The Potential for Vaccine Development against Chlamydial Infection and Disease

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Chlamydia trachomatis and Chlamydia pneumoniae appear to share a common immunobiology with about 80% of their protein coding genes being orthologs. Progress in DNA vaccine development for C. trachomatis suggests that such a subunit approach may prove useful for C. pneumoniae. The recent finding that it is possible to select for chlamydiae with targeted mutations in key metabolic genes together with the new knowledge of the chlamydia genome also suggests that it may be possible to develop live attenuated strains of chlamydiae for use as vaccine.

We hope this information will facilitate the development of a C. pneumoniae vaccine based on similar principles.

Chlamydial Immunity and Pathology

Animal models have been especially helpful in defining the immunobiologic features of C. trachomatis infection [4]. The mouse model is particularly informative, largely because of the ready availability of immune reagents for murine studies and the development of transgenic and knockout (KO) mice. C. trachomatis mouse pneumonitis (MoPn) is the most widely tested biovar among the three C. trachomatis biovars (trachoma, lymphogranuloma venereum, and MoPn). Although human biovars have also been used in animal models, they normally require high inocula or pretreatment with progesterone. MoPn, which was originally isolated from mouse tissues, is thought to be a natural murine pathogen and thus offers an evolutionarily adapted pathogen for analysis of host-pathogen interactions [5].

The significant progress in chlamydial immunobiology based on murine models of MoPn infection has extended and clarified recent immunobiologic studies in humans [6]. In particular, since the discovery of T helper (Th) 1 and 2 subsets, cytokine patterns have been shown to be critical in the regulation of immune responses to a variety of infectious agents including chlamydiae. Clinical investigation has shown that trachoma patients with severe conjunctival scarring have impaired cell-mediated immune responses to C. trachomatis and high IgG antibody titers [7]. Cytokine analysis shows increased interleukin (IL) -4 and reduced interferon (IFN) -γ production in subjects with scarring disease due to C. trachomatis infection compared with controls without scarring disease [8].

Recent findings that elucidate the role of cytokines in granuloma formation and tissue fibrosis induced by parasites are useful in conceptualizing the immunologic basis of T cell cytokine polarization in chlamydial immunopathology. Th2-related cytokines, in particular IL-4, regulate granuloma for-
mation and fibrosis following schistosoma egg challenge [9]. Thus, although granuloma formation was classically described as a consequence of type 1 delayed type hypersensitivity (DTH) responses, granuloma formation and fibrosis are now thought to be associated with Th2 responses. Of relevance to chlamydial infection, we recently reported that, although most wild type mice (C57BL/6) exhibit granulomatous and fibrotic reactions following chlamydial infection, IL-10 KO mice exhibit higher levels of IFN-γ, IL-12, and DTH responses, lower levels of IL-5, and absent granulomatous pathologic changes [10]. These data suggest that Th2 cytokines may indeed mediate fibrosis and scarring due to chlamydial infection and that Th1-related cytokines may suppress these pathologic responses.

DTH and Th1-like responses (IFN-γ production) have been identified as the major protective mechanism against chlamydial infection [11]. Mice with stronger DTH responses clear organisms more quickly with less granuloma formation [10]. Paradoxically, immunopathologic responses (tissue scarring) are also thought to be mediated by DTH responses.

Unsolved in chlamydial immunobiology is whether DTH is a double-edged sword in chlamydia-induced immune responses. Specifically, does DTH clear the organism yet also mediate pathology? Very recent data from IFN-γ KO mice provide a possible explanation [12]. In the study, IFN-γ KO mice showed strong DTH responses to chlamydial infection as demonstrated by remarkable footpad swelling and massive cellular infiltration. The DTH response in IFN-γ KO mice was associated with Th2 cytokine production and with a distinct cellular infiltration. Unlike the mononuclear cellular infiltration observed in wild type controls, the DTH in IFN-γ KO mice showed significant neutrophil and eosinophil infiltration. This DTH response is similar to the type 2 DTH response described by Muller et al. [13] and Mosmann et al. [14], which was induced by local injection of type 2 T cells. The Th2 pattern of DTH responses in IFN-γ KO mice induced by chlamydial infection was partially blocked by anti-IL-4 monoclonal antibody. Of note, IFN-γ KO mice with significant Th2 pattern DTH responses failed to clear chlamydial infection and showed disseminated, multiorgan infection with severe tissue inflammation. These results suggest there are two types of DTH responses to chlamydial infection with one type exhibiting a Th1 pattern dependent on IFN-γ production and a second with a Th2 pattern DTH response.

The finding that either Th1 or Th2 cells mediate DTH responses but that the Th2 pattern DTH fails to control chlamydial infection suggests that DTH may indeed play a dual role in chlamydia immunobiology with the type of DTH response critical in determining whether immunity versus pathology results from infection. Specifically, DTH responses mediated by Th1 cells (Th1 pattern DTH observed in wild type and IL-10 KO mice) are protective during chlamydial infection and inhibitory for immunopathologic reactions, while the DTH induced by Th2 cells (Th2 pattern DTH observed in IFN-γ KO mice) fails to clear infection and may even promote pathology.

Recent identification of Th1 and Th2 cell surface markers has included differential expression of adhesion molecules and chemokine receptors on the surface of Th1 and Th2 cells and their correlation with chemotactic responsiveness of antigen-specific T lymphocytes. Th1, but not Th2, cells express a functional ligand for P- and E-selectin and are therefore selectively recruited to sites where Th1 immune responses occur [15, 16]. Moreover, differential expression of chemokine receptors on human Th1 and Th2 CD4 T cell clones has been reported [17, 18]. In general, Th1 cells show higher CXCR3 and/or CCR5 expression, whereas Th2 cells preferentially express CCR4 and/or CCR3 molecules. The surface expression of these receptors on Th1 and Th2 cells appears to have functional significance in the migration of the cell subsets. For example, macrophage inflammatory protein-1α—a CCR5 agonist and eotaxin—a CCR3 agonist, have chemotactic activity for Th1 and Th2 clones, respectively [18].

The protective value of T cell populations is strongly dependent on their ability to efficiently migrate to infected tissue sites where they perform their effector function. Chemokines directing the recruitment of leukocytes in inflammation and enhancing the expression of adhesion molecules such as integrins are thus involved in the process of protective immunity and pathologic reaction. In the MoPn infection model, T cells, especially CD4 T cells, are essential for host defense against MoPn infection [19]. These cells also appear to need intimate interaction with infected epithelial cells in order to exhibit antimicrobial activity, suggesting the extreme importance of adhesion molecules expressed on chlamydia-specific T cells and on the infected cells in host defense against chlamydial infection [20]. Indeed, leukocyte function antigen-1 and intercellular adhesion molecule-1 interaction play an important role in interaction between chlamydia-specific T cells and chlamydia-infected epithelial cells [21]. We recently showed that although significant numbers of CD4 T cells are seen in the infected organs in IFN-γ KO mice, these cells (which have a Th2 cytokine secretion pattern) fail to localize to sites of chlamydial infection [12]. Thus, in addition to the lack of IFN-γ production by Th2 cells, it is likely that the failure of Th2 cells to target infected cells is also a reason for their inability to control chlamydial infection.

Recent progress in elucidating the developmental basis for Th1/Th2 differentiation resulted from the finding that microenvironmental cytokines are key factors that influence the commitment of Th cell precursors to Th1 or Th2 cells [22]. In particular, the early presence of IFN-γ and IL-12 favors Th1 polarization, whereas the early presence of IL-4 and IL-10 is the potent stimulus for Th2 commitment. In general, the source of these cytokines during the early phases of the immune response often depends on the innate defense mechanisms mobilized by the pathogen. Importantly, chlamydia-infected epi-
thelial cells secrete a variety of proinflammatory and immunoregulatory cytokines [23] among which granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-18 may be most important from the view of canalizing the development of protective Th1-dependent immunity. GM-CSF promotes the maturation of dendritic cells and IL-18 synergistically interacts with IL-12 to promote a Th1 cytokine differentiation pattern. The fact that only actively growing chlamydiae induce epithelial cells to secrete these cytokines likely explains why chlamydial immunity is much better generated following infection than by vaccination with inactive organism [24].

**Immunobiology Lessons Relevant to Vaccine Design**

Collectively these data suggest a paradigm for chlamydial immunity that has great relevance for vaccine design. The paradigm posits that Th1 CD4 T cell responses play the dominant role in protective immunity against chlamydial infection, whereas Th2 cytokine responses, especially IL-10, may be associated with immunopathologic responses. Th2 cells appear to accelerate tissue fibrosis and granulomatous reactions, fail to localize to areas of chlamydial infection, and by definition do not secrete cytokines such as IFN-γ that inhibit the growth of chlamydiae. Furthermore, Th1 but not Th2 effector cells appear to have the appropriate surface receptors that allow them to home to infected cells. On the basis of these results, an effective chlamydial vaccine will need to induce a strong CD4 Th1 cell response if it is to protect against chlamydial infection and prevent tissue pathology.

**DNA Vaccination for Prevention of Chlamydial Infection**

The use of selected genes from pathogenic microorganisms within an eukaryotic expression plasmid as a vaccine has been a fundamental advancement in vaccinology [25]. Expression plasmids contain methylated CpG motifs that elicit innate cytokine responses that promote the canalization of CD4 T cell responses to a Th1 cytokine secretion pattern. The intracellular synthesis of the microbial protein especially within transfected professional antigen-presenting cells facilitates the presentation of antigen on class I and class II molecules and the induction of cell-mediated immunity. The use of one or few microbial protein-coding genes allows the presentation of protective antigens to the immune system to occur in the absence of microbe-directed immune evasion mechanisms and in the absence of competing or pathologic antigens. Immune responses primed by DNA vaccines are also readily amplified by protein-antigen immunization. Thus, for these and other reasons, immunization with DNA vaccines is an important advancement in vaccine technologies that may be particularly relevant to chlamydial vaccine design. We have evaluated the utility of DNA vaccine approaches for chlamydial infection prevention in a mouse model and the published data (summarized below) are encouraging.

The major outer membrane protein (MOMP) is the predominant protein on the surface of *C. trachomatis* elementary bodies (EBs), and infected humans uniformly make strong CD4 and CD8 T cell and antibody responses to it [1]. Since the MOMP is an immunodominant and immunoprotective antigen, we initially evaluated whether DNA vaccination with the MOMP gene protected mice against *C. trachomatis* MoPn lung infection. As previously reported, mice vaccinated with the MOMP gene were protected when compared with control mice in terms of weight loss during infection and as measured by quantitative cultures of the organism during peak in vivo growth [26]. This was true over a range of concentrations of MOMP DNA used in the vaccine [27]. Others report that MOMP DNA immunization is also protective in a mouse model of *C. trachomatis* serovar L2 infection [28] and in an avian model of *Chlamydia psittaci* infection [29]. Recent data also demonstrate that MOMP DNA immunization protects mice against *C. pneumoniae* infection [30]. However, MOMP DNA immunization did not engender immunity in a mouse model of genital MoPn infection—perhaps because of the immunosuppressive effects of progesterone used in that model [31].

Of importance, MOMP DNA immunization also induces Th1 immune responses that correlate with faster clearance of the organism [27]. DNA immunization induces antigen-specific DTH reactions, lymphocyte proliferation responses, and IFN-γ cytokine secretion. In general, antibody responses are weak but demonstrate a Th1 bias with IgG2a titers greater than those of IgG1. No IgA antibodies were elicited by MOMP DNA immunization.

Overall, MOMP DNA vaccination is a promising new approach to chlamydial immunoprophylaxis. However, several known and potential limitations exist with this approach. DNA vaccination induces only partial protection and not sterile immunity. Th1 immune responses and antibody responses evoked by DNA vaccination are generally weaker than those evoked by infection immunity. Lastly, MOMP is a polymorphic protein among human strains of *C. trachomatis* and immunity engendered by it may be immunotype specific. Thus, the use of additional chlamydial antigen genes may be necessary to improve the degree of protection.

The chlamydial genome project has suggested several new candidate chlamydial antigens that might be useful for chlamydial vaccine evaluation. Among the many exciting findings was the identification of genes encoding the components for a complete type III secretion apparatus [32]. Type III secretion systems are triggered by host cell contact and are required to deliver effector enzymes into eukaryotic cells and thus mediate pathogen invasion. Electron microscopy studies of chlamydiae show spike-like surface structures that appear to penetrate the inclusion membrane and that resemble the injectosome complexes that deliver type III secretion effector molecules in other
organisms [33]. Although the structural components of the type III secretion apparatus have a remarkable degree of evolutionary conservation across a diverse array of bacteria, the secreted effector molecules do not. Thus, the identification of chlamydial type III secretion effector molecules is an area of active research. Type III secreted proteins are an exciting potential group of proteins for DNA vaccine development because they are by definition virulence determinants and because their intracytoplasmic location during natural infection facilitates presentation via ubiquitous class I MHC molecules.

One candidate gene that may encode a secreted type III effector protein is located within a chromosomal locus that contains several structural genes of the type III system and has homology to a serine threonine kinase gene. A similar gene is a type III secreted protein in Yersinia organisms and is important in pathogenesis for that species [34]. We therefore evaluated potential type III effector molecules beginning with the chlamydial serine-threonine kinase (STK) gene using the same plasmid system used to characterize the MOMP responses. Figure 1 shows that immunization with STK DNA induced protection (2–3 log_{10}) against MoPn lung infection. However, like MOMP, DNA STK DNA immunization elicited only partial protection. Yet to be tested is whether administering multiple chlamydial genes (e.g., MOMP and STK) together will be necessary to evoke sterile protective immunity.

A Live Attenuated Chlamydial Vaccine

Given that the most successful vaccines from a public health point of view are live attenuated vaccines, the best opportunity for a chlamydial vaccine may well be the development of an attenuated strain. This line of technology has been difficult to contemplate for chlamydia for several reasons including the absence of a gene transfer system, cell-free growth conditions, knowledge concerning basic metabolic capabilities and virulence factors, and difficulty in propagating clonal lineages of the organism. However, recent advancement from the genome project [32] in the development of plaque cloning techniques [35] and the potential for transformation or transduction of chlamydiae by exogenous DNA suggest that the selection of attenuated strains is realistic. The use of a live attenuated strain also takes advantage of the observation that live chlamydia are more effective at engendering protective immunity than dead organisms [24]. Because the intact organism contains many potential protective antigens, live attenuated chlamydiae offers an efficient way of inducing protective immunity against multiple antigens and thus against multiple serovars of the organism.

Live attenuated vaccines are avirulent either because of attenuating mutations or because of replication restrictions associated with their range of hosts. Because the live vaccine replicates in a manner analogous to the target pathogen, it promotes the processing and presentation of antigens in a way that is most similar to the natural infection. Furthermore, while replicating, the live vaccine presumably expresses all or most of its important target immunogens. This may be especially important for chlamydiae, which exist in two developmental forms. A live attenuated chlamydial vaccine can be expected to expose both reticulate body and elementary body-specific antigens to the host’s immune system. Live attenuated vaccines can also stimulate mucosal immune responses and are capable of inducing systemic humoral and cell-mediated responses. Clearly, live attenuated chlamydiae have many advantages. However, a major potential drawback of an attenuated vaccine for use as a chlamydial vaccine is that antigens that could give rise to deleterious autoimmune or immunopathologic responses may also be produced. Understanding the antigen-specific basis for immunopathology may allow for directed deletion of harmful antigen genes from an attenuated chlamydia strain. This remains an important research priority.

*Salmonella* organisms are commonly used for the study of bacterial attenuation because of the availability of advanced genetic systems and animal models with this pathogen. In fact, a live attenuated *Salmonella typhi* vaccine Ty21a is already used in humans in many countries [36]. Attenuating mutations in salmonellae have been identified in many different genes affecting nutrient auxotrophy, virulence traits, global gene expression, and intracellular survival [37]. More than one attenuating mutation to reduce the chance of reversion is normally
required for Salmonella vaccine strains used in humans and likely will also be required for candidate attenuated chlamydial vaccines.

The obligate intracellular growth of chlamydiae has made the isolation of defined mutants a challenge. Drug-resistant mutants of C. psittaci and C. trachomatis have been selected in cell culture, typically by repeated passage of chlamydiae in the presence of increasing drug concentration, and isolated from infected hosts [38–44]. However, only a few of these mutants have been characterized at the genetic level and none has been tested for virulence in an animal infection model.

Temperature-sensitive mutants of an ovine abortion strain of C. psittaci was selected after nitrosoguanidine chemical mutagenesis of infected McCoy cell cultures [45]. Although the genetic basis for temperature sensitivity in this strain was never determined, the mutants are an effective vaccine in ewes [46, 47]. Surprisingly, this exciting work, which shows that attenuated variants of C. psittaci can induce protection, has not been extended to other chlamydia strains where better animal models for virulence assessment are available. This is likely a reflection of perceived difficulties not only in identifying potential targets for attenuating mutagenesis but also in the actual selection procedure. Clearly, recent genome sequencing and plaque-cloning developments should provide impetus for renewed efforts in this area. The availability of the genome sequence has provided insight about putative virulence factors and metabolic capabilities that allow for more informed decisions about potential attenuating targets.

At the molecular level, drug resistance in chlamydiae occurs by mutational mechanisms similar to those of free living bacteria. Missense mutations, which result in amino acid substitutions, give rise to a target protein no longer sensitive to the inhibitor, and regulatory mutations lead to over expression of the target protein [38, 39, 42]. These studies did not address the question of attenuation; however, they indicate that mutant chlamydiae can be isolated by classical procedures if there is appropriate attention to selection conditions. In general, the mutant selection must be done under conditions in which chlamydiae are targeted but not host cell functions. This is relatively straightforward when prokaryotic-specific inhibitors are used as selective agents but becomes more complicated if one is trying to select for a mutation in a target common to both parasite and host [38]. Under these circumstances, it is useful to use host cell lines that have a mutation in the target enzyme that renders them resistant to the selective agent. For example, a hydroxyurea-resistant C. trachomatis isolate was selected by using a hydroxyurea-resistant mouse cell line as host [39]. With genome knowledge in hand, it is now possible to design novel mutant selection protocols.

While the above-described mutant selection procedures are feasible, they still depend on the outgrowth of spontaneous mutants in the bacterial population. Mutation frequencies can readily be enhanced with various chemical or UV mutagens. The difficulty with applying random mutagenesis procedures to an obligate intracellular parasite with a unique growth cycle such as chlamydiae is that many of its genes likely encode proteins essential for survival. In support of this conjecture is the finding the C. trachomatis and C. pneumoniae genomes are very similar. Thus, it may not be possible to do random mutagenesis for chlamydiae. What is clearly needed is the development of a genetic transformation or transduction system that allows for targeted mutagenesis. Once these tools become available it will be possible to systematically approach the question of generating attenuating mutations in chlamydiae. In addition, it may also be feasible to delete genes that are associated with disease pathology (e.g., hsp60) if such genes are nonessential for growth in cell culture [3].

Conclusion

Vaccine development for chlamydiae seems more possible now than in the past. Recent knowledge regarding the molecular and cellular basis for chlamydial immunity and pathology allows for a more targeted approach to eliciting protective immune responses with a vaccine. DNA vaccines are an exciting new tool in engendering protective immunity to chlamydial antigens although they may be limited in eliciting only partial protection when used as single gene constructs. Knowledge of the chlamydial genomes reveals a large number of new potential vaccine candidates that can be exploited in a systematic DNA vaccine approach. The new knowledge of chlamydial metabolism derived from the genome studies has also encouraged speculation about the development of a live attenuated chlamydial vaccine. The shared developmental microbiology and immunobiology between C. trachomatis and C. pneumoniae suggest that the lessons learned in the development of C. trachomatis vaccine will be directly relevant to C. pneumoniae.

References


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