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Induction of protective immunity against a *Chlamydia trachomatis* genital infection in three genetically distinct strains of mice

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SUMMARY

To establish the feasibility of inducing a protective immune response against a chlamydial genital infection in animals with different genetic backgrounds, groups of C3H/HeN (H-2^k), BALB/c (H-2^d) and C57BL/6 (H-2^b) mice, were immunized intranasally with elementary bodies (EB) of the *Chlamydia trachomatis* mouse pneumonitis biovar. Following the intranasal immunization strong *Chlamydia*-specific humoral and cell-mediated immune (CMI) responses were detected in the three strains of mice. Eight weeks following immunization the animals were challenged with *C. trachomatis* in the genital tract. Vaginal cultures showed that the three strains of mice immunized with EB were significantly protected in comparison to the sham immunized animals. To determine the ability of this immunization protocol to protect against infertility six weeks after the genital challenge the animals were mated. Mice of the three strains immunized with EB showed significant protection as demonstrated by the number of animals that were fertile, and the number of embryos present in their uterine horns, in comparison to the sham immunized mice.

INTRODUCTION

Chronic abdominal pain, ectopic pregnancy and infertility are some of the most devastating complications that can occur in women following a *Chlamydia trachomatis* genital infection.[1–7] The reasons for some individuals developing these long-term sequelae are not well understood. The quantity of the infecting inoculum, the hormonal balance and the stage of the menstrual cycle at the time of the infection probably play a significant role on the outcome of the acute infection. In addition, the immunogenetic background of the individual may account for the apparent differences in the susceptibility to develop long-term sequelae.[4,6,7] *C. trachomatis* can be treated with several types of antibiotics, however, a majority of the infections in females are asymptomatic, and even in those that are symptomatic, implementation of therapeutic measures may be too late to prevent the development of sequelae.[4,6,7] For these reasons significant efforts have focused on the development of a vaccine.[8–14]

Experience with several types of vaccines against infectious pathogens has shown that, in addition to the specific immunization protocol, induction of a protective immune response is dependent on the genetic background of the individual.[15,16] As a result of the widespread presence of chlamydial infections throughout the world this consideration is particularly important in the case of a vaccine for *C. trachomatis*. Strains of mice with various genetic backgrounds have been shown to have different susceptibilities to a chlamydial infection.[17–22] For example, when C3H/HeN, BALB/c and C57BL/6 mice where tested for their susceptibility to a *C. trachomatis* intravaginal infection the C3H/HeN were found to be the most susceptible while the C57BL/6 were the most resistant.[19] In addition, the course and outcome of the infection differed markedly between various strains of mice.[17–19,21] Furthermore, significant differences in the innate and acquired immune responses have been reported between these three strains of mice following a genital and a pulmonary infection.[17,18,21,22] We have previously shown that intranasal immunization of BALB/c mice with elementary bodies (EB) of *C. trachomatis* mouse pneumonitis (MoPn) results in an immune response that can protect the animals against a genital challenge.[23,24] Thus, here we wanted to determine if it was possible to induce a protective immune response against a *C. trachomatis* genital challenge in three genetically unique strains of mice that have marked differences in susceptibility and immune response to a chlamydial infection.

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MATERIALS AND METHODS

Organisms

The *C. trachomatis* MoPn strain Nigg II was obtained from the American Type Culture Collection (Manassas, VA) and grown

in HeLa-229 cells as previously described.[23–25] The stocks of EB were titrated in HeLa-229 cells.[23]

Animal immunization and challenge

Seven- to 8-week-old female BALB/c (H-2^d), C3H/HeN (H-2^k) and C57BL/6 (H-2^b) mice were purchased from Simonsen Laboratory (Gilroy, CA). All protocols were approved by, the University of California, Irvine, Animal Care and Use Committee.

BALB/c and C57BL/6 mice were immunized intranasally (i.n) with 10^4 *C. trachomatis* MoPn inclusion-forming units (IFU)/mouse, while C3H/HeN were immunized with 10 IFU/mouse.[23] The number of IFU used for inoculation was 10-fold lower than the LD_{50} for each strain of mice (unpublished data). As a negative control mice were inoculated with HeLa-229 cell extracts. Eight weeks after the initial immunization, animals were anaesthetized with methoxyflurane and a lateral abdominal incision was made to inoculate 10^5 IFU of *C. trachomatis* MoPn into the left ovarian bursa,[20,23,26] The right ovarian bursa was used as a control and inoculated with mock-infected HeLa-229 cell extracts. All experiments were repeated.

Vaginal swabs and genital cultures

Vaginal swabs for *C. trachomatis* MoPn were collected at weekly intervals following the challenge and resuspended in 200 μl of sterile 0·2 M sucrose, 20 mM sodium phosphate (pH 7·2), and 5 mM glutamic acid (SPG).[23] Two aliquots from each specimen (100 and 10 μl of SPG) were cultured in McCoy cells grown in 48-well plates by centrifugation at 1000 ***g*** for 1 hr at room temperature. Following incubation at 37° the chlamydial inclusions were stained at 30 hr with a rabbit polyclonal anti-*C. trachomatis* MoPn serum prepared in our laboratory.[23]

Immunoassays

Blood was collected by periorbital or heart puncture and the serum pooled for each group of animals. Vaginal samples were collected by washing the vagina twice with 20 μl of phosphate-buffered saline (PBS; pH 7·2) and were pooled for each group.[23] All immunoassays were performed with the pooled serum or vaginal washes from each group.

The *C. trachomatis* MoPn specific antibody titre in sera and vaginal washes was determined by an enzyme-linked immunosorbent assay (ELISA) as previously described.[23] Briefly, 96-well plates were coated with 100 μl/well of *C. trachomatis* MoPn EB containing 10 μg/ml of protein in PBS, and 100 μl of serum or 50 μl of vaginal wash was added per well in threefold serial dilutions. After incubation at 37° for 1 hr and washing, the ELISA plates were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG), IgG1, IgG2a, IgG2b, IgG3, IgM or IgA (Southern Biotechnology Associates, Inc. Birmingham, AL). The binding was measured in an ELISA reader (Bio-Rad Laboratories, Richmond, CA) using 2-2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) as the substrate.

The ability of serum to neutralize *in vitro* the infectivity of *C. trachomatis* MoPn EB was determined as described.[27] Briefly, *C. trachomatis* MoPn (10^4 IFU) was added to threefold serial dilutions of the serum made with 5% (v/v) guinea-pig sera in Ca^{2+}-, Mg^{2+}-free PBS. After incubation at 37° for 45 min, the mixture was inoculated by centrifugation onto HeLa-229 cells. The chlamydial inclusions were stained as described above.[23] The inclusions were counted at a magnification of 200×, and the 50% inhibition was calculated using as a control the sera from the animals inoculated with HeLa cell extracts.

For immunoblotting *C. trachomatis* MoPn EB were resolved by sodium dodecyl sulphate– polyacrylamide gel electrophoresis (SDS–PAGE).[28] Approximately, 250 μg of purified EB were loaded on a 7·5-cm wide slab gel. Following transfer to nitrocellulose membranes, antibody binding was detected with horseradish peroxidase-conjugated goat antimouse antibody developed with 0·01% hydrogen peroxide and 4-chloro-1-naphthol.[23]

The lymphocyte proliferation assay (LPA) was performed as previously described.[23] Briefly, the spleens and inguinal and iliac lymph nodes of two to four mice from each group were collected, teased, and splenocytes enriched for T cells by passage over a nylon wool column. Accessory cells for antigen presentation were prepared by irradiating (3000 rad; ^{137}Cs) syngeneic unseparated spleen cells and incubating them with various ratios of *C. trachomatis* MoPn EB. Concanavalin A (Con A) was used as a positive stimulant, and HeLa cell extracts and tissue culture media as negative controls. At the end of 4 days of incubation, 1·0 μCi of [methyl-^{3}H]thymidine (47 Ci/mmol; Amersham; Arlington, Heights, IL) in 25 μl of RPMI-1640 was added per well, and the incorporation of [^{3}H] was measured using a scintillation counter (Beckman Instruments, Fullerton, CA).

To determine the frequency of *Chlamydia*-specific proliferative T and *Chlamydia*-specific B cells, spleens were harvested, teased and single cell suspensions plated. The frequency of *Chlamydia*-specific proliferativeT-cells was determined by limited dilution analysis (LDA) while the antibody secreting B cells were enumerated using an enzyme-linked immunospot (ELISPOT) assay. The LDA was performed as previously described.[29] Briefly, freshly collected lymphocytes were serially diluted eightfold and plated in 12 duplicates in 96-well microtitre plates. Radiated splenocytes were added as feeder cells to all the wells. EB were then added to a set of plates at a ratio of 10 EB for one feeder cell and the control duplicate set of plates received no antigen. The plates were incubated for 4 days and for the last 20 hr of incubation ^{3}H-thymidine (1 μCi/well) was added, the cells harvested and counted on a Beckman scintillation counter. Frequencies of *Chlamydia*-specific proliferative T cells at the 99·9% confidence level were calculated using a software program (courtesy of Dr J. Whittum-Hudson, Wayne State University School of Medicine, Detroit, MI).

To perform the ELISPOT assay sterile flat-bottom 96-well ELISA plates were coated with 50 μl of *C. trachomatis* MoPn EB (1 mg/ml) in 10 mM PBS (pH 7·4) at 4°.[24] The plates were then washed with sterile PBS and the nonspecific sites were blocked with sterile PBS–bovine serum albumin for 1 hr at 37°. To each well 2×10^5 spleen cells were added in 0·1 ml of RPMI-1640 containing 10% fetal bovine serum and 50 μM 2-β-mercaptoethanol. The cells were subsequently incubated at 37° overnight, stained with isotype-specific antibodies and counted.[23]

The levels of interferon-γ (IFN-γ) and interleukin-4 (IL-4) were determined using commercial kits (IFN-γ from

BDPharMingen, San Diego, CA; IL-4 from Endogen, Cambridge, MA) in supernatants from splenic T cells stimulated as described above.[24]

Fertility studies

Six weeks after the intrabursal challenge, groups of four female mice were caged with a proven breeder male mouse for 18 days.[23] Pregnancy was assessed, by determining the increase in weight of the mice. Pregnant mice were euthanized at days 14–17 of gestation. Animals that did not become pregnant during the first mating were mated for a second time. Finally, all non-pregnant mice were killed 2 weeks after the end of the second mating. The number of embryos in both uterine horns was counted at the time the mice were killed.

Statistical analyses

The two-tailed unpaired Student's *t*-test, Mann–Whitney's *U*-test, and the Fisher's exact test were used for analysis with the Statview IV (Abacus, Berkeley, CA) software program.

RESULTS

Immune response to intranasal inoculation with *C. trachomatis* MoPn EB

The three strains of mice were intranasally immunized with a dose of *C. trachomatis* that was 10-fold lower than their respective LD_{50}. The results of the antibody response at 8 weeks following i.n. immunization are shown in Table 1. High titres of antichlamydial IgG and IgA antibodies in the serum were detected in the three strains of mice while the titres of IgM were relatively low. Determination of the different IgG subclasses of antibodies show that all the three strains had a predominant T helper 1 (Th1) response as shown by the ratio of IgG2a/IgG1 antibodies. However, while in the BALB/c and the C3H/HeN mice the ratio was 9 (65 610/7290 and 21 870/2 430, respectively), in the C57BL/6 mice the ratio was 27 (7290/270). Furthermore, while in the BALB/c and C3H/HeN mice the highest titres of antibody were of the IgG2a subtype in the C57BL/6 mice the IgG2b had the highest levels. The vaginal secretions of the BALB/c mice had the highest levels of IgA antibodies while the C57BL/6 mice had the highest levels of IgA antibodies in serum. Neutralizing antibodies were detected in the three strains of mice with the serum in the C3H/HeN having more than a twofold higher neutralization activity.

To further define the specificity of the antibody response, serum samples were tested by immunoblot against *C. trachomatis* EB (Fig. 1). Antibodies from the three strains reacted with a 120 000 MW band, the 60 000 MW cysteine-rich protein (crp) doublet, major outer membrane protein (MOMP), the 28 000 MW protein and lipopolysaccharide (not shown) when tested against EB. In addition, the serum from the C3H/HeN and BALB/c mice reacted with bands at 150 000 and 24 000 MW, while serum from the C57BL/6 and BALB/c mice reacted with a band at 70 000 MW.

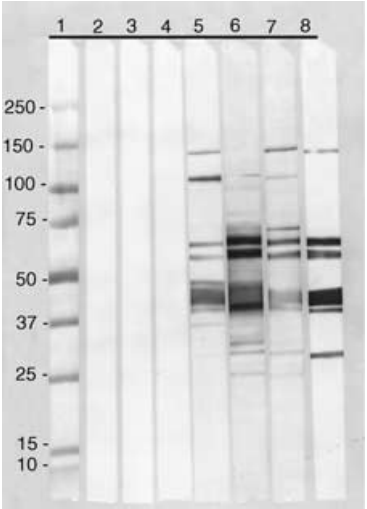

Figure 1. Immunoblot of serum samples using *C. trachomatis* MoPn EB as the antigen performed on a 5–20% gradient SDS–PAGE. Lane 1, MW markers (×1000). Lanes 2, 3, and 4, preimmunization serum samples from C3H/HeN, C57BL/6 and BALB/c mice, respectively. Lanes 5, 6 and 7 serum samples collected 8 weeks after i.n. immunization from C3H/HeN, C57BL/6 and BALB/c mice, respectively. Lane 8, monoclonal antibodies against the 150 000 MW, the 60 000 MW crp, MOMP and the 28 000 MW proteins of the *C. trachomatis* MoPn serovar.

Table 2 depicts the lymphoproliferative responses of splenocytes and lymphocytes from the iliac and inguinal lymph nodes the day before the intrabursal challenge. The negative controls are the mice sham immunized with HeLa cell extracts. When EB were used as the antigenic stimuli, a significant T-cell proliferative response was observed in the splenocytes and the lymph nodes from the three strains of mice immunized i.n. with EB, while the sham immunized animals had no significant response. Overall, the immunized and sham immunized mice had similar T-cell responses to HeLa cells, Con A and the tissue culture medium that were used as controls.

Table 1. Antibody responses in three strains of mice the day before the genital challenge

Mouse strain	Anti-*C. trachomatis* MoPn EB ELISA titre in Serum IgG	IgM	IgA	IgG1	IgG2a	IgG2b	IgG3	Vagina IgG	IgA	Serum neutralizing titre
C3H/HeN	7290	30	2430	2430	21 870	2430	810	81	81	4050
C57BL/6	21 870	30	7290	270	7290	21 870	810	27	81	1350
BALB/c	21 870	30	2430	7290	65 610	2430	2430	81	243	1350

Table 2. Lymphoproliferative responses in T cells from splenocytes and iliac and inguinal lymph nodes the day before the genital challenge

Mouse strain	Group	Enriched T-cells from the spleen (Δ CPM × 1000 ± 1 SD)		Lymphocytes from iliac and inguinal lymph nodes (Δ CPM × 1000 ± 1 SD)	
		EB*	Con A†	EB	Con A
C3H/HeN	Immunized	35 ± 12‡	83 ± 32	7 ± 0·5‡	43 ± 1
	Sham immunized	0·3 ± 0·3	81 ± 18	0·2 ± 0·01	155 ± 168
C57BL/6	Immunized	14 ± 0·8‡	38 ± 3	23 ± 4‡	40 ± 21
	Sham immunized	3 ± 1	43 ± 6	0·01 ± 0·01	77 ± 49
BALB/c	Immunized	23 ± 4‡	139 ± 15	16 ± 2‡	160 ± 10
	Sham immunized	0·8 ± 0·2	107 ± 5	0·8 ± 0·3	204 ± 116

CPM, counts per minute.
*UV-inactivated *C. trachomatis* MoPn EB were added at a ratio of 10 : 1 to the irradiated splenocytes (APC).
†5 μg/ml concanavalin A (Con A).
‡$P < 0{\cdot}05$ by Student's *t*-test compared to the corresponding sham immunized group.

Table 3. Frequency of *Chlamydia*-specific proliferative T-cells and antibody secreting B-cells in three strains of mice the day before the genital challenge

Mouse strain	Frequency of *Chlamydia*-specific proliferative T-cells (±1SD)/10^6		Frequency of *Chlamydia*-specific antibody secreting B-cells (± 1SD)/10^6 splenocytes†	
	Group	Splenocytes*	IgG	IgA
C3H/HeN	Immunized	30 ± 0·6	35 ± 14	170 ± 28
	Sham immunized	2 ± 0·08	0 ± 0	2 ± 3
C57BL/6	Immunized	33 ± 0·6	160 ± 63	173 ± 10
	Sham immunized	4 ± 0·09	0 ± 0	2 ± 3
BALB/c	Immunized	28 ± 0·5	95 ± 0	208 ± 3
	Sham immunized	6 ± 0·14	2 ± 3	5 ± 0

*Precursor frequency of *Chlamydia*-specific proliferative T-cells was measured by limiting dilution analysis.
†Frequency of *Chlamydia*-specific antibody secreting B-cells was enumerated by an ELISPOT assay.

The frequency of *Chlamydia*-specific proliferative T cells was very similar in the three strains of mice (Table 3). Also, the number of antibody secreting B cells was similar in the three strains of mice except for the IgG-secreting cells that were lower in the C3H/HeN mice.

Production of cytokines by splenocytes, harvested 8 weeks after the i.n. inoculation, was measured following stimulation with MoPn EB (Table 4). In the three strains of mice the levels of IFN-γ were significantly higher than the levels of IL-4.

Course of the vaginal shedding of *C. trachomatis* following the intrabursal challenge

Following the intrabursal challenge vaginal cultures were collected from the three strains of mice. As shown in Table 5, none of the C3H/HeN and C57BL/6 mice immunized i.n. with EB had positive vaginal cultures following the intrabursal challenge. In contrast, in the control groups inoculated with HeLa cell extracts, 100% (14 of 14) of the C3H/HeN mice, and

Table 4. *In vitro* production of cytokines by splenocytes in three strains of mice the day before the genital challenge

Mouse strain	Group	IL-4 (pg/ml ± 1SD)		IFN-γ (ng/ml ± 1SD)	
		EB*	Medium	EB	Medium
C3H/HeN	Immunized	<0·1	<0·1	94 ± 16†	<0·03
	Sham immunized	<0·1	<0·1	<0·03	<0·03
C57BL/6	Immunized	2 ± 1	<0·1	112 ± 34†	<0·03
	Sham immunized	<0·1	<0·1	<0·03	<0·03
BALB/c	Immunized	17 ± 0	<0·1	96 ± 9†	<0·03
	Sham immunized	<0·1	<0·1	<0·03	<0·03

*UV-inactivated *C. trachomatis* MoPn EB were added at a ratio of 10 : 1 to the irradiated splenocytes (APC).
†$P < 0{\cdot}05$ by the Student's *t*-test compared to the corresponding sham immunized group.

Table 5. Results of vaginal cultures of three strains of mice following the genital challenge: % mice with positive vaginal culture and mean ± 1 SE IFU of *C. trachomatis* MoPn per mouse per week

Mouse strain	Group	No mice/ group	Week 1		Week 2		Week 3		Week 4		Total % mice with positive culture
			%	IFU	%	IFU	%	IFU	%	IFU	
C3H/HeN	Immunized	17	0*	0†	0*	0†	0*	0†	0	0	0*
	Sham immunized	14	85·7	34 433 ± 26 711	57·1	30 750 ± 26 912	50·0	746 ± 726	14·3	18 ± 10	100·0
C57BL/6	Immunized	18	0*	0†	0*	0†	0*	0†	0	0	0*
	Sham immunized	18	27·8	2424 ± 775	66·7	1006 ± 134	44·4	4156 ± 1373	16·6	9498 ± 3158	66·7
BALB/c	Immunized	16	6·3*	3 ± 3†	6·3*	250 ± 250†	0*	0†	0	0	12·5*
	Sham immunized	20	80·0	8131 ± 1818	65·0	7965 ± 1781	25·0	32 396 ± 7244	15·0	119 ± 27	100·0

*$P < 0.05$ by the Fisher's exact test compared to the sham immunized, challenged mice.
†$P < 0.05$ by the Mann–Whitney *U*-test compared to the sham immunized, challenged mice.

66·7% (12 of 18) of the C57BL/6 mice had positive cultures during the 6-week period of observation ($P < 0.05$). Of the BALB/c mice immunized i.n. with EB only 12·5% (2 of 16) of the animals shed *C. trachomatis* vaginally while 100% (20 of 20) of the controls had positive vaginal cultures ($P < 0.05$). In addition, for the first 3 weeks after challenge the EB immunized BALB/c mice shed significantly less IFU than the mice inoculated with the HeLa cell extracts ($P < 0.05$). Furthermore, in the EB immunized BALB/c mice the length of time the mice had positive vaginal cultures was shorter, 2 weeks versus 4 weeks, than in the HeLa inoculated group. All mice had negative vaginal cultures during the 5th and 6th week of observation.

Fertility studies

The C3H/HeN, C57BL/6 and BALB/c mice immunized i.n. with EB were protected as shown by the fact that when compared with the sham immunized non-challenged group no statistically significant differences in fertility rates were observed ($P > 0.05$) (Table 6). In addition, the immunized challenged animals from the three strains of mice had much higher fertility rates than the sham immunized challenged animals ($P < 0.05$). The most striking differences in this respect were in the C3H/HeN mice. In this group while 100% of the EB immunized mice had bilateral fertility 0% of the sham immunized challenged animals were fertile.

Mice that were i.n. immunized with EB had similar number of embryos in the left uterine horn (challenged) when compared with the sham immunized non-challenged mice ($P > 0.05$) (Table 6). In contrast, the average number of embryos per mouse found in the left uterine horn was statistically significantly lower in the three strains of sham immunized challenged mice when compared with the animals that were sham immunized and non-challenged ($P < 0.05$). Furthermore, no significant differences were observed among these two groups, immunized and challenged and sham immunized non-challenged, when comparing the number of embryos found in the right (control) and left (challenged) uterine horns. Also, only in the C3H/HeN mice the number of embryos in the right horn was significantly decreased in the animals that were sham immunized and challenged, supporting the higher susceptibility of this strain of mice to a chlamydial infection.

DISCUSSION

Here we have for the first time shown that i.n. immunization with *C. trachomatis* EB, of three genetically different strains of mice, can result in the induction of an immune response that is protective against a genital challenge. Mice that were immunized had minimal or no vaginal shedding of *Chlamydia* following the genital challenge. Furthermore, the immunized mice had fertility rates that were equal to control animals that were not challenged. These findings are very encouraging particularly because these three strains of mice differ significantly in susceptibility to a chlamydial infection and the immune responses vary markedly from strain to strain.[17–19,21,22]

Both the cell-mediated and the humoral immune responses play a role in controlling chlamydial infections.[16,30–35] Work using knockout mice and adoptive transfer of lymphocytes

Table 6. Fertility studies of three strains of mice

Mouse strain	Group	No. mice with bilateral fertility/total mice (%)	Mean ± 1 SD no. embryos per mouse per uterine horn	
			Right*	Left†
C3H/HeN	Immunized, challenged	17/17 (100)	3·5 ± 2·0	2·9 ± 1·5
	Sham immunized, challenged	0/14 (0)‡	0·9 ± 2·4§	0·1 ± 0·3§
	Sham immunized, non-challenged	17/19 (89·5)	3·2 ± 1·9	3·4 ± 1·1
C57BL/6	Immunized, challenged	15/18 (83·3)	3·7 ± 2·4	3·2 ± 2·3
	Sham immunized, challenged	3/18 (16·7)‡	2·4 ± 3·0	0·2 ± 0·4§
	Sham immunized, non-challenged	9/11 (81·8)	3·8 ± 2·6	3·8 ± 1·9
BALB/c	Immunized, challenged	16/16 (100)	4·4 ± 2·0	3·4 ± 1·5
	Sham immunized, challenged	3/20 (15)‡	3·1 ± 3·1	0·6 ± 0·6§
	Sham immunized, non-challenged	20/21 (95·2)	4·1 ± 2·0	3·0 ± 1·7

*Inoculated with HeLa cell extracts.
†Inoculated with 10^5 IFU of *C. trachomatis* MoPn.
‡$P < 0{\cdot}05$ by Fisher's exact test compared to the sham immunized, non-challenged mice.
§$P < 0{\cdot}05$ by Student's *t*-test compared to the sham immunized, non-challenged mice.

indicates that $CD4^+$ cells play a greater role than $CD8^+$ cells in resolving a chlamydial genital infection.[31,32] In the three strains of mice that we tested the cell mediated immune response elicited by the intranasal immunization was very strong. In the three groups of mice the frequency of *Chlamydia*-specific proliferative T and B cells, and the lymphoproliferative responses of T cells from the spleen and lymph nodes, were significant following stimulation with EB. Based on the analysis of the levels of the different IgG subtypes in serum it appears that the three strains of mice mounted predominantly a Th1 response. The predominance of a Th1 response was also supported by the measurement of cytokines produced by splenocytes in response to a stimulus with chlamydial EB.

Interestingly, Morrison and Morrison[33] have shown that immunity to a chlamydial genital reinfection does not rely exclusively on $CD4^+$ or $CD8^+$ cells. Mice that were infected in the genital tract with *C. trachomatis* MoPn, and were subsequently depleted of $CD4^+$ and $CD8^+$ cells using monoclonal antibodies, were found to be as resistant to a chlamydial genital reinfection as immunocompetent wild type mice. Based on these findings, these authors concluded that B cells probably play a predominant role in resistance to a chlamydial genital reinfection.[33] A caveat with these experiments is that the mice were treated with progesterone before the primary infection and the secondary challenge with *Chlamydia* and thus, most likely there was a significant bias towards a Th2 response (see below).

In addition to the neutralizing antibodies present in the mucosal surface, the non-specific anatomical defences and the components of the innate immune response, are the only barriers in the initial encounter with the pathogen that can protect against infection.[8,16,30] In this respect, we detected the presence of IgA chlamydial specific antibodies in the vaginal washes from the three strains of mice. Passively transferred neutralizing monoclonal IgA antibodies able to protect in a murine model against a genital chlamydial challenge have been described.[34] Current vaccines however, for the most part do not protect against infection but protect only against disease.[36] In the case of chlamydial genital infections a vaccine that can limit the spread of the organisms to the lower genital tract will probably be efficacious in protecting against the long-term sequelae resulting from infection of the upper genital tract. The ability of this type of vaccine however, to protect from transmission to the sexual partners will be limited. Thus, it is encouraging that the immunized C3H/HeN and C57BL/6 mice had negative vaginal cultures during the 6 weeks of observation. This apparent 'sterilizing immunity' will have to be further validated before we can reach a final conclusion since we can not exclude at this point the possibility that the animals were shedding *Chlamydia* below the detection level.

Based on the results obtained several years ago, using viable and inactivated *C. trachomatis* EB to protect against trachoma, utilizing the whole organism as a vaccine is currently not an acceptable approach.[11] More specifically it was shown, that immunized individuals that were not fully protected when they were subsequently exposed to *C. trachomatis*, developed a severe autoimmune reaction when re-exposed to the organism.[11] Efforts to identify the chlamydial antigen(s) involved in this reaction suggest that the 60 000 MW heat-shock protein (hsp) may play a role in eliciting autoimmunity.[37] The fact that three genes have been detected in *Chlamydia* with similarity to the hsp 60 000 MW family poses interesting questions, not only about their functional role in the organism, but also about their potential role in pathogenesis.[38] A positive aspect of our immunization protocol is that it did not result in functional damage to the genital tract. The fertility rates in the animals that were immunized and challenged were equal to those observed in the fertility control group of mice that were sham immunized and non-challenged.

As a result of the autoimmune reaction observed in previous vaccination trials several attempts have been made at producing a subunit vaccine against genital infections. So far these efforts have provided some positive results. For example, Pal *et al.*[39] showed that a preparation of the chlamydial outer membrane protein can induce a protective immune response against a genital challenge. On the other hand, attempts to use single antigens, in particular MOMP, have yielded contradictory results.[38–43] The apparent discrepancies between the data obtained by several laboratories using various approaches to

prepare the antigen are not well understood. In this respect, it has been proposed that the preservation of conformational epitopes in MOMP may be critical for inducing protection.[14,39] The recent publication indicating that a folded *C. trachomatis* MoPn MOMP preparation purified from EB can induce protection against a genital challenge supports this hypothesis.[41] Furthermore, other antigens may be needed to elicit a protective immune response.[13]

In this respect, in addition to the MOMP two other proteins, as detected by Western blots, elicited antibodies in the three strains of mice including the 60 000 MW crp, and a high molecular weight protein of approximately 120 000 MW that may correspond to one of the polymorphic membrane proteins (PMP).[13,44] Another possible PMP with a molecular weight of 150 000 MW induced antibodies in C3H/HeN and BALB/c mice but no antibodies to this protein were detected in the C57BL/6 mice. There are nine PMP genes in *C. trachomatis* that are thought to code for proteins ranging in size from approximately 900–1500 amino acids in length. Since some of these proteins appear to be surface exposed it has been proposed that they may be able to induce a protective immune response.[13] Relevant to this at least two of these candidate PMPs elicited an immune response in this model. However, whether or not any one of these antigens, that when administered as part of an EB can induce an immune response in animals with different genetic backgrounds, will be able to elicit a protective response when delivered as purified antigens, will have to be tested.

Of the murine models currently available to test vaccine candidates we decided to use the intrabursal model in spite of the obvious shortcoming of the route of inoculation.[19,45,46] This model, originally described by Barron *et al.*[45] offers the advantage that all strains of mice so far tested, independent of age, develop infertility following a relatively low challenge with the *C. trachomatis* MoPn serovar.[23] Furthermore, by inoculating directly the upper genital tract we are delivering a direct and thus, a more stringent challenge to the anatomical site that we are primarily interested in protecting. The intravaginal model, although it follows the natural route of infection, requires a larger dose of *C. trachomatis* MoPn and certain strains of mice appear to be completely resistant to develop infertility if they are inoculated past a certain age.[19,47] To overcome this problem the mice can be treated with progesterone before they are challenged with *Chlamydia.*[46] Treatment with progesterone, however, induces a significant alteration of the immune system including, among others, severe suppression and a shift from a Th1 to a Th2 response.[48–50] Hence, when testing a vaccine candidate this is a significant shortcoming because progesterone may abrogate or mask the protective response induced by the immunization. Thus, currently we do not have an ideal animal model to test vaccine candidates against chlamydial genital infections. However, we expect that if different groups of investigators test vaccine candidates in several models, conclusions will be reached that can be applied to larger segments of the human population than if only one model is characterized.

In conclusion, our results suggest that immunization with EB may induce a protective response in hosts with a variety of genetic backgrounds against a genital challenge with *C. trachomatis*. However, further work is required to extend these observations as immunization protocols utilizing subunit vaccines, or non-viable vaccines, may not be effective at achieving protection in certain hosts with a unique genetic make-up. This animal model however, could help determine the vaccination protocols that are required to stimulate the immune system to generate a protective response in hosts with different genetic backgrounds.

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