

MINIREVIEW

Vaccination against *Chlamydia* Genital Infection Utilizing the Murine *C. muridarum* Model^{∇†}

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***Chlamydia trachomatis* genital infection is a worldwide public health problem, and considerable effort has been expended on developing an efficacious vaccine. The murine model of *C. muridarum* genital infection has been extremely useful for identification of protective immune responses and in vaccine development. Although a number of immunogenic antigens have been assessed for their ability to induce protection, the majority of studies have utilized the whole organism, the major outer membrane protein (MOMP), or the chlamydial protease-like activity factor (CPAF). These antigens, alone and in combination with a variety of immunostimulatory adjuvants, have induced various levels of protection against infectious challenge, ranging from minimal to nearly sterilizing immunity. Understanding of the mechanisms of natural infection-based immunity and advances in adjuvant biology have resulted in studies that are increasingly successful, but a vaccine licensed for use in humans has not yet been brought to fruition. Here we review immunity to chlamydial genital infection and vaccine development using the *C. muridarum* model.**

Chlamydia trachomatis, a Gram-negative obligate intracellular bacterium with a tropism for mucosal epithelial cells, is the most common cause of bacterial sexually transmitted disease in both developed and developing countries, with more than 90 million new cases occurring each year (113–115). More than 4 million new cases of *C. trachomatis* infection occur each year in the United States, where costs associated with treating those infections and associated complications are in excess of \$2 billion annually (107). In the genital tract, infection with *C. trachomatis* is propagated within the single-cell columnar layer of the epithelium in the urethra of men and the endocervix of women. Within the epithelial cells, *C. trachomatis* undergoes a unique biphasic developmental cycle consisting of an infectious, but metabolically inert elementary body (EB) and a noninfectious, but metabolically active reticulate body (RB). After completion of the developmental cycle, the EBs are released and infect neighboring epithelial cells, thereby spreading the infection.

Infection can result in acute inflammation characterized by redness, edema, and mucosal discharge and is diagnosed clinically as mucopurulent cervicitis in women and nongonococcal urethritis in men (10, 85). In women, infection can manifest as abnormal vaginal discharge and/or postcoital bleeding, while the infection is limited to the lower genital tract, and irregular

uterine bleeding and/or pelvic discomfort once the infection ascends to the upper genital tract (85). Symptoms in males are generally limited to dysuria and moderate clear-to-whitish discharge (85). While these symptoms signify an infection, the absence of such symptoms does not necessarily indicate the absence of infection. It is estimated that >70% of women and 50% of men experience asymptomatic infections (15, 113). Without symptoms providing the impetus, asymptomatic individuals may not seek diagnostic testing and the infection will go untreated. Untreated *C. trachomatis* infection can wreak havoc on the reproductive organs, profoundly affecting fertility in women. Taken together, the high rate of asymptomatic infections and the severity of the infection-related pathology indicate that, despite the availability of very effective antimicrobial therapy, control of chlamydial infections will most likely require a vaccine.

HISTORICAL VACCINE STUDIES

Almost immediately following the 1957 isolation of the etiologic agent of trachoma by Tang, human vaccine trials were initiated in areas of trachoma endemicity (5). The outcomes of these trials were mixed, and the results ranged from considerable protection against infection and pathology to partial, short-lived protection (5). In one notable study, researchers vaccinated children with formalin-fixed chlamydial EBs and followed them for 3 years. Vaccination conferred only partial, serovar-specific, short-lived immunity, and compared to their nonvaccinated counterparts a small, but significant, portion of vaccinated individuals experienced an increase in the incidence and severity of infection upon exposure to chlamydiae (5, 94). The exacerbated disease and pathology in these individuals were postulated to be a result of delayed-type hypersensitivity. These experiments were repeated in nonhuman primates, and

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the results mimicked what was seen in the human trials, leading researchers away from the use of whole organisms in immunization and back into animal models (5, 10, 11, 30). To date, no other *C. trachomatis* human vaccine studies targeting ocular or genital infection have been published.

Animal models are instrumental in the study of chlamydial genital infection and essential in characterizing the host response to *C. trachomatis* in females. Mouse, guinea pig, non-human primate, nonprimate monkey, rat, and pig models have all been established (30). However, the availability of inbred mouse lines, transgenic and gene knockout mice, and immunological reagents has made the mouse the preferred model to study chlamydial genital infection. Mice have been used extensively to study acute genital infection, protective immune responses, and vaccine development.

Chlamydia muridarum, formerly the *C. trachomatis* agent of mouse pneumonitis (MoPn), is a murine pathogen that was originally isolated from the lungs of mice and later used to establish a mouse model of genital infection (4, 70). The genomes of *C. muridarum* and *C. trachomatis* serovar D share remarkable similarity in the content and order of genes, with the exception of a region ~50 kb from the origin of termination deemed the plasticity zone (PZ) (92, 98). Within the PZ are genes coding for cytotoxins and a tryptophan operon. Analysis of the cytotoxin genes in *C. muridarum* and *C. trachomatis* has revealed similarities in putative virulence factors (6). The presence of a tryptophan operon in the genome of *C. trachomatis* and the absence of one in the *C. muridarum* genome are important differences between the two biovars and are responsible for their differential sensitivity to gamma interferon (IFN- γ) (69, 87).

In addition to genetic similarities, murine genital infection with *C. muridarum* mimics many aspects of acute genital infection with *C. trachomatis* infection in women. Intra-vaginal inoculation of *C. muridarum* produces a self-limiting infection of the vaginal and cervical epithelial cells that subsequently ascends to the upper genital tract via the epithelial surfaces of the uterine horns and oviducts (57, 58). Resolution of the infection takes approximately 4 weeks and results in long-lived adaptive immunity that protects markedly against reinfection (4, 58). Histopathological analysis has revealed that early infection is characterized by an abundant cellular infiltrate of genital tract tissues predominated by polymorphonuclear neutrophils (4, 58, 60, 102). As the infection resolves, those cells are replaced by macrophages and populations of lymphocytes, including B cells, CD4⁺ T cells, and CD8⁺ T cells. CD4⁺ T cells are present throughout the course of infection, and it has been observed that aggregates of these cells remain in the genital tract submucosae after the infection has cleared (45, 57, 60). Upon resolution of infection, more than 60% of mice are solidly resistant to reinfection with the homologous *Chlamydia* strain, and mice that are susceptible experience infections of considerably shorter duration and lower bacterial burden than primary infection (57). Postinfection sequelae, such as infertility, are observed in humans as well as in the murine model, providing further support for this model.

C. muridarum infects various strains of mice nearly equally (23, 56, 89). Some strains have a higher propensity for the development of hydrosalpinx and shed somewhat greater num-

bers of bacteria, but overall rates of infection are more or less equivalent. In contrast, infection of mice with *C. trachomatis* is highly dependent on mouse strain. For example, C57BL/6 and C57BL/10 mice are highly resistant to genital infection, even when high challenge doses of *C. trachomatis* are used, and the infection is characterized by low bacterial shedding, minimal inflammation, and no hydrosalpinx, and infectious bacteria are only detected for a few days following challenge (23, 88, 89, 116). C3H mice, however, are more susceptible to infection with *C. trachomatis*. When challenged with *C. trachomatis*, genital infection in C3H mice is characterized by moderate shedding of bacteria (10^2 to 10^4 inclusion-forming units [IFU]) for 2 to 5 weeks and minimal to moderate inflammation (23, 89, 100). Postinfection sequelae (i.e., hydrosalpinx) are less common when mice are challenged vaginally with *C. trachomatis*, which necessitates the direct inoculation of the uterine horns and/or ovarian bursa with large doses of chlamydiae (109, 110). Strong adaptive responses are generated when mice are infected with *C. trachomatis* serovars (10, 11, 30), but studies have shown that these infections can clear in the absence of adaptive immunity (87), suggesting that innate immune responses alone can resolve infection.

The observation that *C. trachomatis* infection can resolve in the absence of adaptive immunity should not be used *per se* to invalidate the use of *C. trachomatis* serovars in murine studies of genital tract infection or in vaccine development. Knowledge of the natural course of human genital infection and of the protective responses is very limited because diagnosis of infection mandates treatment. It is, therefore, possible that the rather mild infection seen in murine studies utilizing human *C. trachomatis* biovars may replicate some aspects of human infection; however, no model has been shown to replicate all of the clinical manifestations of human infection. Vaccine studies using *C. trachomatis* have also demonstrated that induction of adaptive responses against human strains can confer a degree of protection against genital challenge, which supports the validity of murine *C. trachomatis* infection for vaccine studies (30). However, because innate immune responses alone resolve murine *C. trachomatis* genital infection (87), it is imperative that all vaccine studies include controls for the contribution of protective innate responses. In contrast to *C. trachomatis*, the resolution of murine *C. muridarum* genital infection, and protection against reinfection, is absolutely dependent on the adaptive immune responses. Thus, the *C. muridarum* genital infection model is more amenable to the study of adaptive immunity and vaccine development. Because the considerable differences between *C. muridarum* and *C. trachomatis* murine genital infection make direct comparisons difficult, we have limited our review to focus primarily on the *C. muridarum* model of infection and immunity, with only mention of notable *C. trachomatis* studies.

PROTECTIVE IMMUNE RESPONSES TO *C. MURIDARUM* INFECTION

Identification of protective responses is a key component of vaccine development. The responses central to chlamydial immunity, both in terms of resolution of primary infection and immunity to reinfection, have been identified through a variety of experimental approaches in the mouse model of genital

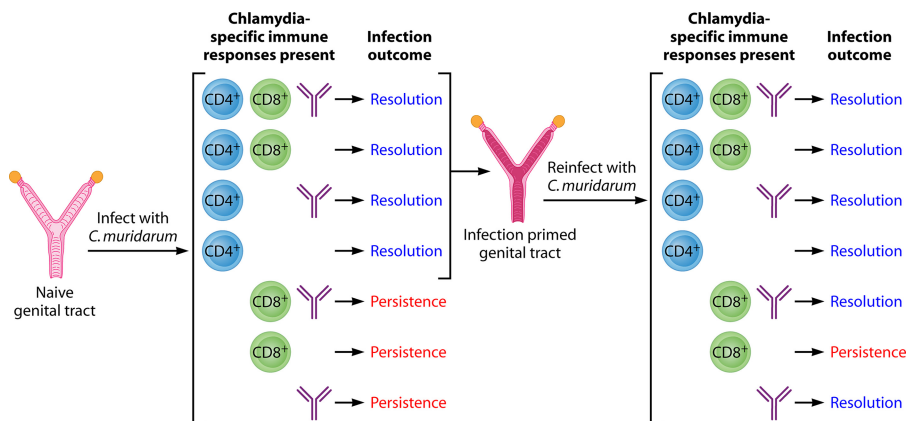


FIG. 1. Immunity to murine *C. muridarum* primary infection and reinfection. Genital infection with *C. muridarum* produces robust long-lived adaptive immunity. The immune responses that are elicited during infection resolve primary infection in approximately 4 to 5 weeks, and upon rechallenge, those adaptive immune responses result in an infection of much shorter duration (3 to 10 days), and far fewer infectious bacteria ($>10^4$ fewer) are shed. Generally speaking, adaptive immune responses elicited during infection consist of CD4⁺ T cells, CD8⁺ T cells, and antibody. CD4⁺ T cells are absolutely essential to bring about the resolution of primary genital infection, whereas CD8⁺ T cells and antibody are dispensable. In the absence of CD4⁺ T cells, primary infection persists. Interestingly, immunity to reinfection is governed by a more complex set of responses. First, as with primary infection, CD4⁺ T cells protect against reinfection and resolve infection in the absence of CD8⁺ T cells and/or antibody responses. However, in the context of reinfection/rechallenge, antibody is now protective and resolves secondary infection in the absence of CD4⁺ and/or CD8⁺ T cells. An indispensable element of the antibody-mediated protective immunity is the priming of the genital tract tissues by CD4⁺ T cells (infection-primed genital tract). Once the genital tract has been primed, CD4⁺ T cells are dispensable. Therefore, while the protective efficacy of antibody is dependent on CD4⁺ T-cell priming of the genital tract, antibody functions independently of CD4⁺ T cells. We do not yet understand the precise mechanism(s) by which antibody functions, but it is highly protective against reinfection and is absolutely dependent upon the activation and/or recruitment of another cell type, by CD4⁺ T cells, that functions with antibody to resolve infection.

infection and have been reviewed previously (57, 93). T cells, particularly major histocompatibility complex (MHC) class II-restricted CD4⁺ T cells, are required for protective immunity (47, 58, 61, 63, 101). MHC class I-restricted CD8⁺ T cells, on the other hand, are not necessary for infection resolution or immunity to reinfection (58, 61, 63, 101). The protective role of antibody is less easily discernible than that of the cellular response (59, 63, 90), but important to vaccine development, it is as protective as CD4⁺ T cells in immunity to reinfection (59, 63). Based on our previous studies (58–63) and a plethora of other studies, we have developed a model depicting immune responses that contribute to the resolution of primary *C. muridarum* genital infection and resistance to reinfection (Fig. 1). Primary *C. muridarum* genital infection resolves in the presence of CD4⁺ T cells, either alone or in concert with CD8⁺ T cells and/or antibody. The infection persists in the absence of CD4⁺ T cells. Changes take place during resolution of the primary *C. muridarum* infection that “prime” the genital tract. Because resolution is dependent on CD4⁺ T cells, “priming” of the genital tract is facilitated by CD4⁺ T cells, although the exact mechanism is unknown. Upon reinfection of a “primed” genital tract, mice are infection resistant if CD4⁺ T cells and/or antibody is present. CD8⁺ T cells appear to be unnecessary in resistance to reinfection. Although not depicted in Fig. 1, cytokines of the Th1 lineage also play an important role in the protective response. Specifically, IFN- γ and interleukin-12 (IL-12) are essential for protection (19, 40, 86, 87), while the Th2 cytokine IL-10 has been associated with a pathological response (39). Taken together, natural immunity to *C. muridarum* genital infection in mice is mediated by CD4⁺ T cells, Th1-type cytokines, and antibody and the effectiveness of a vaccine will likely depend on its ability to induce such responses.

UNIQUENESS OF THE GENITAL TRACT IMMUNE SYSTEM

The mucosal immune system, especially that of the genital tract, has many unique properties that must be taken into account when designing an effective vaccine targeting *C. trachomatis*. The genital tract is unique among mucosal effector sites in that it lacks organized lymphatics (55). Unlike the intestinal tract, which has mucosal inductive sites such as the Peyer’s patches, propagation of immune response for the genital tract occurs at distant sites, such as the spleen and iliac lymph nodes, which can result in a delayed systemic response relative to other sites (55). The female genital tract is also subjected to hormonal regulation, and the effectiveness of intravaginal immunization has been shown to be influenced by the phase of the menstrual cycle (41, 46, 55). Disparity in the dominant Ig class is found between the genital tract and other mucosal sites: the dominant immunoglobulin class in the genital tract is IgG, while IgA is the dominant class at other mucosal sites (55). IgG translocation from the serum to secretions accounts for its dominating presence in the genital tract, rather than an increase in local IgG-secreting cells (48, 55). The immunological properties of the genital tract and the tropism of *Chlamydia* for mucosal epithelial cells emphasize the necessity for a *C. trachomatis* vaccine to induce both systemic and mucosal protective responses.

As such, mucosal vaccination using a number of antigens and multiple mucosal routes has been employed as a strategy to induce protective responses against *C. trachomatis* in the genital tract (17, 22, 33, 35, 75, 78, 79). Although mucosal effector sites are separated by substantial distances in the body,

TABLE 1. Summary of vaccines developed in the murine model of genital infection with *C. muridarum*

Yr	Antigen ^a	Route of vaccination ^b	Route of challenge	Adjuvant/delivery system ^c	Mouse strain	Reference
1994	EBs	i.n.	Ovarian bursa	None	BALB/c	75
1994	MOMP, Hsp60	s.c.	Vagina	IFA	BALB/cByJ	8
1996	EBs	i.n.	Ovarian bursa	None	BALB/c	78
1997	nMOMP, COMC	s.c.	Ovarian bursa	CFA, IFA	BALB/c	84
1998	EBs	Intravaginal	Vagina	Dendritic cells pulsed <i>ex vivo</i>	C57BL/10	103
1999	pMOMP	i.m.	Ovarian bursa, vagina	None	BALB/c, C3H/HeN, C57BL/6	74
2000	EBs, RBs	s.c., i.m.	Ovarian bursa	CFA	BALB/c	82
2000	EBs	Intravaginal	Vagina	Oxytetracycline (subclinical infection)	C57BL/10	104
2001	nMOMP	s.c., i.m.	Ovarian bursa	IFA, CFA	BALB/c	83
2002	rMOMP	Intravaginal	Vagina	Dendritic cells pulsed <i>ex vivo</i>	C57BL/10	95
2003	rMOMP	i.m.	Vagina	VCG	BALB/c	27
2003	EBs	i.n.	Ovarian bursa	None	C3H/HeN, BALB/c, C57BL/6	77
2003	nMOMP	s.c., i.m.	Ovarian bursa	OspA	C3H/HeN, BALB/c	76
2004	rMOMP	Transcutaneous	Vagina	CpG-1826, CT	BALB/c	7
2005	EBs	i.n.	Vagina	None	BALB/c (newborn)	79
2005	nMOMP	s.c., i.m.	Ovarian bursa	CpG-1826, Montanide ISA 720	BALB/c	80
2006	nMOMP	s.c., i.m., i.n.	Ovarian bursa	LT-R72, MF59, LT- K63	C3H/HeN, BALB/c	81
2006	rMOMP	i.n., intravaginal	Vagina	CTB	BALB/c	96
2006	rCPAF	i.n.	Vagina	IL-12	HLA-DR4 (transgenic)	67
2007	EBs (plasmid- deficient)	Intravaginal	Vagina	None	C3H/HeN	71
2007	DNAexpression library	Abdominal	Vagina	Gene gun	BALB/c	53
2007	rCPAF	i.n.	Vagina	IL-12	BALB/c	66
2007	rCPAF	i.n.	Vagina	CpG-1826	BALB/c	18
2007	rCPAF, rMOMP, rIncA	i.n.	Vagina	IL-12	BALB/c	49
2008	pgp3	i.n.	Vagina	None	BALB/c	52
2008	rMOMP	s.c.	Vagina	CAF01	BALB/c, C57BL/6	1
2008	rMOMP	s.c.	Vagina	CAF01	C57BL/6	31
2009	rMOMP	i.n.	Vagina	CTAI-DD	BALB/c	22
2009	rMOMP	i.n.	Vagina	Vault nanoparticles	C57BL/6	17
2009	rMOMP	Transcutaneous	Vagina	CT, CpG-1826, lipid C	BALB/c	34
2009	rTARP, EBs	i.m.	Vagina	CpG-1826, IFA	BALB/c	112
2009	rCPAF	i.n.	Vagina	CpG-1826	C57BL/6, μ MT	65
2009	rMOMP	i.m., intravaginal, transcutaneous	Vagina	CTA2B, VCG	C57BL/6	28
2009	RplF, PmpE/F2	Intravaginal	Vagina	Dendritic cells	C57BL/6	118
2010	rCPAF, inactivated	i.n.	Vagina	IL-12	BALB/c	16
2010	rMOMP, PmpG1, RplF, PmpE/F2	s.c.	Vagina	DDA/TBD, AbISCO, CpG-1826	C57BL/6, BALB/c	119
2010	rMOMP	Oral	Vagina	Lipid C, CpG-1826, CT	BALB/c	33
2010	CTH1	s.c.	Vagina	CAF01	C3H/HeN, CB6F1	73
2010	rCPAF + EBs (UV inactivated)	i.n.	Vagina	CpG-1826	BALB/c	50
2010	nMOMP	s.c., i.m.	Vagina	CpG-1826, Montanide ISA 720	C57BL/6	29

^a MOMP, major outer membrane protein (native, recombinant, plasmid); Hsp60, 60-kDa heat shock protein; COMC, outer membrane complex; CPAF, chlamydial protease-like activity factor; IncA, inclusion membrane protein A; pgp3, secreted plasmid protein encoded by pORF5; TARP, putative type III secretion effector protein; RplF, ribosomal protein L6; Pmp, polymorphic membrane protein; CTH1, fusion protein of OmcB and r116s.

^b i.n., intranasal; s.c., subcutaneous; i.m., intramuscular.

^c IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; VCG, *Vibrio cholerae* ghosts; OspA, *Borrelia burgdorferi* outer surface protein A; CT, cholera toxin; CpG-1826, unmethylated bacterial oligodeoxynucleotide; Montanide ISA 720, water in oil adjuvant; LT-R72, mutated heat-labile enterotoxin from *Escherichia coli*; MF-59, oil in water adjuvant; LT-K63, *E. coli* heat-labile enterotoxin; CAF01 (DDA/TDB), liposomal adjuvant containing the synthetic mycobacterial immunomodulator TDB; CTAI-DD, a toxin-based adjuvant constructed of the cholera toxin A1 subunit directly linked to a dimer of the B cell targeting moiety D from protein A of *Staphylococcus aureus*; lipid C, a lipid-based matrix composed of purified and fractionated triglycerides; CTA2B, modified cholera toxin consisting of A2, the nontoxic cleavage product of the A subunit, and the nontoxic B subunit; AbISCO, an immune-stimulating complex.

the common mucosal immune system homes antigen-specific lymphocytes to mucosal sites other than that of initial exposure (111). Accordingly, immunization at one mucosal site induces generalized mucosal immune responses, although oral immunization induces much more restricted responses than other routes (111). Despite the significant numbers of mucosal pathogens, mucosal vaccination is not widely used. Some mucosal vaccines are currently approved for use in humans, and the pathogens causing diseases such as polio, cholera, typhoid, and tuberculosis are targeted through oral immunization (36, 55, 111). Other mucosally administered vaccines targeting viral pathogens, such as a past rotavirus vaccine, have been recalled due to adverse reactions and demonstrate the difficulty of targeting these sensitive areas (36, 111). There are currently no mucosal vaccines targeting genital tract pathogens approved for use in humans.

VACCINES

Vaccine development in the murine model was initiated in the early 1990s, using both *C. trachomatis* and *C. muridarum*. A host of antigens, adjuvants, and delivery systems have been assessed for their ability to induce protective immunity against genital challenge, with only limited success. A list of vaccine studies utilizing the *C. muridarum* genital infection model published to date is presented in Table 1. The immunogenicity of the vaccines and the protective outcome of the studies are not included in the table due to variations in the methodology that impact the readout and outcome of the studies. Of the studies listed in the table, there are several of particular note because they demonstrated considerable protection against genital challenge. For the purpose of this review, we define protection against genital challenge as a significant decrease (several log₁₀) in IFU recovered from cervicovaginal swabbing. Other attributes of protection such as shortened duration of infection and decreased incidence of infection will be noted separately as these readouts are not included in all studies. Notable studies include the adoptive transfer of dendritic cells pulsed *ex vivo* with inactivated *C. muridarum* EBs (103), parenteral vaccination with native *C. muridarum* major outer membrane protein (nMOMP) with Th1-driving adjuvants (80), and mucosal vaccination with recombinant MOMP (rMOMP) delivered via liposomes or vault nanoparticles (17, 31). Despite the considerable effort that has been focused on vaccine development, no chlamydial vaccine has been tested in humans since the early trachoma vaccine trials.

ANTIGENS

A host of chlamydial antigens have been assessed in mice for their immunogenicity in terms of ability to elicit humoral and cellular immune responses, and in vaccine development with both *C. muridarum* and *C. trachomatis*. Immunoaccessible antigens, such as components of the outer membrane, have received the most attention, but the availability of the genome sequence has led to the identification of new immunogenic antigens. The construction of complete genomic libraries and expression of specific predicted proteins have led to studies to assess the immunogenicity and protective ability of a number of potential vaccine antigens (30, 94). There is significant over-

lap of antigens used in vaccine studies targeting *C. muridarum* and *C. trachomatis*, but some antigens have been tested in only one system, therefore making comparison difficult. As noted above, the emphasis in this overview will therefore be on antigens tested with *C. muridarum* infection, with mention of notable antigens used exclusively with *C. trachomatis*.

EBs. Despite the negative stigma attached to the use of whole EBs from the human trachoma vaccine trials of the 1950s, a considerable number of studies have used whole *C. muridarum* EBs to induce protective immunity (Table 1). Intranasal immunization of mice with *C. muridarum* EBs protects against vaginal challenge and infertility (75, 78, 82). This protection is long lived (78, 79) and protects newborn mice (78, 79), indicating that protection against chlamydial challenge can be conferred at a young age, prior to environmental or sexual exposure. The developmental form of *C. muridarum* also impacts the conference of immunity; intranasal vaccination with RBs does not protect (82). The lack of protection in animals immunized with RBs in contrast to EBs likely occurs because RBs are noninfectious.

The early human field studies utilized intramuscular vaccination with inactivated EBs, which resulted in increased pathology in some of the vaccinated individuals upon reinfection (5). Likewise, intramuscular immunization of mice with a detergent extract of chlamydiae containing MOMP and heat shock protein 60 (Hsp60), also results in increased inflammation in the genital tract and limited protection upon challenge (8). Conversely, when mice are immunized intramuscularly with whole *C. muridarum* EBs by using the adjuvants CpG-1826 and incomplete Freund's adjuvant (112), a level of protection is observed. These mice experience a decrease in bacterial shedding, shorter duration of infection, and significantly less inflammation and pathology than nonvaccinated controls (112). The reasons for differences in the outcomes of the human ocular studies and the murine genital studies are not clear but may relate to the differences in the immune responses between ocular and genital tissues or between mice and humans or may be due to the choice of adjuvant.

Striking protection against *C. muridarum* genital challenge is achieved by the adoptive transfer of dendritic cells pulsed *ex vivo* with inactivated *C. muridarum* EBs (103). This type of personalized health care, while effective, has limited real world applications for prevention of an infection like *C. trachomatis*. The study does, however, support further investigation into the use of EBs as a vaccine antigen and demonstrates the utility of dendritic cells in vaccination strategies.

A live attenuated vaccine is another approach utilizing whole EBs in vaccine strategies. Mice infected with a plasmid-cured strain of *C. muridarum* have a similar course of infection to mice infected with wild-type *C. muridarum* but do not develop oviduct pathology (71). These mice are subsequently protected against challenge with fully virulent *C. muridarum* (71). Since the plasmid-deficient strain of *C. muridarum* establishes a productive infection, it induces a protective immune response that, like infection with fully virulent *C. muridarum*, protects against reinfection (57, 71). Induction of a protective response against genital challenge in the absence of pathology is the goal of vaccine development, and this study indicates the usefulness of investigating a live attenuated vaccine. The safety of a live attenuated vaccine was also demonstrated with the

attenuated plasmidless *C. trachomatis* serovar L2R strain (72). Mice infected with *C. trachomatis* L2R did not develop pathology even after repeated infections. The vaccination, however, failed to protect against pathology when mice were infected with a nonhomologous *C. trachomatis* serovar D strain (72). Collectively, these studies demonstrate the utility of whole EBs in chlamydial vaccine development despite the negative implications of early studies.

MOMP. MOMP is an approximately 40-kDa highly disulfide cross-linked surface-exposed protein that comprises about 60% of the outer membrane of chlamydiae (12, 13, 80, 105). Along with other high-molecular-weight proteins, MOMP maintains the structural rigidity of the outer membrane of the chlamydial EB, which lacks the peptidoglycan found in the outer membrane of other bacteria (32, 117). MOMP is an immunodominant antigen in both humans and animals and contains multiple B- and T-cell epitopes, eliciting both neutralizing antibody and T-cell immunity (9, 80, 91). The structure of MOMP is comprised of four variable domains interspersed with five constant domains (2, 99, 105). *C. trachomatis* serovars are typed according to MOMP, and the uniqueness of each serovar is defined by the amino acid sequence of their variable domains. There are multiple alleles coding for MOMP in *C. trachomatis* but only a single MOMP allele in *C. muridarum* (10). Therefore, vaccine studies targeting MOMP in the *C. muridarum* model of genital infection cannot account for a vaccine's ability to provide cross-serovar protection. However, *C. trachomatis* serovars belong to one of three serogroups, and it has been shown *in vitro* that monoclonal antibodies to serovars in the same serogroup are cross protective (120), so serovar-specific immunity may not be a necessary requirement of a chlamydia vaccine.

MOMP was among the antigens used in the early vaccine studies with the murine model and has seen continuous use throughout the years (8, 30, 108). Included as part of an extracted outer membrane complex, as purified native or recombinant protein, in peptide form, in plasmid form, and as DNA, MOMP is the most frequently used vaccine antigen (Table 1). Initial studies with MOMP as part of a chlamydial detergent extract along with Hsp60 were disappointing and, similar to the early human ocular vaccination studies, resulted in increased pathology (8). A later study immunizing with a detergent-extracted outer membrane complex (COMC) coupled with incomplete Freund's adjuvant showed more promising results with the immunized mice shedding fewer chlamydiae and exhibiting a decrease in pathology and infertility relative to the controls (84). In the same study, several preparations of purified MOMP extracted with different detergents did not protect as well as the MOMP-containing COMC (84). The authors hypothesized that the difference in protection was due to the conformation of the MOMP rather than the presence of other, potentially immunogenic, proteins in the COMC (84). The proteins in the COMC likely retained the native, or close to the native, conformation, whereas the purified MOMP may have lost the native conformation during extraction and purification. Further support for this hypothesis comes from a study comparing the protective efficacies of nMOMP and rMOMP, both combined with equal amounts of the adjuvants CpG-1826 and Montanide ISA 720 in a murine model of *C. muridarum* lung infection (106). The nMOMP resulted in significantly more

protection than the rMOMP. It has also been demonstrated that adoptive transfer of dendritic cells pulsed *ex vivo* with rMOMP does not protect against genital challenge as well as the transfer of dendritic cells pulsed *ex vivo* with whole *C. muridarum* EBs (95).

rMOMP has been used in a number of vaccine studies (Table 1) (30). Vaccination with rMOMP, using a variety of adjuvants, has induced a wide range of levels of immune protection (1, 7, 17, 22, 27, 28, 31, 33, 34, 49, 95, 96, 118, 119). This is also true for studies utilizing nMOMP (76, 77, 81, 83, 84). Most MOMP-based vaccines induce at least partial protection against genital challenge, but the eliciting of a protective response that parallels that achieved by intranasal or genital infection with whole *C. muridarum* EBs is far more infrequent. Two remarkable examples of MOMP-based vaccines that have been shown to protect as well as intranasal infection are a parenteral nMOMP vaccine coupled with the adjuvants CpG-1826 and Montanide ISA 720 (80) and a mucosal vault nanoparticle vaccine with rMOMP as the antigen (17). Both vaccines induce strong Th1 type responses and result in significant protection against challenge. The nMOMP vaccine also provided protection against the development of infertility (80), something which has not yet been demonstrated with the rMOMP nanoparticle vaccine (17). The ability of both an rMOMP vaccine and an nMOMP vaccine to induce such striking immunity may indicate that while the structural conformation is likely important, the correct adjuvant(s) may be able to overcome the conformational defects in rMOMP.

The vaccine preparation containing nMOMP plus CpG-1826 and Montanide ISA 720 has been shown to induce protection not only against upper genital tract challenge in mice (80), but also against vaginal challenge with *C. muridarum* (29). Further studies into the mechanisms of the nMOMP vaccine-induced protective response revealed that both CD4⁺ T cells and antibody are required for the nMOMP vaccine-induced protection (29). The passive transfer of the polyclonal anti-MOMP serum that is generated by vaccination was also shown to protect mice in a model of genital tract reinfection better than convalescent-phase serum from infection-immune animals (29). A recent study assessing the ability of antibody to rMOMP to protect against genital challenge found that while the anti-rMOMP decreased the infectious burden, this treatment accelerated the development of pathology (21). This finding is in contrast to the results of the nMOMP study mentioned above as well as other studies looking at the protective efficacy of anti-MOMP responses (17, 31, 80, 81, 83, 84). The protection elicited by the polyclonal anti-MOMP serum (29) has important implications for vaccine design as it provides further evidence that MOMP is a key protective antigen and anti-MOMP responses provide significant protection against infection.

The vaccine preparation containing nMOMP plus CpG-2395 (macaque-specific CpG) and Montanide ISA 720 also protected nonhuman primates against ocular infection with *C. trachomatis*, although the protection was limited to decreasing bacterial burden and vaccination did not protect against infection-related inflammation and pathology (42). This discrepancy between the findings of the murine and the nonhuman primate studies may be due to differences in the host (mouse versus nonhuman primate), the site of chal-

lenge (genital tract versus eye), or the biovar of *Chlamydia* used (*C. muridarum* versus *C. trachomatis*).

CPAF. The chlamydial protease-like activity factor (CPAF) is a secreted protease conserved among *Chlamydia* species (37). A variety of host proteins are subject to degradation by CPAF, thereby implicating this molecule in evasion of host defenses and in pathogenesis (18, 37, 66, 67). Among the targets of CPAF degradation are host transcription factors such as upstream stimulation factor 1 (USF-1) and regulatory factor X 5 (RFX5), which are required for MHC expression (37, 68). The proapoptotic BH3-only proteins are also degraded by CPAF, implicating this protein in blocking apoptosis in infected cells (18, 37). CPAF has also been shown to cleave the cytoskeletal protein cytokeratin 8, and this cleavage may be a mechanism involved in expansion of the inclusion (37). It has been hypothesized that CPAF is released into the extracellular environment from the cytosol upon rupture of the cell and is then taken up by neighboring cells and processed by the exogenous MHC II pathway (16). As such, CPAF is a dominant antigen in seropositive humans and has been investigated as a possible candidate for a subunit-based vaccine (16).

CPAF is a relative newcomer to the field of chlamydial vaccine studies, but considerable effort has been invested in studying its potential utility as a candidate vaccine antigen. Vaccination with recombinant CPAF (rCPAF) plus IL-12 or CpG-1826 shortens the duration of infection and decreases the cellular infiltrate and oviduct pathology relative to that in unvaccinated animals (18, 66). CPAF exists in both inactive and active forms, but vaccine-induced protection is independent of CPAF's activity state (16). The mechanism of protection induced by CPAF has been investigated and was found to be dependent on IFN- γ (66) and cell-mediated immunity, but not humoral immunity (67). CPAF vaccination has also been shown to induce protective immunity in an HLA-DR4 transgenic strain of mice, which expresses the predominant allele involved in chlamydial antigen presentation to CD4⁺ T cells in humans (67), suggesting that CPAF may also induce a protective response in humans.

While vaccination with CPAF results in a decrease in bacterial burden and a shortened duration of infection relative to naïve animals, the impact of vaccination on the infection is not apparent until 1 week postinfection, despite the use of a mucosal route for vaccination (18, 66). CPAF is secreted into the cytosol of the chlamydia-infected cell during growth and replication. Inactive CPAF is detected within the inclusion as early as 12 h postinfection, but activated, secreted CPAF cannot be detected until 24 h postinfection (37). This requirement for a productive infection may explain the delay in the vaccine-induced protective effect when protection is measured by the shedding of fewer infectious chlamydiae following challenge. However, if the measure of protection is prevention of oviduct pathology, then CPAF vaccination provides significant protection (18, 66). Vaccination with CPAF can induce a protective response against vaginal challenge, but due to the delayed induction of protection, CPAF may be best utilized in conjunction with another antigen. As such, mice vaccinated with rCPAF plus UV-inactivated EBs and CpG-1826 have a shorter duration of infection than those vaccinated with rCPAF and CpG-1826 alone (50). These mice also began clearing the in-

fection sooner than those vaccinated with rCPAF and CpG-1826 alone (50).

Other antigens. Vaccine studies targeting *C. muridarum* have overwhelmingly favored the use of whole EBs, MOMP, and, more recently, CPAF, but protective responses have also been elicited by vaccination with a number of other antigens, some of which are listed in Table 1. Several polymorphic membrane proteins (Pmps) have been assessed as vaccine candidates after both parenteral vaccination (119) and adoptive transfer of dendritic cells pulsed *ex vivo* with the proteins (118). Adoptive transfer of the *ex vivo*-pulsed dendritic cells does not alter the course of infection notably, but liposomal delivery of PmpG leads to a significant decrease in bacterial shedding and a shortened course of infection (118, 119). The putative type III secretion effector protein Tarp induces a *C. muridarum*-specific immune response and offers some protection against inflammation and pathology, but it has little impact on the course of infection (112). Likewise vaccination with inclusion membrane protein A (IncA) does not induce significant protection against genital challenge and only leads to a slight decrease in inflammation (49). When mice are vaccinated with IncA in addition to rCPAF, the protection is more substantial (49). The chlamydial plasmid encodes the secreted protein pgp3 in the open reading frame 5 (pORF5) that is recognized by serum from infected humans, and in that respect is considered immunodominant. Vaccination with pgp3, however, provides minimal protection against genital challenge with *C. muridarum* (51). While many of the chlamydial antigens elicit at least some level of protection against infection or inflammation, none have been able to induce considerable protection on their own, and it is likely that they would fair better in a multisubunit vaccine.

Recent vaccine studies with the murine model, including those utilizing human *C. trachomatis* biovars, were reviewed by Hafner et al. (30). As with the *C. muridarum* vaccine studies, MOMP is the predominant antigen used in the *C. trachomatis* studies, although rMOMP is almost exclusively utilized. Outer membrane proteins other than MOMP, such as OmcB, are immunogenic and induce partial protection against genital challenge with *C. trachomatis* after vaccination (26, 30). Additional Inc proteins as well as putative inclusion proteins have also been assessed for their immunogenicity and protective effect, but the results are not as promising as those obtained with MOMP vaccines (30, 53, 54, 97). Porin protein B (PorB) (43, 44), the small chain of ribonucleoside reductase (NrdB) (3), and PmpD (20) are all recognized by serum from *C. trachomatis*-infected patients, and immune serum and/or monoclonal antibodies specific to these proteins are able to neutralize infection *in vitro* (3, 20). Vaccination with PorB results in partial protection against genital challenge, and protection is increased when MOMP is included as part of a subunit vaccine with *Vibrio cholerae* ghosts (38). Immunization with NrdB induces a robust immune response in mice characterized by high-titer specific antibody in the serum, vaginal lavage, and uterine lavage, as well as high IFN- γ responses by splenocytes (3). NrdB-specific CD4⁺ T cells induce partial protection against genital challenge upon adoptive transfer (3), but testing NrdB directly for vaccine efficacy has not yet been reported. PmpD is a species-specific antigen that elicits pan-neutralizing antibodies (20). If found to be a protective vaccine antigen, it could potentially

provide cross-serovar protection. Importantly, *in vitro* antibodies to chlamydial MOMP and lipopolysaccharide (LPS) block the neutralizing effect of anti-PmpD, indicating a possible mechanism of *C. trachomatis* pathogenesis (20). There have been no published accounts of PmpD used in vaccine studies.

ADJUVANTS AND DELIVERY SYSTEMS

The importance of antigen selection in the design of an effective vaccine is irrefutable, but the choice of adjuvant and/or delivery system is also of utmost importance as this choice can impact the type and strength of the immune response generated. The unique properties of the genital tract also impact vaccine development and indicate that protection requires both mucosal and systemic responses. To address these issues and develop an efficacious vaccine, a variety of adjuvants and delivery systems have been tested (Table 1). The adjuvants tested include the traditional choices of alum, complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), bacterial proteins, including subunits of cholera toxins (CTA1-DD, CTB, CTA2B, and CT), the outer surface protein of *Borrelia burgdorferi* (OspA), and several adjuvants that are currently in clinical trials, such as CpG, Montanide ISA 720, LT-R72, MF-59, LT-K63, and IL-12. Novel delivery systems have also been introduced, and these include lipid C, liposomes (CAF01 and DDA/TDB), dendritic cells, gene guns, *Vibrio cholerae* ghosts, and vault nanoparticles. Vault nanoparticles and liposomes are especially promising as adjuvants for a *C. trachomatis* vaccine as they have been shown to induce significantly protective responses when used with rMOMP (1, 17, 31). Studies of the protective responses involved in immunity to *C. muridarum* demonstrate the importance of promoting a strong Th1-type response. This can be seen in the disparity of protection induced by vaccination with nMOMP plus alum, a Th2-targeting adjuvant, and vaccination with nMOMP plus CpG-1826 and Montanide ISA 720, a Th1-targeting adjuvant combination (80). A study comparing the protective efficacy of vaccination of mice with nMOMP and rMOMP with the same combination of adjuvants demonstrated that while significant protection is induced with rMOMP, nMOMP elicits a far more protective response (106). This suggests that using nMOMP with vault nanoparticles or liposomes as the delivery system has the potential to induce better protection than has already been shown with rMOMP and these systems (1, 17, 31). The caveat to these studies, however, lies in their use of mucosal routes for vaccination and the limited utilization of this route in current human vaccination protocols.

IMPORTANT CONSIDERATIONS

A major challenge for the field of *Chlamydia* vaccine research is the lack of standardization in the procedures used to test *Chlamydia* vaccines, which leads to difficulty in comparing the efficacy of vaccines relative to each other. Several important differences used by various labs engaged in *Chlamydia* vaccine research that significantly impact the results of infection and vaccine studies include mouse strain, route of infectious challenge, dose of infectious challenge, adjuvant, and the antigen used to assess cellular and humoral immune responses *in vitro*. Several studies have made evident that the mouse

strain used for infection impacts the outcome of the infection in terms of infection titer, duration of infection, degree of the upper genital tract infection, severity of the infection-induced pathology, and immune responses generated (23–25). Infectious dose has been shown to impact the course of infection and ascension of bacteria to the upper genital tract (14). Humoral responses after vaccination are measured by enzyme-linked immunosorbent assay (ELISA) to either the antigen used in the vaccination or whole chlamydial EBs. While using the vaccine antigen in the ELISA demonstrates the ability of the vaccine to elicit an antigen-specific response, using whole EBs evaluates whether the antibody elicited by the vaccine will recognize the agent of infection. Vaccines utilizing secreted proteins would not produce antibody that recognize whole EBs, thereby necessitating the use of the vaccine antigen in ELISAs. Collectively, the differences in the criteria and testing strategies listed here as well as other methodologies that are used to assess the protective efficacy of vaccines present an added level of variation that confounds comparison across laboratories.

The route of infectious challenge also impacts vaccine assessment. Vaccines targeting genital infection utilize one of two routes of infection: vaginal and ovarian bursa, which provide two very important models to assess the efficacy of vaccines. Challenging mice at the vagina, the natural route of infection, allows a normal progression of the infection through the genital mucosa. When mice are challenged vaginally, the infection propagates in the lower genital tract at the cervix and then ascends naturally to the upper genital tract, where it leads to pathology and infertility in some mice. Vaginal challenge provides a model to study the ability of a vaccine to prevent infection and the resulting pathology. However, not every mouse that is challenged vaginally develops pathology, and thus the model of upper genital tract challenge is useful in that regard. In the model of upper genital tract challenge, mice are infected directly in the ovarian bursa by a minor surgical procedure. Directly infecting the upper genital tract increases the likelihood of a productive infection of these tissues, thereby increasing the incidence of pathology. Both infection models are of great utility in vaccine development, but given that most studies utilize only one of the models, it is difficult to evaluate and compare the efficacies of vaccines.

CONCLUSIONS

Chlamydial genital infection is a worldwide public health concern. The past several decades have seen significant advancements in chlamydial immunobiology that have made the prospect of a chlamydial vaccine more attainable. Animal models, particularly the mouse model of genital infection, have proven to be enormously useful in identifying candidate vaccine antigens and in elucidating immune responses that contribute to protective immunity. These models have shown that several infection/disease outcomes can be used as measures of vaccine efficacy, including reduced bacterial shedding, shortened duration of infection, and diminished tissue damage (i.e., hydrosalpinx). Using those measures, some vaccines such as the noninfectious nMOMP vaccine are protective as assessed by all three measures (29, 80), whereas other vaccines, such as the noninfectious CPAF vaccine, have some effect on bacterial shedding but provide robust protection against upper genital tract disease (18,

64, 66, 67). Further, recent studies have shown that infection of mice with a plasmid-cured strain of *C. muridarum* produces marked protective immunity without development of deleterious postinfection sequelae (71). These recent advances in chlamydial vaccine biology demonstrate that several options exist for targeting vaccine immunity. A vaccine that produced near-sterilizing immunity would be ideal; however, a vaccine need not necessarily produce sterilizing immunity to be effective. Vaccines that reduced postinfection sequelae, diminished bacterial shedding, and/or shortened the duration of infection would all potentially facilitate the control of chlamydial infections.

The current research activity in the field of chlamydial immunology signifies a renewed interest in vaccine development. Those studies have produced a wealth of new knowledge and research tools, which have made it possible for researchers to home in on and induce the appropriate protective responses with a variety of candidate vaccines. Continued discoveries in defining mechanisms of protection and understanding the pathogenesis of infection will only improve the chance of bringing a licensed chlamydial vaccine to fruition.

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