

A live-attenuated chlamydial vaccine protects against trachoma in nonhuman primates

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Blinding trachoma is an ancient neglected tropical disease caused by *Chlamydia trachomatis* for which a vaccine is needed. We describe a live-attenuated vaccine that is safe and efficacious in preventing trachoma in nonhuman primates, a model with excellent predictive value for humans. *Cynomolgus* macaques infected ocularly with a trachoma strain deficient for the 7.5-kb conserved plasmid presented with short-lived infections that resolved spontaneously without ocular pathology. Multiple infections with the attenuated plasmid-deficient strain produced no inflammatory ocular pathology but induced an anti-chlamydial immune response. Macaques vaccinated with the attenuated strain were either solidly or partially protected after challenge with virulent plasmid-bearing organisms. Partially protected macaques shed markedly less infectious organisms than controls. Immune correlates of protective immunity were not identified, but we did detect a correlation between MHC class II alleles and solid versus partial protection. Epidemiological models of trachoma control indicate that a vaccine with this degree of efficacy would significantly reduce the prevalence of infection and rates of reinfection, known risk factors which drive blinding disease.

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Abbreviations used: EB, elementary body; IFU, inclusion forming unit.

Trachoma, caused by the obligate intracellular bacterium *Chlamydia trachomatis*, is the world's leading cause of infectious blindness. It afflicts millions of people in sub-Saharan Africa and Asia (Schachter, 1978). Trachoma was first described in early Egyptian writings (Taylor, 2008) and remains one of the world's most neglected infectious diseases to this day (Vogel, 2006). Control of trachoma is focused on the implementation of the World Health Organization SAFE strategy that is composed of surgery for trichiasis, antibiotics for infection, facial cleanliness, and environmental improvements (World Health Organization, 1998). Despite its benefits, the SAFE strategy is difficult to implement and sustain effectively, particularly in the most impoverished trachoma endemic communities (Lakew et al., 2009; West et al., 2011). Thus, these socioeconomic environments require sustainable alternative measures to have a lasting impact on trachoma control. Vaccination would be an effective way to accomplish this goal.

Trachoma vaccine development is viewed with considerable skepticism, primarily because

the damaging scarring and blinding complications of the disease are associated with repeated or persistent infections that trigger dominance in pathological rather than protective immunity (Schachter, 1985). In contrast, epidemiological studies have shown the duration of untreated infection becomes shorter with increasing age. This argues that naturally acquired protective immunity eventually develops (Bailey et al., 1999), supporting the idea that an effective trachoma vaccine is achievable. The lack of an experimental trachoma small animal model, the mucosal tropism and biological complexity of chlamydial host interactions, and the inability to genetically manipulate the organism have also been unfavorable to trachoma vaccine development. Moreover, chlamydiae are antigenically complex organisms producing numerous structural and secreted antigenic targets that elicit distinct immune effector mechanisms.

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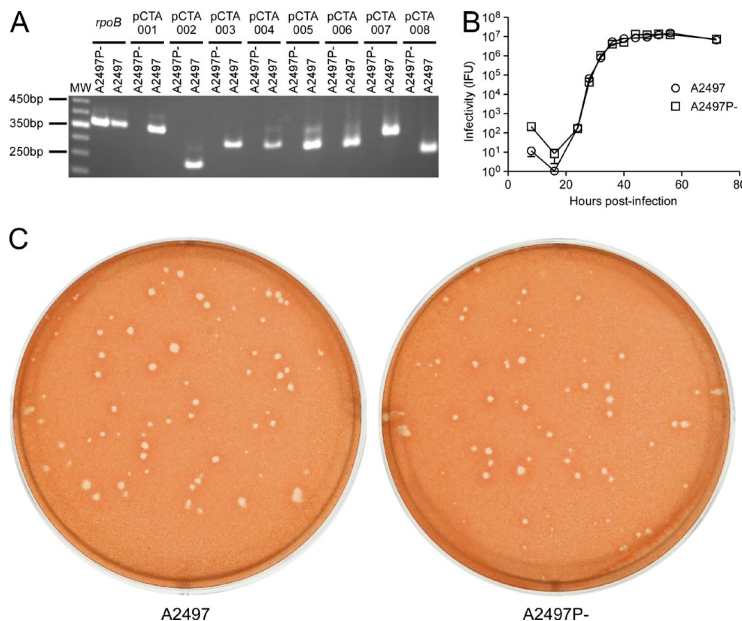


Figure 1. In vitro characterization of the *C. trachomatis* plasmid-deficient trachoma strain A2497P⁻. (A) The presence of eight plasmid open reading frames (pCTA001-008) in A2497 and A2497P⁻ was measured by PCR. The RNA polymerase β subunit (*rpB*) was used as a chromosomal-positive control. (B and C) A2497 and A2497P⁻ were cultured in McCoy cells. (B) In vitro growth. $n = 3$. Error bars indicate standard deviation and results are shown for one of two independent experiments. (C) Plaque size. $n = 2$. Results are shown for one of three independent experiments.

An efficacious vaccine will likely need to satisfy and target these unique infection characteristics without stimulating pathological immunity. Theoretically, this might be best achieved by a live-attenuated vaccine delivered onto the conjunctival surface

that produces protective but not pathological immunity (Brunham et al., 2000; Su et al., 2000; Morrison and Caldwell, 2002). *C. trachomatis* organisms possess a conserved 7.5-kb plasmid of unknown function (Palmer and Falkow, 1986). Naturally occurring (Carlson et al., 2008) or antibiotic-cured (O'Connell et al., 2007) nontrachoma plasmid-deficient strains are attenuated in mouse models of genital tract infection, implicating the plasmid as a key chlamydial virulence factor. We hypothesized that a plasmid-deficient trachoma strain might be similarly attenuated and serve as a novel candidate for a live-attenuated trachoma vaccine. In our study, we test this hypothesis and demonstrate that a plasmid-deficient trachoma strain is highly attenuated for nonhuman primates but induces an immunity that protects against trachoma.

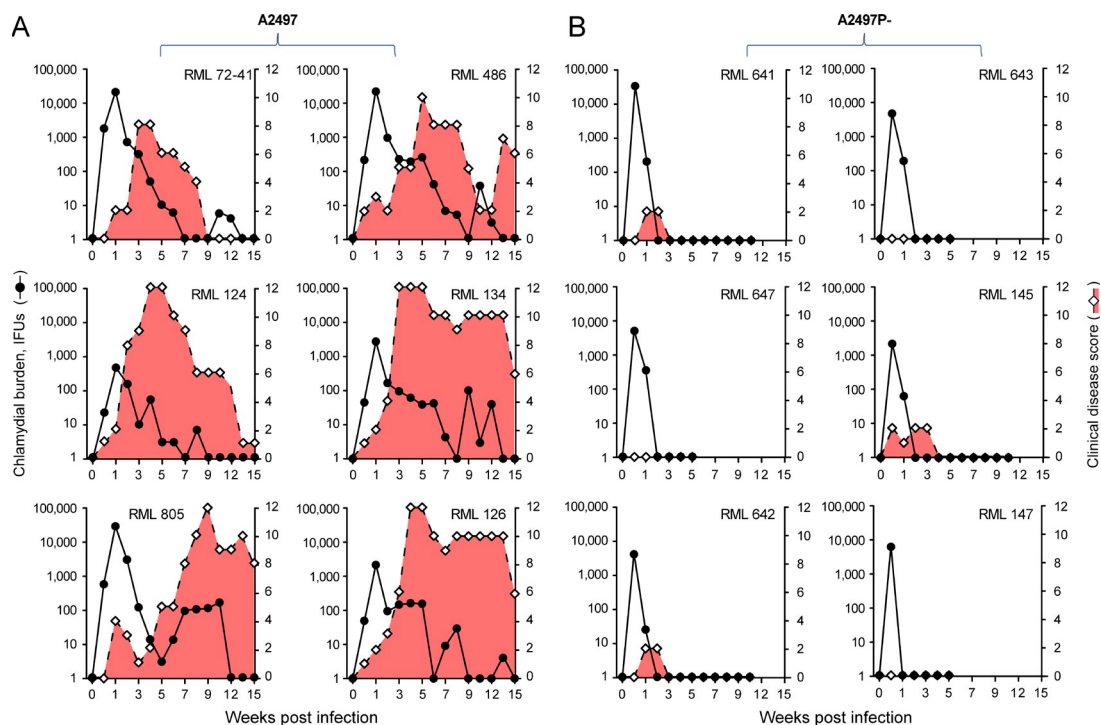


Figure 2. The *C. trachomatis* plasmid-cured trachoma strain A2497P⁻ is highly attenuated for the monkey eye. (A) Six monkeys were infected with A2497 (individual data points are plotted). These monkeys are six historic controls from two recent nonhuman primate studies (Kari et al., 2008, 2009; the information in Fig. 2 A is reprinted with permission from the *Journal of Immunology* and the *Journal of Infectious Diseases*). (B) Six monkeys were infected with A2497P⁻ (individual data points are plotted). RML 145 received 2×10^4 IFU/eye, RML 641 received 2×10^5 IFU/eye, and RML 642, 643, 647, and 147 received 2×10^6 IFU/eye. Day 0 indicates the time of infection of each animal. Chlamydial burden is the geometric mean of recoverable IFU for both eyes of individual monkeys monitored by culture from conjunctival swabs. Clinical disease score is the aggregate clinical score (hyperemia and follicle formation) for both eyes of individual monkeys. The experiment was done once.

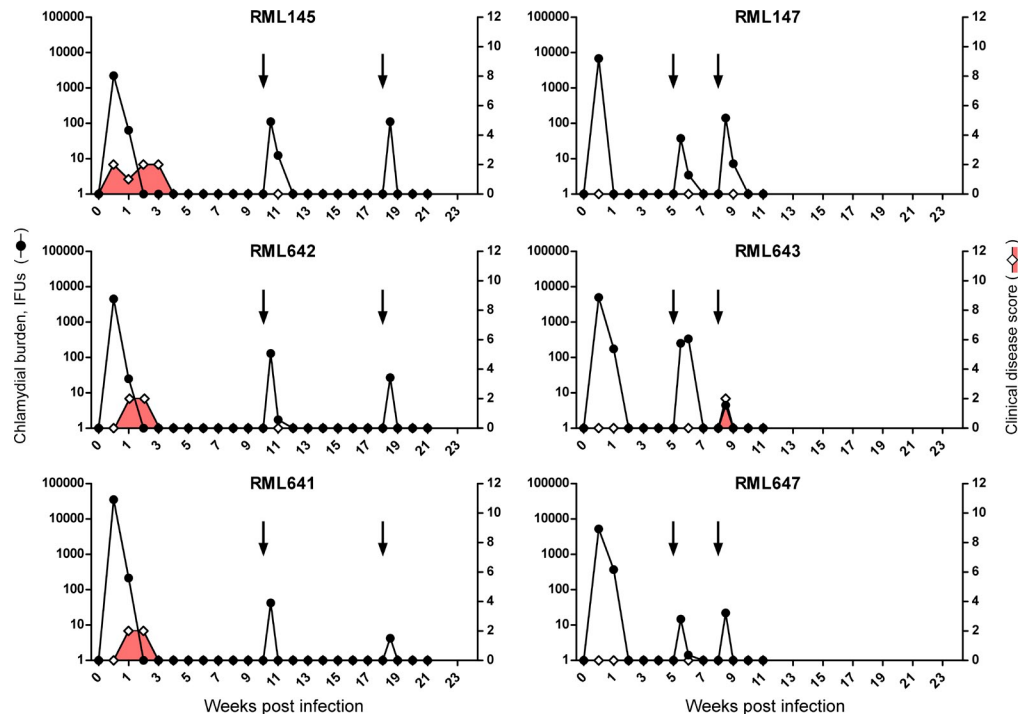


Figure 3. Repeated infections of nonhuman primates with the plasmid-deficient A2497P⁻ strain do not cause ocular pathology. RML 145, 641, and 642 were reinfected at weeks 10 and 18. RML 643, 647, and 147 were reinfected at weeks 5 and 8 (individual data points are plotted). Day 0 indicates the time of the first infection of each animal. Arrows indicate the time of the reinfections. Chlamydial burden is the geometric mean of recoverable IFU for both eyes of individual monkeys monitored by culture from conjunctival swabs. Clinical disease score is the aggregate clinical score (hyperemia and follicle formation) for both eyes of individual monkeys. The experiment was done once.

RESULTS AND DISCUSSION

The plasmid-deficient trachoma strain is attenuated in nonhuman primates

A plasmid-deficient strain was made from a virulent *C. trachomatis* serovar, a Tanzanian trachoma clinical isolate (A2497; Kari et al., 2008), by novobiocin treatment (O'Connell and Nicks, 2006). This plasmid-deficient strain was designated A2497P⁻. The absence of the plasmid in A2497P⁻ was verified by PCR analysis of all eight plasmid open reading frames (Fig. 1 A). De novo genomic sequencing of A2497 and A2497P⁻ revealed no mutations between the two chromosomes and confirmed the absence of the plasmid in A2497P⁻; thus, the two trachoma strains were isogenic with the exception of the 7.5-kb plasmid. The strains did not differ in their in vitro growth characteristics as shown by plaque size, plaque forming kinetics, and one-step growth curves in cultured eukaryotic cells (Fig. 1, B and C).

We next investigated the infection characteristics of the A2497P⁻ strain in a cynomolgus macaque (*Macaca fascicularis*) trachoma model (Taylor et al., 1981; Kari et al., 2008). The conjunctivae of six macaques were inoculated with the A2497P⁻ strain, and after infection chlamydial shedding and ocular pathology they were compared with six historic controls similarly infected with the virulent A2497 strain (Kari et al., 2008, 2009). Animals were infected in two groups. Group 1 (RML 145, 641, and 642) was given primary infections using three different doses of A2497P⁻: 2×10^4 inclusion forming units (IFUs)/eye, 2×10^5 IFU/eye, and 2×10^6 IFU/eye, respectively. Animals in group 2

(RML 643, 647, and 147) all received the highest dose, 2×10^6 IFU/eye (Fig. 2). Infection was assessed by culturing chlamydiae from conjunctival swabs, and ocular clinical disease was monitored by scoring the upper conjunctival lids for subepithelial follicles and hyperemia (Taylor et al., 1981; Kari et al., 2008). Despite no observable differences in the A2497 and A2497P⁻ strains' in vitro infection characteristics (Fig. 1), the strains exhibited a marked difference in pathogenicity for the monkey eye. The A2497P⁻ ocular infections were highly attenuated compared with infections produced by the plasmid-bearing A2497 parental strain (Fig. 2). A2497-infected monkeys were culture positive for an 8–13-wk period after infection before clearance and ocular disease was moderate to severe throughout the culture positive period. In contrast, all monkeys challenged with the A2497P⁻ strain became infected, but the infections were rapidly cleared and spontaneously resolved by day 14 after infection. Most notably, A2497P⁻ infections did not produce measurable ocular clinical pathology. A minimal ocular disease score was observed in several animals but was limited to a mild transient conjunctival hyperemia without follicle formation. The A2497P⁻ monkeys were infected two additional times using the highest dose. Group 1 animals were reinfected at 8–10-wk intervals and group 2 animals at 3–4-wk intervals (Fig. 3). Remarkably, repeated A2497P⁻ infections also failed to evoke ocular pathology (Fig. 3). These findings were unexpected and in complete contrast to previous results showing that repeated infections with virulent plasmid-bearing trachoma

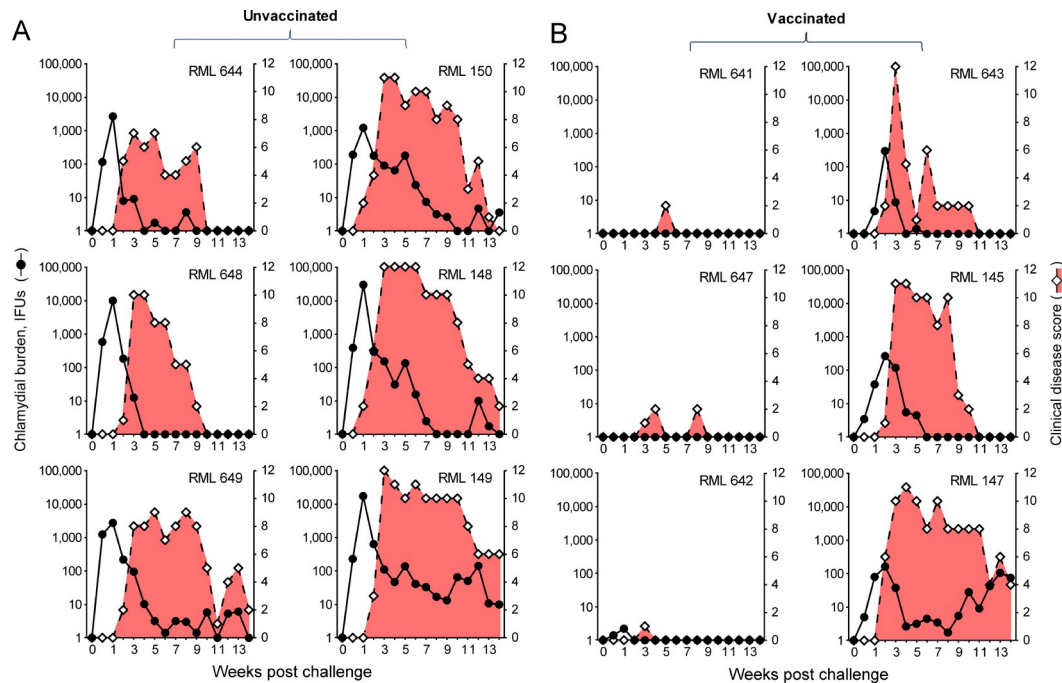


Figure 4. Monkeys vaccinated with the attenuated A2497P⁻ strain are protected against a challenge infection with virulent A2497 organisms. Vaccinated animals and naive unvaccinated controls were challenged 4 wk after the last vaccination. (A) Six unvaccinated control monkeys (individual data points are plotted). (B) Six A2497P⁻ vaccinated monkeys (individual data points are plotted). Solidly protected monkeys were (RML 641, 642, and 647) culture negative and developed no ocular pathology after challenge. The three partially protected monkeys (RML 145, 147, and 643) had reduced chlamydial infection burdens; however, this reduction did not significantly alter the intensity of ocular inflammation. Chlamydial burden is the geometric mean of recoverable IFU for both eyes of individual monkeys monitored by culture from conjunctival swabs. Clinical disease score is the aggregate clinical score (hyperemia and follicle formation) for both eyes of individual monkeys. The experiment was done once.

organisms significantly augmented ocular inflammation and disease in macaques (Taylor et al., 1982). Interestingly, plasmid deficiency did not appear to alter the ability of the organism to infect and replicate within conjunctival epithelial cells, but clearly the presence of the plasmid strongly influenced the organism's ability to persist in ocular tissues once infection had been established. These findings argue that a pathogenic function of the plasmid is to sustain persistent *in vivo* infection in its natural host. The molecular bases underlying this pathogenic strategy are unclear but indicate that the plasmid has coevolved with the organism to promote persistence, perhaps by avoiding immune mediated effectors that are critical to eradication of infection. The infectious yet highly weakened pathogenic phenotype of the A2497P⁻ strain suggested its potential utility as a live-attenuated trachoma vaccine.

Protection against virulent challenge in monkeys immunized with the plasmid-deficient trachoma strain

We challenged the six monkeys that were given multiple A2497P⁻ ocular infections with virulent A2497 trachoma organisms (Fig. 4). Six naive monkeys were similarly challenged and served as unvaccinated controls. Unvaccinated monkeys were uniformly infected and shed chlamydial organisms over a 6–14-wk period after challenge. Infections of naive monkeys resulted in moderate to maximum ocular pathology scores at 3–4 wk after challenge that persisted for 2–4 mo.

In contrast, three of the A2497P⁻ vaccinated monkeys (RML 641, 647, and 642) exhibited complete protection against the virulent challenge. These animals did not become infected and exhibited no ocular pathology after challenge. The other three vaccinated monkeys (RML 643, 145, and 147) were infected after challenge but the infectious burdens were markedly less than unvaccinated controls. Differences in infectious burden were most pronounced during the first 2 wk after challenge, and with the exception of a single animal (RML 147), infections were cleared more rapidly than in control monkeys. Interestingly, despite these lower burdens, we observed no to marginal differences in ocular pathology between partially protected and unvaccinated controls. In no case was a vaccinated animal that shed chlamydiae protected against disease. Thus, even low levels of bacterial replication appear to be associated with pathology, suggesting that preventing disease requires complete or near complete inhibition of bacterial growth. These findings are consistent with earlier observations on human vaccine trials using trachoma organisms (Collier, 1966), where vaccinated humans exhibited a reduction in chlamydial shedding without an associated decrease in ocular pathology. Table I shows a comparison of the aggregate infectious load for protected, partially protected, and unvaccinated controls over the entire culture positive period. Partially protected monkeys shed 95% fewer infectious organisms than unvaccinated controls (partially protected monkeys: IFU range = 1,207–1,890 and

Table I. Weekly and total infectious burdens in A2497P[−] vaccinated and unvaccinated nonhuman primates

Group/monkey number	Weeks of infectious burden														Total infectious burden
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
	IFU	IFU	IFU	IFU	IFU	IFU	IFU	IFU	IFU	IFU	IFU	IFU	IFU	IFU	IFU
Vaccinated															
145	222	554	1,082	13	20	0	0	0	0	0	0	0	0	0	1,891
147	324	376	128	7	7	20	12	3	17	67	19	150	252	166	1,548
641	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
642	7	0	0	0	0	0	0	0	0	0	0	0	0	0	7
643	23	1,153	29	0	2	0	0	0	0	0	0	0	0	0	1,207
647	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unvaccinated															
148	86,244	582	318	125	280	31	5	0	0	0	0	97	3	0	87,685
149	37,186	2,062	226	100	426	117	69	43	61	145	321	290	113	97	41,256
150	2,911	538	352	209	596	53	15	7	7	0	0	22	0	13	4,723
644	7,874	24	18	0	3	0	0	13	0	0	0	0	0	0	7,932
648	23,367	401	25	0	0	0	0	0	0	0	0	0	0	0	23,793
649	10,885	582	466	26	7	0	10	6	2	23	0	28	37	0	12,072

The IFU was recovered from conjunctival swabs at weekly intervals after challenge. The total IFU was recovered for all culture time points. The difference between vaccinated and unvaccinated groups in total infectious burden is highly significant ($P = 0.0026$; Wilcoxon rank-sum test).

mean = 1,549; unvaccinated monkeys: IFU range = 4,723–87,685 and mean = 29,577). The different immunization regimes had no discernible effect on protection.

MHC class II alleles differentiate between solidly and partially protected macaques

Immune correlates of solidly protected versus partially protected macaques were analyzed by testing sera and tears for anti-chlamydial antibodies and PBMC for T cell-specific anti-chlamydial cytokine production. We were not successful in detecting the production of chlamydial specific cytokines by PBMC, possibly because of the low numbers of circulating chlamydial-specific T cells generated after mucosal infection. There was no correlation between anti-chlamydial antibodies measured by ELISA, or serum-neutralizing antibodies and protective immunity (Fig. 5, A–C). However, both ELISA antibody and in vitro neutralization assays use intact elementary bodies (EBs) as antigen and are therefore restricted by their ability to detect a very limited number of surface antigens. We therefore performed a more comprehensive antigen screen using a *C. trachomatis* whole genome proteome array analysis (Wang et al., 2010). We observed no differences in antigen recognition patterns against any of the 908 *C. trachomatis* proteins by proteome array analysis with the sera of solidly and partially protected macaques (Table S1). However, MHC genotyping of monkeys showed that all three solidly protected animals shared the same M1 haplotype in one of their MHC class II alleles. In contrast, none of the partially protected monkeys had the M1 haplotype (Fig. 5 D and Table S2). The small number of animals, and the fact that the experiment was not designed to answer this question, precluded us from determining if this association was statistically significant.

Collectively, these findings support a non-antibody-mediated protection and suggest that either a difference in CD4 T cell antigen recognition or T cell effector function might be the explanation of enhanced vaccine-mediated immunity.

Our findings are consistent with, yet distinct from, those recently reported by O’Connell et al. (2007) using a plasmid-cured *C. muridarum* mouse pathogen in a mouse female

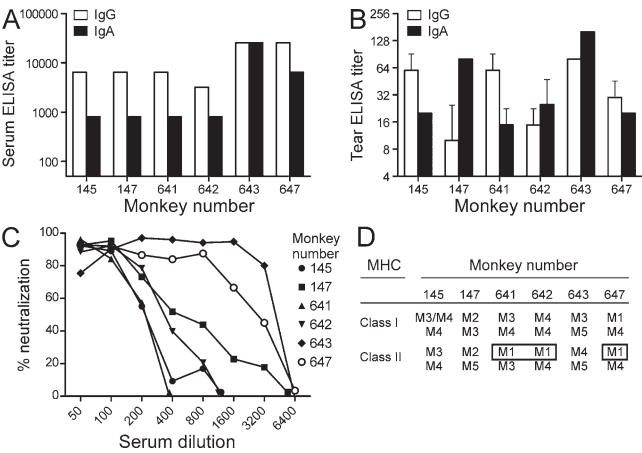


Figure 5. Immune and genetic correlates of vaccine-mediated protective immunity. (A and B) Sera (A) and tear (B) IgG and IgA antibody titer-specific A2497 organisms were measured by ELISA ($n = 2$). Error bars show the standard deviation of the tear antibody titers measured in the left and the right eyes. (C) Serum-neutralizing antibody titers against A2497 organisms. Percentage of specific neutralization for each dilution was calculated as [(preimmune IFUs – immune IFUs)/preimmune IFUs] $\times$ 100. (D) MHC class I and II alleles of immunized monkeys. In A–C, results are shown for one of two independent experiments.

genital tract infection model. These investigators described no difference in infection characteristics between the plasmid-bearing and -deficient mouse strain in the lower and upper (oviduct) genital tract of mice but, curiously, found that infection of oviduct tissue with the plasmid-negative strain failed to induce inflammatory pathology. The molecular basis for this difference in infection-independent pathological outcome in mouse genital tissues was associated with the failure of the plasmid negative strain to stimulate TLR2 dependent cytokine production, a finding implicating differential TLR pattern recognition expression between cervico-vaginal and oviduct tissues. Lastly, the *C. muridarum* plasmid-deficient strain did not afford protection against infection after challenge with virulent plasmid-bearing organisms but did protect against upper genital tract pathology. In our study, we similarly show that the human *C. trachomatis* serovar A plasmid-deficient and plasmid-bearing strains have similar infection potency for conjunctival epithelial cells of nonhuman primates and that infection with plasmid-deficient organisms fails to produce measurable ocular inflammatory pathology (Fig. 2). Of note, however, as it is important to vaccine development, we show that infection with the plasmid-deficient human strain provides significant protection against ocular infection (Fig. 4). We also describe an additional hereto unrecognized pathogenic property of the chlamydial plasmid: the propensity of plasmid-bearing organisms to cause persistent infection that results in a sustained ocular inflammatory response (Fig. 2), a property which might be unique to human strains and infection. Nevertheless, the studies conducted in both mice and nonhuman primate models support an important role of the plasmid in chlamydial pathogenesis and the use of plasmid-deficient strains as live-attenuated vaccines.

Concluding remarks

We have shown the plasmid-deficient trachoma strain is highly attenuated for the monkey eye, demonstrating that the plasmid is a key virulence factor important to the pathogenesis of infection. As the nonhuman primate model has an excellent predictive value for human infection and disease, these findings strongly support a similar pathogenic role for the plasmid in human infections. We have also shown that vaccination with the attenuated strain generated an immune response that protected macaques against challenge with virulent trachoma organisms. Extrapolation of these findings to endemic trachoma regions suggests that a live-attenuated vaccine with this degree of efficacy would significantly reduce trachoma infection rates and transmission frequency, which would interrupt the cycles of reinfection, the primary epidemiological risk factor which drives scarring inflammatory disease and blindness. As the protective effect of vaccination would be sustainable in the population, the live-attenuated trachoma vaccine described herein could ultimately help control this ancient and scouring blinding disease. Our findings may also be beneficial in the design and development of a live-attenuated vaccine for the prevention of chlamydial sexually transmitted infections, which are a leading cause of bacterial sexually transmitted diseases and a risk factor for HIV transmission.

MATERIALS AND METHODS

Isolation of the plasmid-deficient A2497P⁻ strain. A2497-infected McCoy cells (MOI 1) were treated with 7 µg/ml novobiocin 4 h after infection and harvested 42 h after infection with surviving organism plaque cloned (Matsumoto et al., 1998). 200 individual plaques were picked and screened by PCR for plasmid. Plasmid negative clones were expanded and rescreened. A plasmid-negative clone, termed A2497P⁻, was selected for experimentation. A2497P⁻ was propagated in McCoy cells, and EBs were purified by density gradient centrifugation (Caldwell et al., 1981). Genomic DNA was isolated from purified EBs of A2497P⁻ and the parent A2497 strain (Carlson et al., 2005), and genomes were sequenced by 454 technology as previously described (Sturdevant et al., 2010). Assembled contigs of A2497, A2497P⁻, and the *C. trachomatis* serovar A reference strain A/HAR-13 were analyzed and compared (Carlson et al., 2005). Gap closing and confirmation were done by PCR and capillary sequencing as previously described (Sturdevant et al., 2010). The annotated A2497 genome was deposited into GenBank, National Center for Biotechnology Information, under accession nos. CP002401 and CP002402.

In vitro characterization of A2497P⁻. One-step growth curves and plaqueing were performed in McCoy cells as previously described (Kari et al., 2008).

Nonhuman primates. Healthy adult cynomolgus macaques (*M. fascicularis*) maintained at the Rocky Mountain Laboratories were cared for under standard practices implemented by the Rocky Mountain Veterinary Branch. Monkeys were housed separately when being used for experimental studies. All handling procedures were reviewed and affirmed by the Animal Care and Use Committee at Rocky Mountain Laboratories and work was conducted in full compliance with the Guide for Care and Use of Laboratory Animals. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Infection of macaques. Infections were done by direct instillation of chlamydiae onto the upper and lower conjunctival surfaces (20 µl per eye) of both eyes (Kari et al., 2008). Infections with A2497P⁻ for the evaluation of attenuation were done as follows: one monkey received 2×10^4 IFU/eye (RML 145), one animal 2×10^5 IFU/eye (RML 641), and four animals 2×10^6 IFU/eye (RML 642, 643, 647, and 147). After the primary infection, all six monkeys received two additional infections of 2×10^6 IFU/eye at 4- or 8-wk intervals. For comparison of virulence, A2497P⁻ infected monkeys were compared with six historic control animals similarly infected with the A2497 plasmid-positive parent strain at 2×10^4 IFU/eye in two recent nonhuman primate studies (Kari et al., 2008, 2009). 30 d after the last infection, the six A2497P⁻ immunized monkeys and six unimmunized naive control monkeys were simultaneously challenged conjunctivally with the virulent A2497 parental strain at 2×10^4 IFU/eye.

Evaluation of infection and disease. Clinical evaluation of infected monkeys was performed weekly. Evaluations were done in a blinded manner by a veterinarian primatologist. Monkeys were scored for hyperemia and follicle formation on the upper conjunctival surfaces in both eyes, as described by Taylor et al. (1981). Hyperemia was scored as follows: 0, no hyperemia; 1, mild hyperemia; and 2, severe hyperemia. Subepithelial conjunctival follicles were scored as follows: 0, no follicles; 1, 1–3 follicles; 2, 4–10 follicles; 3, >10 follicles; and 4, follicles too numerous to count. The scores recorded for the upper conjunctival surfaces of both eyes were added for each animal and termed the clinical response score. The highest possible score was 12 (Kari et al., 2009). Chlamydial shedding was monitored by culturing organisms in monolayers of cycloheximide-treated McCoy cells from swabs taken from the upper and lower conjunctival surfaces (Kari et al., 2009). Day 0 samples were taken before infection. Tears and blood were collected weekly for serological analysis.

ELISA of serum and tear antibody responses. Serum and tear chlamydial-specific IgA and IgG antibody titers were assayed by ELISA using formalin fixed serovar A EBs as previously described (Kari et al., 2009). Antibody titers were expressed as the highest dilution giving an absorbance (OD) value of at least three times above the background.

In vitro serum neutralization assays. In vitro infection neutralization activities of serum antibodies against serovar A EBs were measured as previously described (Su et al., 1990), except that antibody dilutions were incubated with 10^5 IFU and during the infection plates were centrifuged for 1 h and rocked for 30 min at 37 °C. Percent specific neutralization for each dilution was calculated as [(preimmune IFUs – immune IFUs)/preimmune IFUs] $\times$ 100.

MHC class I and II haplotype determination. MHC class I and class II haplotypes were determined from whole blood by the Wisconsin National Primate Research Center University of Wisconsin-Madison.

Proteome array. A *C. trachomatis* whole genome scale proteome array ELISA was used to profile serum antibody responses in monkeys as described previously (Wang et al., 2010). Primate antisera were preabsorbed with bacterial lysates containing glutathione S-transferase (GST) alone before reacting with the microplate-immobilized *C. trachomatis* GST fusion proteins. The reactivity was detected using a secondary antibody conjugate and measured as OD at 405 nm as described elsewhere (Rodgers et al., 2010).

Statistical analyses. Statistical analyses were done using the nonparametric Wilcoxon rank-sum test. Differences were considered significant at a value of $P < 0.05$.

Online supplemental material. Table S1 shows the result of the *C. trachomatis* whole genome proteome array analysis. Table S2 shows the MHC alleles of the vaccinated monkeys. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20111266/DC1>.

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