, R., and R. DeMars. 1995. Human T cell epitopes of the *Chlamydia trachomatis* MOMP and HLA class II restriction elements for the epitopes. Abstract presented at the Eleventh meeting of the International Society for STD Research, New Orleans, Louisiana.

Osmond, D.G. 1986. B lymphocyte genesis in the bone marrow. Cinader, B and R. Miller. (eds.), In, Progress in Immunology, vol. VI. 49-58. Academic Press, New York. Paige, C., and G. Wu. 1989. The B cell repertoire. Faseb J. 3, 1818-1824.

Pal, S., Fielder, T.J., Peterson, E.M., and L.M. de la Maza.1994. Protection against infertility in a BALB/c mouse salpingitis model by intranasal immunization with the mouse pneumonitis biovar of *Chlamydia trachomatis*. Infect. Immun.62, 3354-62.

Pal, S., Peterson, E.M., L.M. de la Maza. 1996. Intranasal immunization induces long-term protection in mice against a *Chlamydia trachomatis* genital challenge. Infect-Immun. 64, 5341-8.

Pal., S., Theodor, I., Peterson, E.M., and L.M. de la Maza. 1997a. Monoclonal immunoglobulin A antibody to the major outer membrane protein of the *Chlamydia trachomatis* mouse pneumonitis biovar protects mice against a chlamydial genital challenge. Vaccine 15, 575-582.

Pal., S., Theodor, I., Peterson, E.M., and L.M. de la Maza. 1997b. Immunization with an acellular vaccine concisting of the major outer membrane complex of *Chlamydia trachomatis* induces protection against a genital challenge. Infect. Immun. 65, 3361-3369.

Pannetier, C., Cochet, M., Darche, S., Casrouge, A., Zoller, M., and P. Kourilsky. 1993. The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains as a function of the recombined germ-line segments. Proc. Natl. Acad. Sci. USA 90, 4319

Parhami-Seren, B., Wysocki, L., and M. Margolies. 1989. The amino acid residues at the VH-D-JH junctions affect the affinity of anti-p-azophenylarsonate antibodies. J. Immunol. 143, 4090-4097.

Parry, N., Ouldridge, E., Barnett, P., Clarke, B., Francis, M., Fox, J., Rowlands, D., and F. Brown. Serological prospects for peptide vaccines against foot-and-mouth disease virus. J. gen. Virol. 70, 2919-2930. 1989.

Patarroyo, M.E., Amador, R., Clavijo, P., Moreno, A., Guzman, F., Romero, P., Tascon, R., Franco, A., Murillo, L.A., Ponton, G., and Trujillo, G. 1988. A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum* malaria. Nature 332, 158-161.

Patera, A., Graham, C., Thomas, D., and C. Smith. Immunodominance with progenitor B cell diversity in the neutralizing antibody repertoire to influenza infection. Eur. J. Immunol. 25, 1803-1809.

Pederson, L., Birkelund, S., Holm, A., Ostergaard, S., and G. Christiansen. 1996. The 18 kd Chlamydia trachomatis histone H1-like protein (Hc1) contains a potential N -terminal dimerization site and a C-terminal nucleic acid binding domain. J. Bacteriol. 178, 994-1002.

Peeling, R.W., and R.C. Brunham. 1996a. Treatment of chlamydial infections. Rec. Res. Dev. Antimicrob. Agents Chemother. 1, 455-469.

Peeling, R.W., and R.C. Brunham. 1996b. Chlamydiae as Pathogens: New species and new issues. Emerg. Infect. Dis. 2, 307-319.

Peeling, R., Kimani, J., Plummer, F., Maclean, I., Cheang, M., Bwayo, J., and R. brunham. 1997. Antibody to chlamydial hsp60predicts an increased risk for chlamydial pelvic inflammatory disease. J. Infect. Dis. 175, 1153-1158.

Pernis, B., Forni, L., and A.R. Lawton. 1976. Synthesis of multiple immunoglobulin classes by single lymphocytes. Cold Spring Harbour Symp.Quant. Biol. 41, 175

Perry, L., Feilzer, K., and H. Caldwell. 1997. Immunity to *Chlamydia trachomatis* is mediated by T helper 1 cells through IFN-gamma dependent and independent pathoways. J. Immunol. 158, 3444-3352.

Persson, C., Nordfelth, R., Holstrom, A., Hakansson, S., Rosqvist, R., and H. Wolf-Watz. 1995. Cell-bound Yersinia translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. Molec. Microbiology 18, 135-150.

Peterson, E., de la maza, L., Brade, L., and H. Brade. 1998. Characterization of a neutralizing monoclonal antibody directed at the lipopolysaccharide of *Chlamydia pneumoniae*. Infect. Immun. 66, 3848-3855.

Pincus, S., Rosa, P., Spangrude, G., and J. Heinemann. 1992. The interplay of microbes and their hosts. Immuno. Today 13, 471-473.

Planz, O., Seiler, P., Hengartner, H., and R.M. Zinkernagel. 1996. Specific cytotoxic T cells eliminate cells producing neutralizing antibodies. Nature 382, 726-729.

Pollock, R.R., French, D., Gefter, M.L., and M.D. Scharff. 1988. Identification of mutant monoclonal antibodies with increased antigen binding. Proc. Natl. Acad. Sci. USA 85, 2298-2302.

Press, J.L., and C.A. Giorgetti. 1993. Molecular and kinetic analysis of an epitope-specific shift in the B-cell memory response to a multideterminant antigen. *J. Immunol.* 151. 1998-2013.

Pride, M., Shi, J., Anchin, D., Linthicum, P., LoVerde, A., Thakur, A., and Y. Thanavala. 1992. Molecular mimicry of hepatitis B surface antigen by an anti-idiotope derived synthetic peptide . Proc. Natl. Acad. Sci. USA 89, 11900-11904.

Puel, A., Mevel, J., Bouthillie, Y., Decreusefond, C., Fridman, W., Feingold, N., and D. Mouton. 1998. Identification of two quantitative trait loci involved in antibody production on mouse chromosome 8. Immunogenetics 47, 326-331.

Putkonen, P., Rolander, M., Charlotte, A., Schwartz, S., Thorstensson, R., Okuda, K., and J. Hinkula. 1998. Immune responses but no protection against SHIV by gene gun delivery of HIV-1 DNA followed by recombinant subunit protein boosts. Virology 250, 293-301.

Qu, Z., Cheng, X., de la maza, L., and E. Peterson. 1994. Analysis of the humoral response elicited in mice by a chimeric peptide representing variable segments I and IV of the major outer membrane protein of *Chlamydia trachomatis*. Vaccine 12, 557-564.

Quiding, M., Nordstrom, I., Kilander, A., Andersson, G., Hanson, L.A., Holmgren, J., and C. Czerkinsky. 1991. Intestinal immune responses in humans. Oral cholera vaccination induces strong intestinal antibody responses and interferon-gamma production and evokes local immunological memory. J. Clin. Invest. 88, 143-148.

Quiding-Jarbrink, M., Granstrom, G., Nordstrom, I., Holmgren, J., and C. Czerkinsky. 1995. Induction of compartmentalized B-cell responses in human tonsils. Infect. Immun. 63, 853-857.

Radbruck, A., Burger, S., Klein, S., and W. Muller. 1986. Immunol. Rev. 89, 69.

Radic, M.Z., and M. Zouali. 1996. Receptor Editing, Immune diversification, and self tolerance. Immunity 5, 505-511.

Rajewsky, K., Forster, I., and A. Cumano. 1987. Evolutionary and somatic selection of the antibody repertoire in the mouse. Science 218, 1088-1094.

Rajewsky, K. 1996. Clonal selection and learning in the antibody system. Nature 381, 751-757.

Rank, R.G., White, H.J., and A.L., Barron. 1979. Humoral immunity in the resolution of genital infection in femle guinea pigs infected with the agent of guinea pig inclusion conjuctivitis. Infect. Immuni. 26, 573-579.

Rank, R.G., and A.L., Barron. 1983. Humoral immune response in acquired immunity to chlamydial genital infection of female guinea pigs . Infect. Immun. 39, 463-465.

Rank, G., and P. Bavoil. 1996. Prospects for a vaccine against *Chlamydia* gential diseaseII. Immunity and vaccine development. Bull. Inst. Pasteur 94, 55-82.

Rapley, R., Flora, S., and M. Walker 1992. Direct PCR sequencing of murine immunoglobulin genes using E. coli single-stranded DNA-binding protein. PCR Meth. Appl. 2, 99-101.

Rassenti, L.Z., Kohsaka, H., and Kipps, T.J. 1995. Analysis of immunoglobulin V_H gene repertoire by an anchored PCR-ELISA. Ann. N.Y. Acad. Sci. 764, 463-473.

Ratech, H. 1992. Rapid cloning of rearranged immunoglobulin heavy chain genes from human B-cell lines using anchored polymerase chain reaction. Biochem. Biophys. Res. Commun. 182, 1260-1263.

Raulston, J. 1995. Chlamydial envelope components and pathogen-host cell interactions. Mol. Microbiol. 15, 607-616.

Renegar, K.B., and P.A. Small. 1991. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. J. Virol. 65. 2146-2148.

Reth, M., Gehrmann, P., Petrac, E., and P. Wiese. 1986. A novel VH to VHDJH joining

mechanism in heavy chain negative null. pre B cells results in heavy chain production. Nature 322. 840-

Reth, M.G., Jackson, S., and F. Alt. 1986. $V_H D_H J_H$ formation and $D_H J_H$ replacement during pre-B differentiation: non-random usage of gene segments. EMBO J. 5, 2131-2140.

Reynolds, S.R., Dahl, C.E., and Harn, D.A. 1994. T and B epitope determination and analysis of multiple antigenic peptides for the *Schistosoma mansoni* experimantal vaccine triose-phosphate isomerase. J. Immunol. 152, 193-200.

Richman, D.D., Murphy, B.R., Tierney, E., and R.M. Chanock. 1974. Specificity of the local secretory antibody to influenza A virus infection. J. Immunol. 113, 1654-1656.

Robinson, K., Bellaby, T., Chan, W., and D. Wakelin. High levels of protection induced by a 40-mer synthetic peptide vaccine against the intestinal nematode parasite *Trichinella spiralis*. Immunology 86, 495-498, 1995.

Rocca-Serra, J., Tonnelle, C., and M. Fougereau. 1983. Two monoclonal antibodies against different antigens using the same VH germ line gene. Nature 304, 353-355.

Roost, H., Bachmann, M., Haag, A., Kalinke, U., Pliska, V., Hengartner, H., and R. Zinkernagel. 1995. Early high-affinity neutralizing anti-viral IgG responses without further overall improvement of affinity. Proc. Natl. Acad. Sci. USA. 92, 1257-1261.

Rossqvist, R., Magnusson, K., and H. Wolf-Watz. 1994. Target cell contact triggers expression and polarized transfer of Yersinia YopE cytotoxin into mammalian cells. EMBO J. 13, 964-972.

Rowland-Jones, S. 1997. Dimers are a girl's best friend. Nature Med. 3, 1199-1200.

Rowlands, D.J. 1992. How can peptide vaccines work? FEMS Microbiol. Lett. 100, 479-482.

Ruberti, F., Cattaneo, A., and Bradbury, A. 1994. The use of the RACE method to clone hybridoma cDNA when v-region primers fail. J. Immunol Meth. 173, 33-39.

Rudikoff, S., Giusti, A., Cook, W., and M.D. Scharff. 1982. Single amino acid substitution altering antigen binding specificity. Proc. NAtl. Acad. Sci. USA. 79. 1979-

Rudikoff, S., Pawlita, M., Pumphrey, J., and M. Heller. 1984. Somatic diversification of immunoglobulins. Proc. Natl. Acad. Sci. USA. 81, 2162-2166.

Russel, M.W., Brown, T.A., and J. Mestecky. 1982. Preferential transport of IgA and OgA-immune complexes to bile compared with other external secretions. Mol. Immunol. 19, 677-682.

Sambrook, J., Fritsch, E., and T. Maniatis. 1989. Molecular cloning. A laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press. New York, USA.

Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Sanz, I., 1991. Multiple mechanisms participate in the generation of diversity of human H chain CDR3 diversity. J. Immunol. 147, 1720-1729.

Sassano, M., Repetto, M., Cassani, G., and Corti, A. 1994. PCR amplification of antibody variable regions using primers that anneal to constant regions. Nucleic Acids Res. 22, 1768-1769.

Sasso, E.H., Buckner, J.H., and L.A. Suzuki. (1995) Ethnic differences in VH gene polymorphism, Ann. N.Y. Acad. Sci. 764, 72-73.

Sastry, L., Alting-Mees, M., Huse, W., Short, J., Sorge, J., Hay, B., Janda, K., Benkovic, S., and Lerner, R.A. 1989. Cloning of the immunological repertoire in *Escherichia coli* for generation of monoclonal catalytic antibodies: Construction of a heavy chain variable region-specific cDNA library. Proc. Natl. Acad. Sci USA 86, 5728-5732.

Schatz, Oettinger, M., and D. Baltimore. 1989. Cell 59, 1035-1048.

Schaefer, B. 1995. Revolutions in rapid amplification of cDNA ends: New strategies for polymerase chain reaction cloning of full-length cDNA ends. Anal. Biochem. 227, 255-273.

Schilling, J., Clevinger, B., Davie, J., and L. Hood. 1980. Amino acid sequence of homogeneous antibodies to dextran and DNA rearrangements in heavy chain V-region gene segments. Nature 283, 35-40.

Schittek, B., and K. Rajewsky. 1992. Natural occurence and origin of somatically mutated memory B cells in mice. J. Exp. Med. 176, 427-438.

Schodel, F., Wirtz, R., Peterson, D., Hughes, J., Warren, R., Sadoff, J., and D. Milich. Immunity to malaria elicited by hybrid hepatitis B virus core particles carrying circumsporozoite protein epitopes. J. Exp. Med. 180, 1037-1046, 1994.

Schulman, J.L., and E.D. Kilbourne. 1965. Induction of partial specific heterotypic

immunity in mice by a single infection with influenza A virus. J. Bact. 89, 170-174.

Schulze-Gahmen, U., Klenk, H., and Beyreuther, K. 1986. Immunogenicity of loop-structured short synthetic peptides mimicking the antigenic site A of influenza virus hemagglutinin. Eur. J. Biochem. 159, 283-289. Early conformation LOOPED PAPER with improved immuogenicity.

Schwartzkoff, C.L., J. Egerton, D. Stewart, P. Lehrbach, T. Elleman, and P.A. Hoyne. 1993. The effects of antigenic competition on the efficacy of multivalent footrot vaccines. *Aust. Vet. J.* 70: 123-126.

Scott, G.H., and R.J. Sydiskis. 1976. Infect. Immun. 13, 696.

Scott, M., Crimmins, D., McCourt, D., Zocher, R., Thiebe, R., and H. Zachau. 1989. Clonal characterization of the human IgG antibody repertoire to H. influenza b polysaccharide. II. J. Immunol. 143, 293.

Scott, J.K., and G.P. Smith. 1990. Searching for peptide ligands with an epitope library. Science 249, 386-390.

Seidl, K.J., MacKenzie, J., Wang, D., Kantor, A., Kabat, E., Herzenberg, L., and L.A. Herzenberg. 1997. Frequent occurence of identical heavy and light chain Ig rearrangements. Int. Immunol. 9, 689-702.

Seidl, K.J., MacKenzie, J., Wang, D., Kantor, A., Kabat, E., Herzenberg, L., and L.A. Herzenberg. 1997b. Recurrent identical rearrangement and repeated expression of identical heavy and light chains in single anti-phosphatidylcholine B cells. Ann. N.Y. Acad. Sci. 815, 484-488..

Sela, M. 1969. Antigenicity: Some molecular aspects. Science 166, 1365-1374.

Sercarz, E. 1989. The architechtonics of Immune dominance: The alelatory effects of molecular position on the choice of antigenic determinants. In: Antigenic determinants and Immune regulation. Chem. Immunol. 46, 169-185. Sercarz, E. ed.. Basel, Karger.

Shapiro, M.A., and M. Weigert. 1987. How immunoglobulin genes rearrange. J. Immunol. 139, 3834.

Sharon, J., Gefter, M., Wysocki, L., abd M. Margolies. 1989. Recurrent somatic mutations in mouse antibodies to p-azophenylarsonate increase affinity for hapten. J. Immunol. 142, 596-601.

Shaw, D., Stanley, C., Partidos, C., and Steward, M. 1993. Influence of the T-helper

epitope on the titre and affinity of antibodies to B-cell epitopes after coimmunization. Mol. Immunol. 30, 961-968.

Shen, G.L., Liu, E.X., and A.R. Wu. 1986. Relationship between three different B cell types and the specific efficiency of hybridoma formation. Sci. Sin. B. 29, 165-72.

Shvartsman, Y., Agranovskaya, E.N., and M.P. Zykov. 1977. J. Infect. Dis. 135, 697.

Siber, G.R., Santosham, M., Reid, G., Thompson, C., Almeido, J., Morrel, A., deLange, G., Ketcham, J., and E. Callahan. 1990. Impaired antibody response to *Haemophilus influenza* type b polysaccharide and low IgG2 and IgG4 concentrations in Apache children. N. Eng. J. Med. 323, 1415-1416.

Siekevitz, M., Huang, Z., and M. Gefter. 1983. The genetic basis for antibody production: a single VH gene encodes all mabs to arsonate. Eurr. J. Immunol. 13, 123-132.

Siekevitz, M., C. Kocks, K. Rajewsky, and R. Dildrop. 1987. Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. *Cell* 48. 757-770.

Silverman, G. and A. Lucas. 1991. Variable region diversity in human circulating antibodies specific for the capsular polysaccharide of Haemophilus influenza type b. J. Clin. Invest. 88, 911-920.

Silverman, G. 1995. Unconventional B-cell antigens and human immune repertoires. Ann. N.Y. Acad. Sci. 764, 342-355.

Sinclair, M.C., and Aitken, R. 1995. PCR strategies for the isolation of the 5' end of an immunoglobulin-encoding bovine cDNA. Gene 167, 285-289.

Slifka, M.K., Antia, R., Whitmire, J., and R. Ahmed. 1998. Humoral immunity due to long lived plasma cells. Immunity 8, 363-372.

Smith, G.P., Hood, L., and W.M. Fitch. 1971. Antibody Diversity. Annu. Rev. Biochem. 40, 778-1011.

Smith, G.P. 1985. Filamentous fusion phage: Novelexpression vectors that display cloned antigens on the virion surface. Science 228, 1315-1317.

Smith, G.P. 1994. The progeny of sexual PCR. Nature 370, 389-391.

Snapper, S., Lugosi, L., Jekkel, A., Melton, R., Keiser, T., Bloom, B., and W.R. Jacobs. Lysogeny and transformation in mycobacteria: stable expression of foreign genes. Proc.

Natl. Acad. Sci. 85, 6987-6991, 1988.

Snijders, A., Benaissa-Trouw, B., Oosterlaken, T., Puijk, W., Posthumus, W., Meleon, R., Boere, W., Oosting, J., Kraaijeveld, C., and Snippe, H.. Identification of linear epitopes on semliki forest virus E2 membrane protein and their effectiveness as a synthetic peptide vaccine. J. gen. Virol. 72, 557-565, 1991.

Snijders, A., Benaissa-Trouw, B., Snippe, H., and C. Kraaijeveld. Immunogenicity and vaccine efficacy of synthetic peptides containing semliki forest virus B and T epitopes. J. gen. Virol. 73, 2267-2272, 1992.

Solin, M-L., and M. Kaartinen. 1992. Allelic polymorphism of mouse Igh-J locus, which encodes immunoglobulin heavy chain joining JH. segments. Immunogenet. 36, 306-313.

Sollazzo, M., Haseman, C., Meek, K., Glotz, D., Capra, J., and M. Zanetti. 1989. Molecular characterization of the VH region of murine autoantibodies from neonatal and adult BALB/c mice. Eur. J. Immunol. 19, 453-457.

Sompuram, S., Den, W., and J. Sharon. 1996. Analysis of antigen binding and idiotypic expression by antibodies with polyglycine-replaced complementary determining regions. J. Immunol. 156, 1071-1081.
CDR1 critical for hapten binding

Sood, A.K., Cheng, H., and Kohler, H. 1986. An efficient and general method for sequencing immunoglobulin mRNAs. J. Immunol. Meth. 95, 227-235.

Spalatin, J., Fraser, C.E., Connell, R., Hanson, R., and D.T. Berman. 1966. Agents of psittacosis-lymphogranuloma venereum group isolated from muskrats and snowshoe hare in Saskatchewan. Can. J. Comp. Med. Vet. Sci. 30, 260-264.

Spalatin, J., Iverson, J.O., and R.P. Hanson. 1971. Properties of a *Chlamydia psittaci* isolated from muskrats and snowshoe hares in Canada. Can. J. Microbiol. 17, 935-942.

Spangler, B. 1991. Binding to native proteins by antipeptide monoclonal antibodies. J. Immunol. 146, 1591-1595.

Spanopoulou, E., Zaitseva, F., Wang, F., Santagata, S., Baltimore, D., and G. Panayotou. 1996. The homeodomain region of Rag-1 reveals the parallel mechanisms of bacterial and V D.J recombination. Cell 87, 263-276.

Stanfield, R.S., Fieser, T.M., Lerner, R.A., and I.A. Wilson. 1990.. Crystal structures of an antibody bound to a peptide and its complex with peptide antigen at 2.8 amgstroms. Science 248, 712-719.

Stanfield, R.S., Takimoto-Kamimura, M., Rini, J.M., Profy, A.T., and I.A. Wilson. 1993. Major antigen induced domain rearrangements in an antibody. Curr. Biology 1, 83-93.

Stanhope-Baker, P., Hudson, A., Shaffer, A., Constantinescu, A., and M. Schlissel. 1996. Cell-type specific chromatin structure determines the targetting of V(D)J recombinase activity in vitro. Cell 85, 887.

Stark, S.E., and A.J. Caton. 1991.. Antibodies that are specific for a single amino acid interchange in a protein epitope use structurally distinct variable regions. J. Exp. Med. 174. 613-624.

Steele. E.J. 1979. Somatic selection and Adaptive evolution: on the inheritance of acquired characters. Williams-Wallace, Toronto.

Steele, E.J., Rothenfluh, H., Ada, G.L., and R.V. Blanden. 1993. Affinity maturation of lymphocyte receptors and positive selection of T cells in the thymus. Immunol. Rev. 135. 5-49.

Stephens, R., Wagar, E., and U. Edman. 1988. Developmental regulation of tandem promoters for the MOMP gene of *Chlamydia trachomatis* . J. Bacteriol. 170, 744-750.

Stephens, R.S., Wagar, E.A, and G.K. Schoolnik. 1988. High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of *Chlamydia trachomatis*. J. Exp. Med. 167, 817-31.

Stephens, R.S. 1998. Phylogenetic analysis of a multigene family conserved between *Chlamydia trachomatis* and *Chlamydia pneumoniae*. In, Chlamydial Infections. Proceeding of the 9th International symposium on human chlamydial infection. Napa, California.

Steward, M.W., and C.R. Howard. 1987. The potential of synthetic peptides for vaccines. Med. Lab. Sci. 42, 376-387.

Stillman, C.A., Linton, P.J., Koutz, P., Decker, D., Klinman, N.R., and Gingeras, T.R. 1994. Specific immunoglobulin cDNA clones produced from hybridoma cell lines and murine spleen fragment cultures by 3SR amplification. PCR Meth. Appl. 3, 320-331.

Stothard, D., Buguslawski, G., and R. Jones. 1998. Phylogenetic analysis of the *Chlamydia trachomatis* major outer membrane protein and examination of potential pathogenic determinants. Infect. Immun. 66, 3618-3625.

Stover, C., de la Cruz, V., Fuerst, T., Burlein, J., Benson, L., Bennet, L., Bansal, G.,

Young, J., Lee, M., Hatfull, G., Snapper, S., Barletta, R., Jacobs, W., and B. R. Bloom. New use of BCG for recombinant vaccines. Nature 351, 456-460, 1991.

Su, H., Watkins, N., Zhang, X., and H. Caldwell. 1990. *Chlamydia trachomatis*-host cell interactions: role of the chlamydial major outer membrane protein as an adhesin. Infect. Immun. 58, 1017-1025.

Su, H., Morrison, R., Watkins, N., and H. Caldwell. 1990b. Identification and characterization of T helper epitopes of the major outer membrane protein of *Chlamydia trachomatis*. J. Exp. Med. 172, 203-212.

Su., H., and H.D. Caldwell. 1992. Immunogenicity of a chimeric peptide corresponding to T helper and B cell epitopes of the *Chlamydia trachomatis* major outer membrane protein. J. Exp. Med. 175, 227-235.

Su, H., Parnell, M., and H.D. Caldwell. 1995. Protective efficacy of a parenterally administered MOMP-derived synthetic oligopeptide vaccine in a murine model of *Chlamydia trachomatis* genital tract infection: serum neutralizing IgG antibodies do not protect against chlamydial genital tract infection. Vaccine 13, 1023-1032.

Su, H., Feilzer, K., Caldwell, H., and R. Morrison. 1997. Chlamydia trachomatis genital tract infection of antibody deficient gene knockout mice. Infect. Immun. 65, 1993-1999.

Suga, H., Ersoy, O., Williams, S., Tsumuraya, T., Margolies, M., Sinskey, A., and S. Masamune. 1994.. Catalytic antibodies generated via heterologous immunization. J. Am. Chem. Soc. 116. 6025-6026.

Sutcliffe, A., Shinnick, T., Green, N., and R.A. Lerner. 1983. Antibodies that react with predeterm,ined sites on proteins. Science 219, 660-666.

Svennerholm, A.M., Holmgren, J., Hanson, L.A., Lindblad, B., Quereshi, F., and R.J. Rahimtoola. 1977. Boosting of secretory IgA antibody responses in man by parenteral cholera. Scand. J. Immunol. 6, 1345-1349.

Tam, J.P. Recent advances in multiple antigen peptides. J. Immunol. Meth. 196, 17-32, 1996.

Tang, D., DeVit, M., and S. Johnston. 1992. Genetic immunization is a simple method for eliciting an immune response. Nature 356, 152-154.

Tarlinton, D., Stall, A., and L.A. Herzenberg. 1988. Repetitive usage of immunoglobulin VH and DH gene segments in CD5+ Ly-1 B clones of (NZB X NZW)F1 mice. The EMBO J. 7, 3705-3710.

Taylor, H.P. and N.J. Dimmock. 1985. Mechanism of neutralization of influenza virus by scretory IgA is different from that of monomeric IgA or IgG. J. Exp. Med. 161, 198-209.

Taylor, H.R. 1990. Development of immunity to ocular chlamydial infection. *Am. J. Trop. Med. Hyg.* 42. 358-364.

Teale, J., and E.G. Morris. 1989. J. Immunol. 143. 2768.

ten Boekel, E., Melchers, F., and A. Rolink. 1997. Changes in the VH repertoire of developing precursor B lymphocytes in mouse bone marrow mediated by the pre-B cell receptor. Immunity 7, 357-368.

Thiry, G., Clippe, A., Scarcez, T., and J. Petre. 1989. Cloning of DNA sequences encoding foreign peptides and their expression in the K88 pili. Appl. Env. Microbiol. 55, 984-993.

Thompson, C. 1995 New insights into VDJ recombination and its role in the evolution of the immune system. Cell 3, 531-539.

Thunberg, U., Rosenquist, R., Lindstrom, A., Lindh, J., Sundstrom, C., Roos, G., and J. Sallstrom. 1997. Comparative analysis of detection systems for evaluation of PCR amplified immunoglobulin heavy-chain gene rearrangements. Diag. Mol. Path. 6, 140-146.

Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature lond.. 302, 575-

Tomasi, T.B. Jr., ans S.D. Zigelbaum. 1963. The selective occurrence of gamma-IA globulins in certain body fluids. Clin. Invest. 42, 1552-1560.

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

Toye, B., Zhong, G., Peeling, R, and R. Brunham. 1990. Immunologic characterization of a cloned fragment containing the species-specific epitope from the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. 58, 3909-3913.

Trentin, L., Zambello, R., Facco, M., Sancetta, R., Cerutti, A., Milani, A., Tassarini, C., Crivellaro, C., Cipriani, A., Agostini, C., and G. Semenzato. 1996. Skewing of the T cell receptor repertoire in the lung of patients with HIV-1 infection. AIDS 10, 729-237.

Tribbick, G., Triantafyllou, B., Lauricella, R., Rodda, S.J., Mason, T., and H.M. Geysen.

Systematic fractionation of serum antibodies using multiple antigen homologous peptides as affinity ligands. J. Immunol. Meth. 139, 155-166, 1991.

Triantafyllou, B., Tribbick, G., Maeji, N., and H.M. Geysen. Use of the multiple peptide synthesis technique for the generation of antipeptide sera. Cell Biophys. 21, 33-52, 1993.

Troutt, A.B., McHeyzer-Williams, M.G., Pulendran, B., and Nossal, G.J.V. 1992. Ligation-anchored PCR: A simple amplification technique with single sided specificity. Proc. Natl. Acad. Sci. USA 89, 9823-9825.

Tumas-Brundage, K., and T. Manser. 1997. The transcriptional promoter regulates hypermutation of the antibody heavy chain locus. J. Exp. med. 185, 239-250.

Ulrich, H., Moore, F., and P. Schulz 1997. Germline diversity within the mouse Igk-V9 genefamily. Immunogenetics 47, 91-95.

van Regenmortel, M.H.V. 1998. From absolute to exquisite specificity.Reflections on the fuzzy nature of species, specificity and antigenic sites. J. Immunol. Meth. 216, 37-48.

Villaverda, M., Carol, H., Hjulstrom, M., Holmgren, J., and C. Czerkinsky. 1997. "PERFEXT": a direct method for quantitative assessment of cytokine production in vivo at the local level. Res. Immunol. 148, 257-266.

Villeneuve, A., Brossay, L., Paradis, G., and J. Hebert. 1994. Determination of neutralizing epitopes in the variable domains I and IV of the major outer membrane protein from *Chlamydia trachomatis* serovar K. Microbiology 140, 2481-2487.

Vora, K., and T. Manser. 1995. Altering the antibody repertoire via transgene homologous recombination: Evidence for global and clone-autonomous regulation of antigen-driven B cell differentiation. J. Exp. Med. 181,271-281.

Wagner, S., Milstein, C., and M. Neuberger. 1995. Codon bias targets mutation. Nature 376, 732.

Wagner, S., and M. Neuberger. 1996. Somatic mutation of immunoglobulin genes. Annu. Rev. Immunol. 14. 441-457.

Waldman, R.H., Wood, S.H., Torres, E., and P.A. Small. 1970. Influenza antibody response following aerosol administration of inactivated virus. Am. J. Epidemiol. 91, 575-584.

Waldman, R.H., Wigley, F.M., and P.A. Small. 1970. Am. J. Epidemiol. 91, 575.

Walter, G., Tomlinson, I., Dear, P., Sonhammer, L., Cook, G., and G. Winter. 1995. Comparison of the human germline and rearranged V_H repertoire reveals complementarity between germline variation and somatic mutation. Ann. N.Y. Acad. Sci. 764, 180-182.

Wang, S., and J.T. Grayston. 1970. Local and systemic antibody response to trachoma eye infection in monkeys. *In* Trachoma and Related Disorders Caused By Chlamydial Agents, *Excerpta Medica Int. Congress Series 223.* 217-232.

Wang, S., and J.T. Grayston. 1974. Human serology in *Chlamydia trachomatis* infection with microimmunofluorescence. *J. Infect. Dis.* 130. 388-397.

Wang, S., and J.T. Grayston. 1982. Micro imunofluorecence antibody responses in *Chlamydia trachomatis* infection, a review. *In* Chlamydial Infections. P.-A. Mardh et al. editors. Elsevier Biomedical Press 301-316.

Wang, S.P., and J.T. Grayston. 1984 Micro-immunofluoresence serology of *Chlamydia trachomatis.* In Medical Virology III, dela Maza and Peterson, eds. Elsevier Science Publishing Co. 87-118.

Wang, Y., and M. Yeh. 1996. Molecular characterization of the V genes of four IgE antibodies to trichosanthin. Immunology 89, 316-323.

Ward, E.S., Gussow, D., Griffiths, A., Jones, P.T., and Winter, G. 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature 341, 544-546.

Ward, M.E. 1997. Chlamydia host and host cell interactions. In, Molecular aspects of host pathogen interactions. M.A. McCrae et al., eds. Cambridge University Press, New York.

Wasniowska, K., Duk, M., Czerwinski, M., Steuden, I., Dus, D., Radzikowski, C., Bartosz-Bechowski, H., Konopinska, D., and Lisowska, E. 1992. Analysis of peptidic epitopes recognized by the three monoclonal antibodies specific for the same region of glycophorin A but showing different properties. Mol. Immunol. 29,783-791.

Wassen, L., Schon, K., Holmgren, J., Jertborn, M., and N. Lycke. 1996. Local intravaginal vaccination of the female genital tract. Scand. J. Immunol. 44, 408-414.

Weber, J., Blaison, T., Martin, T., Knapp, A., and J. Pasquali. 1994. Evidence that the VkIII gene usage is non-stochastic in both adult and newborn peripheral B cells. J. Clin. Invest. 93, 2093-2105.

Webster, R.G., and B.A. Askonas. 1980. Cross-protection and cross-reactive cytotoxic T

cells induced by influenza virus vaccines in mice. Eur. J. Immunol. 10, 396-401.

Weiss, K.M. 1993. Genetic variation and human disease: Principles and evolutionary approaches. Cambridge University Press. New York.

Williams, M., Rose,J., Rott, L., Franco, M., Greenberg, H., and E. Butcher. 1998. The memory B cell subset responsible for the secretory IgA response and protective humoral immunity to rotavirus expresses the intestinal homing receptor alpha4beta7. J. Immunol. 161, 4227-4235.

Williams, W.V., Kieber-Emmons, T., VonFeldt, J., Greene, M.I., and Weiner, D.B. 1991. Design of bioactive peptides based on antibody hypervariable region structures. Development of conformationally constrained and dimeric peptides with enhanced affinity. J. Biol. Chem. 266, 5182-5190.

Wilson PC, de Bouteiller O, Liu YJ, Potter K, Banchereau J, Capra JD, and V. Pascual. 1998. Somatic hypermutation introduces insertions and deletions into immunoglobulin V genes. J. Exp. Med. 187,59-70.

Wimmer, E., and B. Jameson. Poliovirus antigenic sites and vaccines. Nature 308, 19, 1984.

Wimmer, E., Emini, E., and B. Jameson. Peptide priming of a poliovirus neutralizing antibody response. J. Infect. Dis. 6 S2., S505-S509, 1984.

Wisdom, G.B., Peptide antigens, Biochem. Soc. Trans. 20, 226-229, 1992.

Wolff, J.A., Malone, R., Williams, P., Chong, W., Ascadi, G. Jani, A., and P. Felgner. 1990. Direct gene transfer into mouse muscle in vivo. Science 247, 1465-1468.

Wright, P.F., Murphy, B.R., Kervina, M., Lawrence, E.M., Phelan, M.A. and D.T. Karzon. 1983. Secretory immunological response after intranasal inactivated influenza A virus vaccinations: evidence for immunoglobulin A memory. Infect. Immun. 40, 1092-1095.

Wu, T.T., and E.A. Kabat.1970. An analysis of the sequences of the variable regions of bence jones proteins and myeloma light chains and their implicationsfor complementarity.J. Exp. Med. 132, 211-250.

Wyllie, S., Ashley, R.H., Longbottom, D., and A.J. Herring. 1998. The major outer membrane protein of *Chlamydia psittaci* functions as a porin-like ion channel. Infect Immun 11: 5202-7.

Wysocki, L.J., Manser, T., Gridley, T., and M. Gefter. 1986. Molecular limitatons on variable region gene junctional diversity. J. Immunol. 137, 3699-3701.

Xiang, J.H., Roder, J., Pan, Z., Roifman, C., and Hozumi, N. 1991. Modification in framework region 1 results in a decreased affinity of chimeric anti-TAG72 antibody. Mol. Immunol. 28, 141-148.

Xiang, J., Chen, Z., Delbaere, L., and E. Liu. 1993. Differences in antigen binding affinity caused by a singleamino acid substitution in the variable region of the heavy chain. Immunol. Cell. Biol. 71, 239-247.

Xuejun, Z., and W. Jiahua. 1986. Homology of trichosanthin and ricin A chain. Nature 321, 477-478.

Yancopolous, G., Desiderio, M., Paskind, J., Kearney, J, Baltimore, D., and F. Alt. 1984. Preferential utilization of the most JH proximal VH gene segments in pre B cell lines. Nature 311, 727.

Yancopolous, G., Malynn, B., and F. Alt. 1988. Developmentally regulated and strain-specific expression of murine VH gene families. J. Exp. Med. 168, 417-435.

Yang, X., and R.C. Brunham. 1998. T lymphocyte immunity in host defence against *Chlamydia trachomatis* and its implication for vaccine development. Can. J. Infect. Dis. 9, 99-109.

Yetter, R.A., Lehrer, S., Ramphal, R., and P.A. Small. 1980a. Outcome of influenza infection: Effect of site of initial infection and heterotypic immunity. Infect. Immun. 29, 654-662.

Yetter, R.A., Barber, W.H., and P.A. Small Jr. 1980b. Heterotypic immunity toinfluenza in ferrets. Infect. Immun. 29, 650-653.

Yu, C.C.K., Larijani, M., Miljanic, I., and G. Wu. 1998. Differential usage of VH gene segments is mediated by cis elements. J. Immunol. 161, 3444-3454.

Yuan, D., and P.W. Tucker. 1984. Regulation of IgM and IgD synthesis in B lymphocytes I. Changes in biosynthesis of mRNA for mu and delta chains. J. Immunol. 132. 1561-1565.

Yuan, Y., Y. Zhang, N. Watkins, and H.D. Caldwell. 1989. Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. *Infect. Immun.* 57. 1040-1049.

Zanetti, M., Sercarz, E., and J. Salk. The immunology of new generation vaccines. Immunol. Today 8, 18-25, 1987.

Zhang, Y., Stewart, S., and H. Caldwell. 1989. Protective monoclonal antibodies to CHlamydia trachomatis serovar-, and serogroup specific MOMP protein determinants. Infect. Immun. 57, 636-638.

Zhang, J.P., and R. Stephens. 1992. Mechanism of *C. trachomatis* attachment to eukaryotic host cells. Cell 69, 861-869.

Zhang, D., Yang, X., Berry, J.D., Shen, C., McClarty, G., and R.C. Brunham. 1996a. Mucosal DNA immunization with the outer membrane protein MOMP. gene protects against *Chlamydia trachomatis* infection. Abstract In: Molecular Approaches to the Control of Infectious Diseases, September 9-13, Cold Spring Harbour, New York.

Zhang, D., Yang, X., Shen, C., Berry, J., McClarty, G., and R.C. Brunham. 1996b. Intramuscular immunization with a DNA vaccine produces partial immunity to *Chlamydia trachomatis* infection. In: Brown, F., Burton, D., Doherty, P., Mekalanos, J., Norrby, E. eds. <u>Vaccines 97</u>. New York: Cold Spring Harbour Laboratory Press.

Zhang, D., Yang, X., Berry, J., Shen, C., McClarty, G., and Brunham, R.C. 1997. DNA vaccination with the MOMP gene protects against *Chlamydia trachomatis* infection. J. Infect. Dis. 176, 1035-1040.

Zhang, J., and W. Liu. 1992. The mechanism of action of trichosanthin on eukaryotic ribosomes. Nucleic Acids Res. 20, 1271-1275.

Zhao, Q., C. Lammel, E. Lindquist, and R.S. Stephens. 1996. Recall of original serologic response after challenge with homologous and heterologous *Chlamydia trachomatis* serovars. *J. Infect. Dis.* 173. 609-618.

Zhong, G., and R.C. Brunham. 1990a. Immunoaccessible peptide sequences of thgene subfamily is highly polymorphic. J. Immunol. 151, 800-809.

Adderson, E.E., Wilson, P.M., Cunningham, M., and P. Shackelford. 1998. *Haemophilus influenza* type b polysaccharide-protein conjugate vaccine elicits a more diverse antibody repertoire in infants than in adults. J. Immunol. 161, 4177-4182.

Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: Understanding their relation. *Science* 272. 54-60.

Akolkar, P., Sikder, S., Bhd, R., and R.C. Brunham. 1993. Immunogenicity evaluation of

a lipidic amino acid-based synthetic peptide vaccine for *Chlamydia trachomatis*. J. Immunol. 151, 3728-3736.

Zhong, G., Berry, J.D., and R.C. Brunham. 1994a. Antibody recognition of a neutralization epitope on the major outer membrane protein on *Chlamydia trachomatis*. Infect. Immun. 62. 1576-1583

Zhong, G., Smith, G.P., Berry, J.D., and R.C. Brunham. 1994b. Conformational mimicry of a chlamydial neutralization epitope on filamentous phage. J. Biol. Chem. 269, 24183-24188.

Zhong, G., Berry, J.D., and S. Choukri. 1997. Mapping epitopes of neutralizing monoclonal antibodies using phage random peptide libraries. J. Ind. Microbiol. Biotechnol. 19, 71-76.