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MINIREVIEW

Update on *Chlamydia trachomatis* Vaccinology

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ABSTRACT Attempts to produce a vaccine to protect against *Chlamydia trachomatis*-induced trachoma were initiated more than 100 years ago and continued for several decades. Using whole organisms, protective responses were obtained. However, upon exposure to *C. trachomatis*, disease exacerbation developed in some immunized individuals, precluding the implementation of the vaccine. Evidence of the role of *C. trachomatis* as a sexually transmitted pathogen started to emerge in the 1960s, and it soon became evident that it can cause acute infections and long-term sequelae in women, men, and newborns. The main focus of this minireview is to summarize recent findings and discuss formulations, including antigens, adjuvants, routes, and delivery systems for immunization, primarily explored in the female mouse model, with the goal of implementing a vaccine against *C. trachomatis* genital infections.

KEYWORDS *Chlamydia trachomatis* vaccines, *Chlamydia muridarum*, antigens, adjuvants, routes of immunization, delivery systems, *Chlamydia trachomatis*, vaccinology

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Chlamydiae infections are widespread throughout the animal kingdom (1–3). Until recently, trachoma was thought to be the most common clinical manifestation in humans (1, 2). Trachoma likely first appeared in China and Mesopotamia (~2,700 B.C.) and then spread to the Middle East and the Mediterranean region (1, 2, 4).

HISTORICAL BACKGROUND

In 1907, Halberstaedter and von Prowazek (5), using Giemsa stain, first described the presence of intracytoplasmic inclusions in conjunctival scrapings from monkeys. However, it was not until 1957 that Tang et al. (6, 7), utilizing chicken embryos, isolated *Chlamydia* from humans with trachoma, although the lymphogranuloma strains have been grown in the yolk sac since 1942 (2, 8).

In 1913, a group in Tunis, led by Nicolle, initiated vaccine studies in humans and nonhuman primates (9). Resistance to rechallenge was observed in some individuals, but in others, inconclusive results occurred. Major efforts to produce a vaccine using live or inactivated *Chlamydia* occurred after the isolation of the organisms by Tang et al. (6, 7). Four research groups (1, 2, 4) performed vaccine trials and reached similar conclusions: some vaccine formulations were protective for a period of 1 to 3 years, the protection was serovar/serogroup specific, and certain immunized individuals, upon reexposure, developed more-severe disease than the placebo-treated groups (8, 10–16). In a few instances, a higher attack rate was observed in vaccinated children than in controls (17, 18). Although Bietti's laboratory manufactured a vaccine, it was never implemented in humans (16).

Improvements in sanitary and hygienic conditions have resulted in the disappearance of trachoma from most parts of the world. In 1993, with the goal to eradicate trachoma by 2020, the WHO implemented the SAFE strategy (2, 19). This SAFE plan

includes *s*urgery for trichiasis, *a*ntibiotic treatment of active trachoma, *f*acial cleanliness, and *e*nvironmental improvements focusing on providing clean water supply and toilets and reducing the number of flies in the affected areas.

The presence of *Chlamydia trachomatis* in the genital tract was first indirectly described when a nongonococcal, or amicrobiana, ophthalmia was observed in newborns (20). In 1910, Heymann (21) noted the presence of inclusions in samples from the cervix of the mother and the urethra of the father of an infant with inclusion blennorrhea. It is even possible that the identification of *Chlamydia* in the genital tract was described earlier as the cause of an amicrobial urethritis (22). Using the yolk sac technique, Jones et al. (23) first recovered *C. trachomatis* from the cervix of a mother with pelvic inflammatory disease (PID). In the 1970s, the introduction of tissue culture techniques greatly improved the ability to isolate *Chlamydia* from the genital tracts of males and females and the eyes of newborns (1, 2).

C. trachomatis is now recognized as the most common sexually transmitted bacterial infection, with approximately 100 to 150 million new cases occurring each year worldwide, affecting 68 million females (24–26). Most of the infections (70 to 80%) in women are asymptomatic; however, it is estimated that 5 out of 1,000 will develop tubal factor infertility (27–29). The long-term sequelae of a *C. trachomatis* infection in females with PID include chronic abdominal pain, ectopic pregnancy, and infertility (27, 29–37). Screening and treating females may increase the rates of reinfection but reduces the incidence of PID (31, 38–40). In males, epididymitis, but not infertility, is the most severe complication (1, 41). Reactive arthritis (Reiter's syndrome [urethritis, conjunctivitis, and arthritis]) has also been associated with *Chlamydia* (1, 41). In females, most cases of upper genital pathology are due to a single episode of PID. For example, in the pioneering work by Westrom et al. (30), 1,844 women with laparoscopically verified PID and 657 women with normal laparoscopic results were studied over a period of several years. Of the 1,241 women who had episodes of PID, 141 were not able to conceive. Tubal factor infertility was associated with the severity and number of PID episodes. The majority of women who were not able to conceive, 56% (79/141), had a single PID episode, and in most of them, 62% (49/79), the episode was severe. The number of PID episodes also affected the chances of developing tubal infertility. Of the individuals who had a single episode, 8.0% (79/991) became infertile, while 19.5% (36/185) of those who had two episodes and 40.0% (26/65) of patients who had three or more episodes were infertile. The ectopic pregnancy rates for the first pregnancy after index laparoscopy were 1.4% in controls and 9.1% among patients. Prevention of these long-term sequelae is the principal goal of a vaccine (26, 42–51).

IMMUNITY TO *C. TRACHOMATIS* GENITAL INFECTIONS

Natural immunity in humans. It is well-known that genital chlamydial infections are more frequent in young sexually active individuals, between the ages of 15 to 20, than in older persons (24, 52, 53). This finding has been interpreted as evidence of naturally induced protective immunity. Some clinical and experimental evidence supports this suggestion. For example, in sex workers, resistance to infection correlates with duration of prostitution, independently of age, indicating development of acquired immunity (54). Geisler et al. (55) also showed that natural immunity occurs in females with a *C. trachomatis* genital tract infection. Because antibiotic treatment interfered with the development of natural immunity, individuals who were not treated with antibiotics had fewer reinfections than those who were treated with antibiotics. However, it is important to note that, at least in mice, resistance to a primary genital infection increases with age, suggesting that some physiological processes may account for a lower prevalence of chlamydial infections in older individuals (56, 57).

Use of *Chlamydia muridarum* versus *C. trachomatis* in vaccinology studies. The *C. muridarum* isolate, previously called *C. trachomatis* mouse pneumonitis (MoPn), has been extensively utilized to perform studies in mice (44, 45, 58–60). At the genomic level, *C. trachomatis* and *C. muridarum* are highly orthologous, and thus, it is thought that findings in mice will correlate well with observations in humans (61). There are,

however, significant differences in the pathogenicity of *C. trachomatis* versus *C. muridarum* in the mouse model.

Following a vaginal challenge, *C. trachomatis* serovars (A to K) and *C. muridarum* produce quite different acute and chronic diseases in mice. In animals not pretreated with depot-medroxyprogesterone acetate (DMPA), depending on the strain, stage of the estrous cycle, and age of the mice, different doses of *C. muridarum* (50% infective doses [ID_{50}] of $\sim10^4$ to $>10^7$ inclusion-forming units [IFU]) produce severe infection and infertility, while *C. trachomatis* does not do so (56, 57, 60, 62). When pretreated with DMPA, all strains of mice tested so far, with the exception of A/J and DBA/2J mice, develop severe infection and long-term sequelae following a vaginal challenge with a low dose of *C. muridarum* (ID_{50} of $\sim$200 IFU) (45, 63–67). In contrast, a vaginal infection with *C. trachomatis* in mice pretreated with DMPA induces only mild, short infections and no long-term upper genital pathology, i.e., hydrosalpinx and/or infertility (32, 63, 68–72). Even in the highly susceptible C3H/HeJ mice that have a mutated nonfunctional Toll-like receptor 4 (TLR4), a vaginal infection with *C. trachomatis* serovar D (strain UW-3Cx) does not elicit long-term tubal pathology (73).

Two publications have reported infertility, without hydrosalpinx formation, following single or multiple vaginal infections with very high doses ($\sim10^6$ or 10^7 IFU) of *C. trachomatis* serovar D or E in mice pretreated with DMPA (74, 75). The lack of hydrosalpinx in these mice suggests that no gross long-term damage was produced in the fallopian tube, and therefore, another mechanism(s), which may or may not be relevant to humans, had to account for the infertility. Hadad et al. (76) recently reported infertility in mice following a vaginal challenge with 3×10^4 IFU of a clinical serovar D isolate. The histopathological findings were not discussed. The apparent high virulence of this serovar D isolate is unusual and needs confirmation.

Sturdevant and Caldwell challenged the validity of using *C. trachomatis* in vaccinology (77). These authors reported that C57BL/6 $Rag1^{-/-}$ mice, which lack mature T- and B-cell immunity, treated with DMPA cleared a vaginal infection with *C. trachomatis* serovar D (D-LC), suggesting that innate immunity is sufficient to control the infection. Interestingly, C3H/HeN male mice with severe combined immunodeficiency (SCID) cannot control a urethral challenge with *C. trachomatis* serovar D (UW-3/Cx), while the wild-type (WT) mice can (78). Finally, following transcervical, intrauterine, or intrabursal infections, in mice pretreated with DMPA, not all *C. trachomatis* serovars/isolates induce infertility in most strains of animals, while *C. muridarum* does (79–87).

In conclusion, in our opinion, further testing of *C. trachomatis* isolates is needed to validate their use in vaccine studies. The production of a humanized mouse model could help to address some of the limitations (88). In the meantime, *C. muridarum* allows us to reproduce in mice the mild, severe acute, and chronic infections that *C. trachomatis* produces in humans. The main shortcoming of *C. muridarum* is the lack of isolates that will allow testing for cross-serovar protection.

Immunity to *C. muridarum* in the mouse model. Characterizing immune responses in humans is extremely difficult for many reasons (1, 32, 59). Each individual has a different immunogenetic background. Infection can occur at birth or shortly thereafter. The infection can be asymptomatic. There is cross-reactivity among different species of *Chlamydia*. Keeping track of sexual behavior in humans is difficult. Therefore, most of what we know about protective immunity against chlamydial infections is based on work performed in the mouse model. Using WT and knockout (KO) mice for genes involved in cellular and humoral immune responses, cytokines and chemokines, and passive transfer of T cells and antibodies, immune components that play a role in protection against primary and secondary genital *C. muridarum* challenges have been determined (44, 45). From these studies, it was found that major histocompatibility complex class II (MHC-II)-restricted $CD4^+$ T cells are required for protective immunity, while MHC-I-restricted $CD8^+$ T cells are not essential for resolution of a primary infection or protection against a genital reinfection (64, 89–91). Human data support these findings. For example, HIV-1-infected patients, who have low $CD4^+$ T-cell counts,

are at a higher risk of becoming genitally infected with *C. trachomatis* and developing long-term sequelae than healthy individuals (92). Although $CD8^+$ T cells play a critical role in protection against most intracellular pathogens, they are not required to control chlamydial infections. Interestingly, Igietseme et al. (93) showed that clones of $CD8^+$ T cells, likely by producing gamma interferon (IFN-γ), enhance clearance of a *C. muridarum* genital infection, and Nogueira et al. (94) demonstrated that cross-mucosa protection (respiratory and genital) is mediated by $CD4^+$ and $CD8^+$ T cells and not by antibodies.

After vaccination, some of the circulating Th1 lymphocytes may become mucosal tissue-resident memory cells (Trm) rapidly reducing *C. muridarum* load upon reinfection (85, 95). This hypothesis is consistent with the detection of $CD4^+$ T cells in the mouse genital tract tissues long after the infection was cleared (96). Once these lymphocytes have been deposited locally, they may protect against microbial invasion by both antigen-specific and -nonspecific means and even undergo autonomous proliferation (97–99). Thus, as first proposed by Morrison and Morrison (96), an effective *C. trachomatis* vaccine may need to induce Trm in the genital tract mucosa (95).

The role of B cells and/or antibodies against a primary infection is not clear. However, antibodies have been found to be as protective against reinfection as $CD4^+$ T cells, making them important in vaccine development (90, 100–102). Following a primary *C. muridarum* infection of the genital tract, mice are resistant to a rechallenge if $CD4^+$ T cells and/or antibodies are present (100, 103, 104). Antibody-deficient mice and those that lack Fc receptors resolve a primary *C. muridarum* genital infection, but not a secondary infection, as well as WT animals do (44, 64, 90, 100).

Considering the extraordinary complexity of the antibody response, it is not surprising that in humans and animal models, some antibodies have been found to be protective, while others correlated with pathogenesis. For example, high levels of antibodies to the *C. trachomatis* 60-kDa heat shock protein (Hsp60) have more often been detected in patients with long-term sequelae than in individuals without complications (105–107). However, high IgA levels in the genital tract protect mice against a *C. muridarum* infection and correlate with protection against the long-term sequelae (108, 109). In humans, mucosal antibodies, especially IgA, correlate with reduced bacterial shedding (108). Also, even in the absence of T cells, passive immunization of mice with monoclonal antibodies can control a primary *C. muridarum* infection (110, 111).

Th1 cytokines, particularly IFN-γ and interleukin 12 (IL-12), are needed for protection against *C. muridarum* (112, 113). In contrast, IL-10, a Th2 cytokine, was found to be associated with pathological responses (114). The critical role of IFN-γ against chlamydial infections has been demonstrated using neutralizing antibodies and by showing that mice deficient in IFN-γ or its receptor do not resolve a primary *C. muridarum* genital infection (112, 115). These animals develop systemic dissemination but are partially protected against reinfection (101, 113, 116). In conclusion, based on the evidence we have in mice infected with *C. muridarum*, to elicit optimal protection, a chlamydial vaccine will have to induce $CD4^+$ T cells with Th1-biased cytokines, in particular IFN-γ, and humoral responses, especially mucosal antibodies.

C. TRACHOMATIS VACCINE ANTIGENS

Whole-cell vaccines. In mice, intranasal immunization with live elementary bodies (EB) was the first approach shown to elicit very robust protection against a *C. muridarum* genital infection (117, 118). Following an intrabursal challenge, vaccinated mice showed a significant decrease in vaginal shedding and, more importantly, protection against infertility. This protection was found to be long-lived and also protected newborn mice, suggesting that a live-chlamydia vaccine could be effective in very young individuals before sexual maturity (119, 120). As determined by vaginal shedding, intranasal inoculation with live EB was also found to protect mice against a vaginal challenge with *C. trachomatis* serovar E (Bour) (121). Protection with *C. muridarum* UV-inactivated EB was not as effective as that obtained with viable organisms,

and reticulate bodies were not protective (121–124). UV-inactivated serovar E (Bour) EB, with AS01B as an adjuvant, were shown to induce cross protection against vaginal and intrauterine challenges with serovar K (UW-31/Cx) as determined by shedding (125). Adoptive transfer of dendritic cells pulsed *ex vivo* with inactivated *C. muridarum* EB achieved very strong protection that was considered to depend on IL-12 production by the pulsed dendritic cells (126, 127). These results support further investigation on testing nonviable whole *Chlamydia* organisms as vaccines.

The use of a live attenuated EB vaccine is another potential approach. Mice inoculated vaginally with a plasmid-cured isolate of *C. muridarum* developed an infection with a course similar to that following inoculation with the WT isolate, but no upper genital pathology was observed (128, 129). Following a subsequent challenge with the WT isolate, animals first infected with plasmid-cured *C. muridarum* had less upper genital pathology than those first inoculated with the WT. However, there was minimal protection against vaginal shedding, and the fertility of the mice was not tested. Similar findings were reported when plasmid-cured *C. muridarum* was inoculated into the ovarian bursa (129). Mice vaccinated with a plasmid-cured *C. trachomatis* serovar L2R had reduced shedding but no upper genital tract protection against a challenge with serovar D (UW-3Cx) (130).

Studies of nonhuman primates have also shown that vaccination with plasmid-cured *C. trachomatis* serovar A, at least in animals that shared a common MHC-II class allele, can induce protection against ocular infection and disease (131). However, additional work is required before this approach is tested in humans. For example, Qu et al. (132) showed that cervical inoculation of rhesus macaques with WT or plasmid-deficient *C. trachomatis* serovar D (UW-3Cx) resulted in the same levels of infection, immunity, and pathology in the genital tract. This suggests that the plasmid is not required for *C. trachomatis* to colonize the genital tracts of rhesus macaques. However, the plasmid appears to be important for *C. trachomatis* infection of the human genital tract, since most human isolates are plasmid competent (133, 134).

It is not clear why live *Chlamydia* induced better protective immunity than killed organisms (122–124). Replication and dissemination of live *Chlamydia* will broaden and expand the antigenic load (135). Also, one distinguishing feature is that inactivated organisms load far fewer peptides onto dendritic cell MHC-II molecules than do viable organisms (123). However, it will be worth comparing the levels and types of Trm in the genital tracts of mice immunized with live or dead EB, since it was recently demonstrated that the requirement for viability to induce protective immunity could be overcome by using adjuvantized nanoparticles, which correlated with the induction of transmucosal Trm (85).

Subunit vaccines. (i) Introduction. Safety concerns about the negative effects observed during the trachoma vaccine trials and costs of production have encouraged the formulation of a *C. trachomatis* subunit vaccine. Based on the trachoma vaccine trials, protection against an ocular challenge was found to be serovar/serogroup specific, an observation supported in experiments with mice (1, 2, 136, 137). DNA sequencing of the major outer membrane protein (MOMP) and computational analysis established that MOMP was likely responsible for the protection observed during the trachoma vaccine trials (137–140). Initial attempts to vaccinate animals with *C. muridarum* recombinant MOMP (rMOMP), peptides corresponding to the variable domains (VD), or with DNA plasmids that expressed this protein were not encouraging (65, 141–145). In addition, the expected serovar/serogroup specificity of the protection elicited with MOMP was, and still is, a significant concern. For these reasons, the search for additional protective antigens was undertaken. Initially, an empirical approach was used, and several proteins, particularly those localized to the outer membrane of *Chlamydia*, were tested. Since the availability of the *Chlamydia* genome sequence and implementation of protein microarrays, it became possible to identify chlamydial antigens that can induce antibody- and/or cell-mediated immune responses in infected individuals (107, 146–158). Identification of immunogenic components using these

approaches, however, does not imply that they will be protective. Therefore, it is necessary to test these antigens in animal models to demonstrate their protective ability.

(ii) Major outer membrane protein. The major outer membrane protein (MOMP) is a 40-kDa protein that accounts for approximately 60% of the mass of the outer membrane of *Chlamydia* (159, 160). Structurally, MOMP has four VD, which define the uniqueness of each serovar, alternating with five constant domains (CD) (138, 139). Like porins from other Gram-negative bacteria, MOMP has a trimeric structure (161). However, unlike most bacterial membrane proteins, MOMP is highly disulfide cross-linked with other cysteine-rich proteins, forming an intermolecular network that may account for the structural rigidity of the outer membrane of EB, since *Chlamydia* has limited amounts of peptidoglycan (162–165). In humans and animals, MOMP is an immunodominant antigen that has multiple T-cell and B-cell epitopes and can induce T-cell immunity and neutralizing antibodies (166–170).

The first evidence that a subunit *Chlamydia* vaccine could protect mice against a genital challenge was reported in 1997 (171). Mice immunized using a detergent-extracted chlamydial outer membrane complex (COMC) and incomplete Freund's adjuvant were protected as determined by the numbers of *C. muridarum* IFU recovered and fertility rates (171). This study also showed that MOMP, extracted with several detergents, was not as protective as the COMC. The authors postulated that the enhanced protection elicited by the COMC was due to the conservation of the native conformation of MOMP and not due to the additional antigens present in the COMC. In 2001, mice vaccinated with MOMP in its native trimeric conformation (nMOMP) with Freund's adjuvant were significantly protected against shedding and infertility, while those vaccinated with denatured nMOMP were not, supporting the critical role of the structure of MOMP in protection (172). The humoral immune responses were lower, but the cell-mediated immune responses were higher, in mice immunized with nMOMP versus denatured nMOMP. Pal et al. (169, 173) vaccinated mice with nMOMP, using CpG plus Montanide ISA 720 as adjuvants, and showed very robust protection against an intrabursal challenge with *C. muridarum*. Animals immunized with nMOMP were as protected against shedding and infertility as those immunized intranasally with live EB. The importance of the conformation of MOMP for protection was further demonstrated when rMOMP and nMOMP were directly compared, using CpG plus Montanide ISA 720 as adjuvants, for their ability to elicit protection against a respiratory *C. muridarum* challenge in mice (174). Based on changes in body weight, lung weight, and number of IFU recovered from the lungs, nMOMP was significantly more protective than rMOMP was. These results have now been confirmed in the genital challenge model (103, 175, 176). Vaccination with nMOMP can protect mice against intrabursal and intravaginal challenges, with significant decreases in the number of mice that have positive vaginal cultures, the number of IFU recovered, length of shedding, and infertility rates. In the vaginal model, it was shown that nMOMP-induced protection requires both $CD4^+$ T cells and antibodies (103). Importantly, vaccination of nonhuman primates with the *C. trachomatis* serovar A (A2497) nMOMP, formulated with CpG-2395 and Montanide ISA 720, protected against an ocular challenge with the same serovar, as shown by a significant decrease in shedding (177). However, no protection against inflammatory responses was observed.

Immunization with *C. muridarum* rMOMP using various adjuvants has shown promising results in animal models (174, 176, 178, 179). For example, Hansen et al. (178) immunized mice with rMOMP and the cationic liposome 1 (CAF01) and, as determined by a reduced chlamydial load, obtained protection against a vaginal challenge, but hydrosalpinx formation was not prevented. Hickey et al. (179) administered rMOMP orally with a novel lipid-based adjuvant, resulting in significant protection against vaginal shedding, although *C. muridarum* was still present on the last culture day (18 days postinfection). No difference in the inflammatory response in the genital tract was noticed at 28 days postinfection. The authors proposed that the lack of protection

against tissue pathology in the rMOMP-immunized animals was due to the influence of the treatment with DMPA before the vaginal challenge (179). Carmichael et al. (176), using *C. muridarum* rMOMP, CpG plus Montanide ISA 720 as adjuvants and a combination of mucosal and systemic routes for immunization, showed for the first time protection against vaginal shedding and infertility using a recombinant antigen. Badamchi-Zadeh et al. (180) constructed a consensus MOMP from variant sequences of *C. trachomatis* serovar E. The consensus MOMP transgene was delivered using plasmid DNA, human adenovirus 5 (HuAd5) and/or modified vaccinia Ankara (MVA). The DNA-HuAd5-MVA-protein regimen was used to immunize BALB/c and B6C351 mice. Based on vaginal shedding, minimal protection against an intravaginal challenge with serovar D (UW-3/Cx) was observed.

Olsen et al. (181) recently tested a multivalent vaccine containing the VD4 and surrounding CD of MOMP from *C. trachomatis* serovars D, E, and F. This construct, adjuvanted with CAF01, elicited immune responses in mice; some of the immune responses were serovar specific, while other responses were to the conserved VD4 epitope (LNPTIAG) and neutralized serovars D, E, and F *in vitro*. Vaginally challenged mice were protected as determined by the number of *C. trachomatis* serovar D (UW-3/Cx) IFU recovered from the vagina and prevention of the inflammatory response in the upper genital tract. T-cell depletion and passive transfer of serum experiments provided evidence of the critical role of $CD4^+$ T cells and antibodies in protection. Using the same MOMP construct, plus CT043 and CT414 antigens, investigators from the same laboratory elicited neutralizing antibodies and weak protection against vaginal shedding in minipigs challenged with serovar D (182). Hadad et al. (76) have reported that a construct consisting of the VD2/VD4 regions of *C. trachomatis* serovar E, adjuvanted with cholera toxin (CT), protected mice against a vaginal challenge with a clinical serovar D isolate. The authors observed a decrease in vaginal shedding during the first 2 weeks after the challenge and partial protection against infertility.

(iii) Polymorphic membrane proteins. Genome sequencing uncovered the presence of a family of polymorphic membrane proteins (Pmps) unique to *Chlamydiales* (138). In *C. trachomatis* and *C. muridarum*, there are nine *pmp* genes, and based on the presence of a C-terminal phenylalanine and a peptide leader sequence and the results of proteomic studies, these proteins were predicted to be located in the outer membrane (183–185). Pmps have molecular masses ranging from ~100 to 150 kDa, contain β-barrel domains in the C terminus, and exhibit motifs that have been associated with adhesion to the host cells in their N-terminal regions (186). On the basis of their structure, they are considered to be type V secretion autotransporters, which mediate the translocation of the N terminus to the bacterial surface. In addition, some of these proteins contain poly(G) tracts that control the phase-variable expression of the genes, providing a means by which organisms can evade host immune responses (187). Pmps have been found to be immunogenic in humans, nonhuman primates, and mice infected with *C. trachomatis* or *C. muridarum* (151, 153, 156, 188, 189). Antibodies to PmpA have been associated with decreases in fertility and live births (190).

In 2006, Crane et al. (191) reported that antibodies to PmpD could neutralize all the *C. trachomatis* serovars *in vitro*. Paes et al. (192) vaccinated mice with PmpD from *C. trachomatis* serovar E (Bour), using a synthetic agonist to TLR4 as adjuvant, and based on vaginal shedding, observed protection against a challenge with serovar D (UW-3/Cx). In 2008, Karunakaran et al. (193) using an immunoproteomic approach discovered novel T-cell epitopes in several *C. muridarum* Pmps (serovars E, F, G, and H). Vaccination of C57BL/6, BALB/c, and C3H/HeN mice with N-terminal fragments from each of these four proteins was shown to confer protection against a vaginal challenge as determined by accelerated clearance of *C. muridarum*. In particular, PmpG elicited the best protection. A polyvalent recombinant vaccine, consisting of the four Pmps from serovar D and MOMPs from serovars D, F, and J, formulated with the Th1-polarizing adjuvant dimethyldioctadecylammonium (DDA)-monophosphoryl lipid A (MPL), as determined by vaginal shedding, protected C57BL/6 mice against a transcervical challenge with *C.*

trachomatis serovar D (86). Interestingly, Inic-Kanada et al. (194) found that PmpC from *C. trachomatis* serovar E elicited limited protection against an ocular challenge with *Chlamydia caviae* in guinea pigs.

(iv) Chlamydial protease-like activity factor. Chlamydial protease-like activity factor (CPAF) is a secreted protease highly conserved among all the *Chlamydia* species (99% amino acid identity among the *C. trachomatis* serovars). CPAF is produced as a zymogen and as an internal segment and inhibits proteolytic activity (195). Following dimerization and trans-autocatalytic cleavage, active CPAF behaves as a serine protease consisting of a homodimer of catalytic domains, each with two distinct subunits (196). Once in the host cell cytosol and/or released extracellularly, CPAF may act as a virulence factor by degrading eukaryotic proteins that potentially affect chlamydial colonization (197–201).

CPAF has been found to be a dominant antigen in *Chlamydia*-seropositive humans and mice (107, 151). Vaccination of mice with *C. muridarum* CPAF, using IL-12 or CpG as an adjuvant, shortens the duration of infection, decreases oviduct pathology, and elicits cross-serovar protection (202, 203). No protection against infertility was obtained following a primary *C. muridarum* vaginal challenge, but it was observed against a secondary challenge (204). The protection against vaginal shedding elicited by CPAF starts 1 to 2 weeks postchallenge. The delay may be because activated secreted CPAF is detected only after 24 h postinfection. A T-cell epitope from *C. muridarum* CPAF has been found to be protective in HLA-DR4 transgenic mice, suggesting that this antigen may be protective in humans (205). The protection elicited by CPAF is conformation independent, since both the active and inactive forms of the protein elicit the same levels of protection, and protection is mainly dependent on $CD4^+$ T cells, producing IFN-γ, with contribution from $CD8^+$ T cells and antibodies (124, 206, 207).

(v) Plasmid glycoprotein 3. Most *C. trachomatis* isolates have a plasmid that codes for eight proteins, including plasmid glycoprotein 3 (Pgp3) (molecular mass of 28 kDa) (134, 208, 209). Crystallization of this protein from *C. trachomatis* serovar D showed it to be an ~84-kDa homotrimer (210). Pgp3 was found in the chlamydial envelope and also in the cytoplasm of the host cell (211, 212). This protein appears to be the major virulence factor encoded in the chlamydial plasmid. Pgp3-deficient *C. trachomatis* and *C. muridarum* were highly attenuated, and this protein may promote chlamydial colonization by neutralizing host immune effectors (129, 213). However, the precise mechanism by which this protein promotes chlamydial pathogenicity remains unclear. Sera from humans and mice identified the homotrimer but not the monomer as an immunodominant antigen (151, 214). Donati et al. (215) vaccinated C3H/HeN mice with a DNA plasmid expressing *C. trachomatis* serovar D Pgp3 and a control group with the same plasmid containing an irrelevant insert. As determined by the number of positive salpinx cultures, mice vaccinated with the Pgp3 plasmid were partially protected against a genital challenge with *C. trachomatis* serovar D. Vaccination of mice with *C. muridarum* Pgp3, using CpG as an adjuvant, elicited protection against a genital challenge (216). Although the role of the plasmid in chlamydial pathogenesis is still not fully elucidated, the possibility that some of the plasmid gene-encoded proteins may be pathogenic casts doubts about their use as a vaccine. In addition, although relatively rare, plasmidless or with variant plasmids, *C. trachomatis* has been isolated from humans that may not be protected by vaccines using only plasmid antigens such as Pgp3 (134, 208, 217).

Multivalent vaccines. Antigens formulated in a multivalent vaccine may have synergistic, additive, neutral, or antagonistic effects (155, 218, 219). Several *C. muridarum* antigen combinations have been tested in the mouse model. For example, Finco et al. (155) identified chlamydial antigens that elicited humoral and cell-mediated immune responses, and combinations of these proteins were tested for their ability to protect mice against an intranasal *C. muridarum* challenge. Mice immunized with TC0106, TC0210, TC0313, or TC0741, adjuvanted with LTK63 plus CpG, had a 0.5 to 0.9 $\log_{10}$ reduction in the number of IFU recovered from the lungs. Two four-antigen

combinations (one combination was TC0106, TC0210, TC0313, and TC0741, and the other combination was TC0106, TC0431, TC0551, and TC0890) were also tested. The first antigen combination resulted in a 4.1 $\log_{10}$ reduction and the second antigen combination resulted in a 2.2 $\log_{10}$ reduction in the yield of *C. muridarum* from the lungs, indicative of additive effects. Yu et al. (219) also found additive effects in the genital model. Three *C. muridarum* proteins, PmpE/F, PmpG, and MOMP, and a combination of these proteins were formulated with three different adjuvants, CpG, Abisco, and CAF01, and used to immunize mice. The combination of the three proteins, adjuvanted with CAF01, as determined by vaginal shedding, exhibited the greatest degree of protection against a genital challenge. C57BL/6, BALB/c, and C3H/HeN mice immunized with a multisubunit recombinant vaccine that included PmpE, PmpF, PmpG, PmpH, and MOMP, and DDA-MPL as the adjuvant, also conferred better protection against a genital challenge than immunization with single antigens (220). Coler et al. (125) also showed that MOMP combined with CT875 and adjuvanted with AS01B elicited better protection against a vaginal challenge with *C. trachomatis* serovar K (UW-31/Cx) than the individual antigens.

Neutral effects by combining *C. muridarum* antigens have also been reported. Yu et al. (221) vaccinated mice with PmpE, PmpF, PmpG, Aasf, RplF, TC0420, or TC0825, adjuvanted with DDA-MPL, and challenged the animals in the genital tract. PmpG elicited the most robust immune response and the best protection as shown by a decrease in the number of IFU recovered. However, three combinations of antigens (PmpE-PmpF-PmpG-PmpH-Tarp, Aasf-RplF-Rec0, and TC0420-TC0825-TC0285) only protected as well as the best individual protein in the formulation. Similar neutral findings were reported when testing combinations of antigens in the respiratory model (222). Mice were immunized with components of the *C. muridarum* putative ATP synthase complex TC0580, TC0581, TC0582, and TC0584 or with MOMP. In addition, TC0582 was formulated in combination with TC0580, TC0581, or MOMP. Animals immunized with combinations of two of these three antigens were protected only as well as mice vaccinated with MOMP, the most protective protein in the formulation. Also, Li et al. (203) reported that the addition of CPAF to MOMP, or IncA, from *C. trachomatis* serovar D (UW3-Cx) did not enhance the CPAF-induced effect on *C. muridarum* clearance or oviduct pathology. On the basis of these results, when formulating a multivalent subunit chlamydial vaccine, it will be necessary to evaluate the interactions between the antigens before implementation.

Other potential vaccine antigen candidates. In addition to the above-discussed antigens, other chlamydial proteins have been tested for their ability to induce immune responses and/or protection against infection and/or disease. Several excellent reviews have discussed these findings (45–47, 223–228). Some of the antigens that have been found to elicit protection in the mouse model include the outer membrane protein B (OmcB), putative type III secretion effector protein Tarp, macrophage infectivity potentiator (MIP), CopB, CopD, Cap1, and CT584, the inclusion membrane protein (IncA), porin protein B (PorB), the ribonucleoside reductase (NrdB), and glycogen phosphorylase (229–238). The ability of these antigens to induce robust protective immune responses is limited and requires additional testing.

ADJUVANTS

Most of the current vaccines against microbial pathogens are formulated using live attenuated or whole inactivated organisms (239, 240). Since the discovery of Toll-like receptors (TLRs), it has become clear that microbes contain not only antigenic components but also other products that, by activating these eukaryotic cell receptors, have an adjuvant effect and help stimulate robust and well-defined innate and adaptive host immune responses. According to C. A. Janeway, this mechanism explained the efficacy of whole-cell vaccines: "the immunologist's dirty little secret" (241–245). Thus, when the vaccinated host is exposed to a pathogen, rapid and focused immune responses occur.

The implementation of subunit vaccines, like those for hepatitis B virus (HBV) and human papillomavirus (HPV), has required the inclusion of several adjuvants in their

formulation to replicate the robust immunological responses elicited by whole-organism vaccines (239, 240, 246, 247). Because individual adjuvants are only moderately effective at eliciting broad long-lasting protective immune responses and are often targeted to a single type of TLR, there is a need to use several adjuvants for subunit vaccines. In addition, the distribution of TLRs varies among tissues and thus the need to test adjuvant combinations against pathogens, such as *C. trachomatis*, that have multiple portals of entry.

The bacterial components with TLR immune adjuvant activity are called pathogen-associated molecular patterns (PAMPs) (248–254). Examples of PAMPs include membrane components, i.e., lipopolysaccharide (LPS), flagellin, lipoteichoic acid, porins, and intracellular components, such as nucleic acids (241, 243). Following a primary infection, some pathogens elicit solid protective immune responses. For these responses, we can assume that the native antigens and PAMPs present in the organisms are inducing innate and adaptive immune responses that protect the host against reinfection (239, 240, 248–254). However, many bacterial, parasitic, and viral primary infections do not elicit robust protection against a secondary infection. Therefore, against these pathogens, including *Chlamydia*, it may be necessary to formulate vaccines that elicit "unnatural immunity" (255). During a *Chlamydia* infection, TLR- and nucleotide-binding oligomerization domain 1 (NOD1)-mediated activation occurs (256–258). Several chlamydial PAMPs, including LPS, MOMP, macrophage infectivity potentiator (MIP), and heat shock protein 60 (Hsp60), are involved in this process, resulting in signaling mainly through TLR2 and TLR4 (257, 259–262). However, all these chlamydial PAMPs, including LPS, induce weak immune responses, and this may explain the lack of robust protection elicited by a primary infection (262).

Initial chlamydial vaccine trials were performed using alum and oil in water adjuvants, now known to be Th2-biased adjuvants. When it was found that, at least in the mouse model, Th1-biased immune responses were necessary to induce protection, new adjuvants were tested. Several studies have used single adjuvants and adjuvant combinations in vaccine formulations to protect mice against a *C. muridarum* challenge.

To determine the relative efficacy of single adjuvants, the following nine ligands to TLR or NOD receptors were screened for their ability to protect against a *C. muridarum* respiratory infection: Pam_2CSK_4 (TLR2/6), poly(I·C) (TLR3), monophosphoryl lipid A (MPL) (TLR4), *Bacillus subtilis* flagellin (TLR5), imiquimod R837 (TLR7), imidazoquinoline R848 (TLR7/8), CpG-1826 (TLR9), M-Tri-DAP (InvivoGen) (NOD1/2), and muramyl dipeptide (NOD2) (263). Mice were vaccinated with rMOMP, adjuvanted with each individual agonist, and challenged intranasally, and protection was determined based on changes in the weight of the body and lungs and the number of IFU recovered from the lungs. On the basis of these parameters, the most efficacious adjuvants were Pam_2CSK_4 and poly(I·C). CpG-1826 elicited intermediate protection, while monophosphoryl lipid A induced minimal protection (263). Based on the IgG2a/IgG1 ratio in serum, Pam_2CSK_4 and MPL elicited Th2-biased immune responses, while CpG-1826 induced strong Th1 responses and poly(I·C) induced balanced Th1/Th2 responses. The lack of correlation between Th1/Th2 responses, as determined by the IgG2a/IgG1 ratio, and protection suggests that, by itself, this may not be an adequate immunological parameter to predict protection. CPAF formulated with CpG-1826 or recombinant murine IL-12, as single adjuvants, elicited similar Th1 immune responses and limited protection against a genital challenge (124, 203, 264, 265).

Several studies have characterized the use of adjuvant combinations to protect against *C. muridarum* infections. For example, Yu et al. (219) formulated recombinant PmpG-1, PmpE/F-2, and MOMP individually or in combination with three different adjuvants, CpG-1826, AbISco-100 or CAF01. The combination of PmpG-1, PmpE/F-2, and MOMP plus CAF01, as determined by *C. muridarum* shedding, was the most efficacious to protect C57BL/6 and BALB/c mice against a vaginal challenge. The CpG-1826 formulation failed to protect, supporting the need for the use of adjuvant combinations. This group of investigators confirmed those results by testing four combinations of adjuvants, DDA-MPL, CAF01, CAF04, and Montanide ISA 720 VG plus

CpG-1826, in addition to alum. PmpG was used as the test antigen. The most robust immune responses and best protection against vaginal shedding were observed when DDA-MPL or CAF01 was utilized as the adjuvant (221). These two adjuvant combinations elicited the highest frequency of multifunctional CD4 T cells coexpressing IFN-γ and tumor necrosis factor alpha (TNF-α), supporting the role of these cytokines in protection.

Adjuvant combinations have also been tested using MOMP as the antigen. In the genital model, the combination of CpG-1826 plus Montanide ISA 720 VG, a non-Toll receptor adjuvant, has been found to be quite effective at eliciting protective immune responses, using both nMOMP and rMOMP as antigens (169, 176, 266). MF59, a detergent-stabilized oil-in-water emulsion containing squalene and currently in human use, was found to elicit better protection against a genital *C. muridarum* challenge in mice than when single adjuvants, LT-K63 or LT-RT2, derived from *Escherichia coli* enterotoxin, were formulated with nMOMP (267). Cheng et al. (268) compared combinations of CpG-1826 with Pam_2CSK_4 or with Montanide ISA 720 VG. The Pam_2CSK_4 plus CpG-1826 combination was found to elicit the most robust protection against a *C. muridarum* respiratory challenge (268). IC31 is an adjuvant that combines the oligodeoxynucleotide $d(IC)_{13}$ (ODN1a) with the peptide KLKLLLLLKLK (KLK) (269). IC31 generates a depot at the injection site and provides immune stimulation via the TLR9/MyD88 pathway (270–272). This adjuvant combination formulated with nMOMP elicited significant protection against a respiratory challenge with *C. muridarum* (273).

ROUTES OF IMMUNIZATION AND DELIVERY SYSTEMS

The utilization of a mucosal route for immunization appears to be relevant for protection against mucosal pathogens such as *C. trachomatis*. Mucosal tissues comprise the largest source of immunity in the body and are the first line of defense against many pathogens (274–280). The genital tract is considered to be a component of the common mucosal immune system (CMIS). It is assumed that stimulation of the CMIS results in immune responses at remote mucosal effector sites, but stimulation of the various mucosal inductive sites results in uneven distribution of immune responses at the effector sites. Overall, the most effective way to induce immune responses at a specific effector site is to locally administer the vaccine or, perhaps, stimulate sites with related lymph drainages (117, 176, 279, 281–283).

In addition to eliciting a solid immune response at the entry site, deciding what route to use for vaccination requires evaluation of other factors, including safety, ease of use, cost, societal acceptance, and adjuvant availability. Immunization by the intravaginal route seems the most appropriate choice for eliciting protection against genital pathogens. Intravaginal immunization through use of a gel has been successful against cholera, and both intravaginal and rectal immunizations have been utilized against HIV (239, 240, 284–287). The main limitation of this route is patient acceptance. Furthermore, low levels of IgA and IgG in vaginal fluid after vaginal vaccination alone have been reported (279, 281). Oral immunization is readily acceptable (286). A good example is the live-poliovirus vaccine. Yet, for subunit vaccines, the need for a successful delivery system and the large amounts of antigen required for robust immune responses severely limit this approach. The sublingual route may be a more desirable alternative and has been successful in mouse models (176, 283, 288). Colonic immunization may also be a possibility, since it has been shown to induce higher vaginal and serum IgA levels than oral and intramuscular immunizations (176, 289). The ease of use and patient acceptance will have to be improved by utilizing oral delivery systems. The intranasal route induces mucosal IgA and stimulates immunity not only in local tissues but also in the genital tract (117, 290, 291). Unfortunately, intranasal immunization may negatively affect the central nervous system, and this safety issue is why some vaccines have been pulled from the market (287, 292).

Systemic (intramuscular and subcutaneous) and mucosal (sublingual and colonic) routes, alone and in combination, were tested by Ralli-Jain et al. (283) for their ability to protect mice against a *C. muridarum* respiratory challenge. The best protection was achieved when mucosal, followed by systemic, immunizations were utilized. Carmichael

et al. (176) also tested combinations of mucosal (intravaginal, colonic, and intranasal) and systemic (intramuscular and subcutaneous) routes to immunize mice against a vaginal challenge with *C. muridarum*. The strongest *Chlamydia*-specific humoral and cell-mediated immune responses were obtained in the groups immunized by a combination of mucosal and systemic routes. As determined by vaginal shedding and fertility rates, animals vaccinated by a combination of mucosal and systemic routes were also the best protected. Mucosal immunization by itself elicited weak immune responses and minimal protection.

Work by Stary et al. (85) supports enhanced protection using a mucosal route for immunization. These authors vaccinated mice with UV-inactivated *C. trachomatis* serovar D (UW-3/Cx) EB by the intranasal or intrauterine route using nanoparticles coupled to R848. Mucosal immunization elicited Trm that migrated to the uterine mucosa, eliciting protection against a transcervical challenge, while subcutaneous immunization failed to induce Trm and protect.

Several delivery systems, including DNA plasmids, poliovirus, adenovirus, hepatitis B virus, influenza virus, *Vibrio cholerae* ghosts, and nanoparticles, have been used to vaccinate mice with various chlamydial antigens (65, 141, 144, 293–300). Although some of these delivery systems offer interesting opportunities, such as the ability to carry antigens from several pathogens, so far the results obtained do not appear to offer significant advantages over well-established approaches. Furthermore, including antigens that are not medically relevant from the delivery system in a vaccine introduces unknown safety issues and may divert immune responses. The use of edible plants is certainly an interesting approach but needs to be carefully evaluated for its possible negative impact (301). Therefore, more work is needed before any of these delivery systems is implemented.

CROSS-SEROVAR PROTECTION

The low virulence of *C. trachomatis* in mice and the lack of *C. muridarum* serovars present a major challenge for testing cross-serovar protection in the mouse model. However, several investigators have addressed this problem. For example, Tuffrey et al. (302) used a recombinant fragment of *C. trachomatis* serovar L1 MOMP to immunize mice and challenged them by the vaginal or uterine route with serovar F (NI1). In some groups of vaccinated mice, there was a reduction in the duration of colonization and the severity of salpingitis. Li et al. (203) constructed a multivalent vaccine that included three recombinant proteins, CPAF, MOMP, and IncA, from serovar D. Vaccinated mice were challenged vaginally with *C. muridarum*. Immunized mice had less shedding and upper genital tract pathology than the negative-control animals did. Eko et al. (303) immunized mice with *V. cholerae* ghosts expressing PorB and/or PmpD from serovar D and challenged them vaginally with *C. muridarum*. The groups vaccinated with one or both antigens had a significant decrease in vaginal shedding. A different approach was taken by Olivares-Zabaleta et al. (130). Mice were vaccinated intravaginally with the plasmid-free *C. trachomatis* serovar L2 (25667R) and subsequently challenged vaginally with serovar D (UW-3/Cx). Immunized mice were not protected against infection or inflammatory disease but had a reduced infectious burden. By taking advantage of the respiratory model, Tifrea et al. (304) showed that mice immunized with *C. trachomatis* recombinant MOMP from serovars D (UW-3/Cx), E (Bour), and F (IC-Cal-3) can be protected against disease and bacterial burden from a challenge with *C. muridarum*. In conclusion, although we do not have a good mouse model, these results suggest that by using a multivalent vaccine, it may be possible to elicit solid cross-serovar protective responses.

CAN THE IMMUNOLOGICAL AND PROTECTION FINDINGS IN MICE BE DIRECTLY TRANSLATED TO HUMANS?

In evolutionary terms, differences in innate and adaptive immune responses between humans and mice have developed over the last 100 million years when primates and rodents were separating (305–307). Living in contrasting habitats and being exposed to different microorganisms have resulted in significant divergence of their

immune systems (308–313). In the last 100 years, the establishment of inbred strains of mice has added another layer of immunological differences (314). As a result, we cannot be certain that the findings obtained in the mouse model will be directly applicable to humans. Even more, results in one strain of mice may not directly extrapolate to another strain. Here, we will briefly discuss some differences between the innate and adaptive immune systems of humans and mice that may impact the development and implementation of a *C. trachomatis* vaccine.

One of the main defense mechanisms against pathogens in humans and mice is the presence of pattern recognition receptors (PRR), such as the TLRs (10 present in humans and 13 in mice) that activate the innate immune system by binding molecular patterns of microorganisms, such as LPS (312, 313, 315, 316). In humans, binding of ligands to TLRs causes significant inflammatory responses that help protect against pathogens by limiting their burden. In contrast, mice respond very weakly to TLR agonists. For example, in humans, a dose of ~15 μg of LPS/kg of body weight causes severe diseases, including shock, while in mice, the 50% lethal dose (LD_{50}) is ~10 mg/kg (309, 317). Furthermore, significant differences are also found between different strains of inbred mice. For example, agonists of TLR3 were the most effective adjuvants to elicit IFN-γ-producing NK, CD4, and CD8 T cells in C57BL/6 mice, while in BALB/c mice, TLR7/8 was found to be the most effective (318, 319).

IFN-γ is released in humans and mice during inflammation to trigger innate immune responses. In mice, IFN-γ induces the expression of the p47 immunity-related GTPase (IRG) proteins that include proteins encoded by 18 genes, and IRG knockout mice are highly susceptible to pathogens (320). Humans express only two IRG proteins, and neither of the two proteins is induced by IFN-γ. In mice, IFN-γ induces three different IRG genes against *C. trachomatis*, which leads to rerouting of the inclusions to autophagosomes (321). In contrast, *C. muridarum* has a large toxin with homology to Yop7 that blocks the interaction of IRG proteins with the inclusion membrane (112). In addition, in human epithelial cells, IFN-γ elicits expression of indoleamine 2,3-dioxigenase, which, by depleting the host tryptophan pools, inhibits the growth of *C. muridarum*. However, *C. trachomatis* can grow by producing a tryptophan synthase that may use indole generated by microbes in the human genital tract to produce tryptophan (88, 322).

The leukocyte compositions of humans and mice are also significantly different. Humans have 50 to 70% neutrophils and 50 to 30% lymphocytes, while mice have only 10 to 30% neutrophils and 90 to 70% lymphocytes (312, 313). In addition to differences in the proportions, there are functional differences between human and mouse neutrophils. For example, human neutrophils express many defensins, while mouse neutrophils express only a few (323–325). Similarly, myeloperoxidase levels in mouse neutrophils are 80 to 90% lower than in human neutrophils (326). Significant differences also exist between human and mouse eosinophils, granulocytes, monocytes/macrophages, NK cells, and mast cells (313).

Fc receptor (FcR) represents a link between innate and adaptive immune responses. FcαR1 (CD89) is an important IgA receptor in humans and is expressed in neutrophils, eosinophils, monocytes/macrophages, and dendritic and Kupffer cells, while mice lack FcαR1 and use alternative receptors (310).

There are also clear differences in the adaptive immune responses between humans and mice (310). For example, IL-7 receptor (IL-7R) deficiency in mice blocks development of both B and T cells (327). In humans, however, IL-7R deficiency blocks only the development of T cells, suggesting that B-cell development is not dependent on IL-7 (328). Also, mice, but not humans, exclusively express CD5 and CD23 in B cells, while CD38 is expressed in human, but not mouse, plasma cells (329). Mice produce IgA, IgD, IgE, IgG (IgG1, IgG2a, IgG2b, and IgG3) and IgM, while humans express IgA (IgA1 and IgA2), IgD, IgE, and IgG (IgG1, IgG2, IgG3 and IgG4). Interestingly, C57BL/6, C57BL/10, SJL, and NOD mice produce IgG2c instead of IgG2a (330). In mice, IL-4 induces IgG1 and IgE, while in humans, it elicits IgG4 and IgE.

An important component of adaptive immunity is the differentiation of T cells

toward the Th1 or Th2 phenotype. Mosmann and Coffman (331) first demonstrated in mice the polarization of T cells. While Th1 and Th2 cells can be found in human diseases, the polarization is not so clear. For example, in mice, IL-10 is a Th2 cytokine, while in humans, both Th1 and Th2 cells can make IL-10 (332). Also, in humans, IFN-α is produced by several types of cells in response to viral infections inducing Th1 development by activation of STAT4. In contrast, in mice, IFN-α does not induce Th1 cells and does not activate STAT4 (333).

From this brief analysis, we can conclude that there are many differences between the immune systems of humans and mice and even between various strains of inbred mice. From the vaccinology perspective, it is likely that the antigens that are effective in mice will also be effective in humans. In particular, MOMP, already indirectly identified in the original trachoma vaccine trials as a dominant protective antigen, will likely be protective in humans. Other chlamydial antigens, such as Pmps and CAF, known to elicit humoral and cell-mediated immune responses in humans and protection in mice will also likely be protective in humans. However, an adjuvant(s) found to be the most efficacious in mice might not be the most effective in humans. To broaden the chances of success, testing adjuvants in inbred and outbred mice is recommended. Routes and delivery systems for vaccines are fairly restricted in humans. Utilizing one of the most common routes currently used for delivery of human vaccines, the intramuscular or subcutaneous route, will greatly facilitate implementation. Combining mucosal and systemic routes will likely improve *Chlamydia* vaccine efficacy, but it may pose significant safety and practical challenges.

In summary, as Gibbons and Spencer (311) concluded after reviewing human and mouse intestinal immunity: "Although the value of animal models should not be underestimated, close attention should be given to the detail; the functional outcome may seem the same but the mechanism might be different." Recent genomic studies support this conclusion (308, 309).

PROSPECTS FOR A HUMAN *C. TRACHOMATIS* VACCINE

Although great progress has been made over the last 4 decades, the prospects of implementing a *Chlamydia* vaccine against genital infections in the near future are still under discussion. *C. trachomatis* causes significant morbidity but no mortality. Therefore, the first priority for the implementation of a *C. trachomatis* vaccine is safety. Based on the findings observed during the vaccine trachoma trials, the development of a delayed-type hypersensitivity reaction and increase in susceptibility to infection, or any other negative effect, has to be avoided. The use of a live attenuated or inactivated whole-organism vaccine may be justifiable to protect against trachoma, but it is highly unlikely that it will be used against genital infections. In addition to safety issues, the costs of producing such a vaccine will be difficult to justify. Therefore, a subunit recombinant vaccine will probably be deployed.

A therapeutic *C. trachomatis* vaccine may have some efficacy if it is delivered before irreversible damage occurs in the female genital tract. Yet, a therapeutic vaccine, although possible, is highly unlikely to be implemented, since at least with our current methodologies, detection of early onset of upper genital tract pathology is a major challenge (334). In the case of trachoma, it may be worthwhile to explore the effects of a therapeutic vaccine, particularly since there is already evidence that it can be effective in humans (335–337).

It is likely that most of our current vaccines do not induce sterilizing immunity (239, 240). To block chlamydial infection, a vaccine will have to elicit high levels of mucosal/systemic protective antibodies that are maintained throughout the life of the individual, or at least their reproductive life, a goal not easily achievable. Existing vaccines control the pathogen at the site of entry and therefore minimize disease, including long-term sequelae. Additionally, they decrease shedding and therefore transmission. At the population level, this is a critical effect, since the number of newly infected individuals can rapidly decline. Using a computer model, it was determined that in the case of a *C. trachomatis* vaccine, over a period of at least 10 years, even when its efficacy was only

50%, the decline in new infections will be dramatic (338). This conclusion was supported by other studies that also determined that vaccination was cost-effective (339, 340). Vaccinating both males and females will further expedite the control of this pathogen (341).

Based on the experimental work done with single-antigen vaccines, nMOMP induces the most robust protection against shedding and infertility. Producing enough nMOMP, by direct extraction from *Chlamydia*, will be extremely expensive. In addition, due to the serovar/serogroup specificity of the protection, a vaccine may need to be formulated with MOMPs from two to four serovars (45, 137, 342). As shown by Olsen et al. (181), a synthetic MOMP vaccine that includes specific epitopes from various serovars is a potentially attractive alternative. Another possibility is to formulate a multivalent vaccine with rMOMP and other well-conserved recombinant antigens, such as CPAF and/or the Pmps. Advantages of CPAF include cross-serovar protection and a lack of dependence on its native conformation for protection, which greatly facilitate production and formulation. Shortcomings include limited protection against vaginal shedding and infertility. The Pmps are large proteins (>100 kDa), and the recombinant constructs tested so far have used only N-terminal fragments, usually less than 50% of the entire protein. The N-terminal fragments of Pmps are thought to represent the surface-exposed domains. Full-size Pmps, particularly if they can be refolded into their native conformation, will likely induce robust cross-serovar protective immune responses, since they are well conserved among all the human serovars. However, phase variation expression could limit the protective ability of Pmps, especially for PmpG (187, 189). Vaccines based on Pmps will therefore require the addition of other antigens. Also, there is no evidence yet that Pmps can protect mice against infertility.

In addition to the antigens, evaluation of adjuvants, routes, and delivery systems for immunization that are not only effective but also safe and convenient is going to be critical before a *C. trachomatis* vaccine is implemented. On the basis of the findings we have in the mouse model, the adjuvants will have to elicit robust cell-mediated and humoral immune responses. The number of adjuvants currently licensed for human use is quite limited. The recent positive experience with the implementation of a highly efficacious systemic subunit vaccine to protect against HPV-induced cervical cancer is highly encouraging. HPV infects the transitional zone of the human cervix, the same area of the epithelium *C. trachomatis* infects. This observation implies that it may also be possible to formulate an efficacious *Chlamydia* vaccine that is delivered by a systemic route (intramuscular or subcutaneous) and not a mucosal route, using the appropriate adjuvants. However, in the case of HPV, humoral immune responses appear to play the major role in protection, while for *C. trachomatis*, cell-mediated and humoral immune responses will likely be required to elicit optimal protection (59, 246, 343, 344). Therefore, the adjuvants and routes of immunization for *C. trachomatis* may differ significantly from those needed for HPV.

Once a vaccine that elicits robust protection in animal models has been found, a set of criteria will need to be defined to measure protection during the human clinical trials. Having a single parameter to assess protection may not be possible. To determine whether or not protection against tubal factor infertility is achieved by vaccination is not a realistic criterion, since it will require many years and a very large number of subjects to ascertain that effect. Assessment of PID episodes in the vaccinated and control populations will likely be the most robust endpoint to evaluate the efficacy of the vaccine. As shown by Westrom et al. (30), single, not multiple, severe PID episodes are the leading cause of infertility in women.

There are other parameters that can assist in evaluating the efficacy of the vaccine. In the mouse model, there is a good correlation between vaginal shedding and development of upper genital pathology. The number of animals with positive cultures and the length and severity of vaginal shedding are parameters that could be used to evaluate the efficacy of the vaccine. With these parameters, even if we do not have specific data to determine protection against upper genital pathology in a particular

individual, we could estimate the impact on transmission rates and, therefore, control of the infection in the population (338, 340).

During the vaccination trials, noninvasive radiological techniques, such as magnetic resonance imaging (MRI), could also help to assess the development of upper genital pathology (345–347). However, in addition to the high cost, the lack of sensitivity and specificity may limit the usefulness of our current methods. Serological testing of individuals before and after vaccination, using whole-genome *Chlamydia* protein microarrays to test serum and vaginal samples, could provide supportive information (148, 151, 154, 156). Measurement of inflammatory cytokines and chemokines in cervical vaginal secretions may also assist assess vaccine efficacy (348–350). Importantly, careful short-term and long-term follow-up will be required to make sure no negative effects occur at the time of vaccination and later on when the individual is exposed to *Chlamydia*.

CONCLUDING REMARKS

Work over the last 4 decades has identified vaccine components, including antigens, adjuvants, and immunization routes, that can induce significant protection against a *C. muridarum* genital challenge in the mouse model. In our opinion, to expedite the implementation of *C. trachomatis* vaccines, the next step should be to test in parallel the most promising formulations reported by various investigators. The two or three most efficacious vaccine formulations could then be evaluated in various inbred and outbred mice and other animal models, such as the guinea pig, and, if necessary, in nonhuman primates. A cooperative international effort between vaccinologists, government agencies, and private companies could result in the implementation of a *C. trachomatis* vaccine in the next decade. Currently, a MOMP-based vaccine is in phase I clinical trials (26).

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