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In Vitro and In Vivo Properties of Chemically Induced Temperature-Sensitive Mutants of *Chlamydia psittaci* var. *ovis*: Screening in a Murine Model

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Two temperature-sensitive (*ts*) strains, 1B and 1H, were obtained after nitrosoguanidine treatment of the wild-type virulent abortive ovine strain *Chlamydia psittaci* AB7. Optimum growth temperature on McCoy cells was 38°C for the three strains, but at the restrictive temperature, 39.5°C, *ts* strains differed from the parental strain in their total infective yield of chlamydiae, their efficiency of plating, and the morphology of the plaques and cytoplasmic inclusions. Their survival at 51°C was also reduced compared with that of the wild field strains. The virulence of the two *ts* strains was attenuated for pregnant mice, but these two *ts* strains were able to multiply in mice and to induce a strong immunity to virulent challenge with either the parental AB7 strain or the caprine or bovine abortive field strains.

Chlamydia psittaci, an obligate intracellular bacterium, induces placental infections and abortions in various mammals, in particular ewes. Although the disease induces a good immunity (20, 22), vaccines prepared with inactivated chlamydiae emulsified in adjuvant have not really been efficient (11, 13, 19). An avirulent live vaccine administered before breeding could be the key to control of chlamydial abortion in ewes (20). Attempts to attenuate the chlamydial agent of ovine abortion by serial passages in the yolk sac of chicken embryos or in cell cultures were unsuccessful (2, 3). We then tried to isolate temperature-sensitive (*ts*) mutants. Two *ts* mutants were isolated in tissue culture after treatment of a virulent abortive strain of *C. psittaci* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). This study describes the in vitro and in vivo properties of these two *ts* mutants.

MATERIALS AND METHODS

Cell culture. McCoy cells (7) were grown at 38°C in monolayer cultures in Eagle minimum essential medium containing 10% fetal calf serum (MEM).

Strains of *C. psittaci*. *C. psittaci* AB7 (6) in its second passage in chicken embryos after isolation from an aborted lamb was used as the parental strain for isolation of *ts* mutants.

The following *C. psittaci* strains were used for comparative thermal inactivation studies or as infectious challenge: ovine abortive strains AB4, AB6, AB8, AB9, and AB10; caprine abortive strain AC1; bovine abortive strain AV1; ovine intestinal strains IB1 and IB2; and the ovine pneumonitis strain 109-75, which was a generous gift supplied by P. Russo, Laboratoire National de Pathologie des Petits Ruminants, Nice, France. The abortive strains were isolated in cell culture from vaginal swabs from the aborted animal, and the intestinal strains were obtained from the feces of healthy sheep (5).

Stocks of chlamydiae were propagated in the yolk sacs of developing chicken embryos as previously described (16) and stored at −70°C.

Infectivity titration. The titration of chlamydial infectivity for McCoy cells was performed by a plaque assay (1). Briefly, it was carried out on McCoy monolayers under a solid overlay medium containing 1.5% Noble agar. Counts were made on day 14 after staining with 1:10,000 neutral red.

Isolation of *ts* mutants in tissue culture. McCoy monolayers in flat-bottom tubes were infected with the aid of centrifugation with *C. psittaci* AB7 and incubated in MEM. After 24 h, the medium was removed, and 1 ml of Dulbecco phosphate-buffered saline (PBS) containing 10 μg of NTG was added. The NTG-treated infected cells were incubated for 0.5 h at 35°C. The NTG was removed, and the infected cells were washed twice in PBS. Then 1 ml of MEM was added, and the incubation was continued at 35°C for 72 h.

Chlamydiae from mutagenized infected cultures were harvested with glass beads and frozen at −70°C.

Mutagenized stock suspensions of NTG-treated chlamydiae were plated on Disposo-Tray multidishes (Linbro; model FB 6TC) to yield approximately 10 plaques per dish. All isolated plaques were picked up, inoculated on McCoy cell tube cultures, and incubated at 35°C for 5 days. Infected cultures were than harvested with glass beads and screened for temperature sensitivity on McCoy cells. Tenfold dilutions of each isolate were prepared in PBS and inoculated in duplicate dishes of Disposo-Tray multidishes; one multidish

was placed at 35°C, and the other was at 39.5°C. Isolates which failed to produce plaques at 39.5°C in a dilution at least 10-fold less than the highest dilution which produced plaques at 35°C were considered to be potential *ts* mutants. These isolates were plaque purified two additional times at 35°C, and plating efficiencies (35°C/39.5°C) were reexamined. Identical mutants were avoided by isolating only a single mutant from each mutagenized stock. Two *ts* mutants, *ts* 1B and *ts* 1H, were selected by NTG treatment. After plaque purification, the two *ts* strains were propagated in yolk sacs of developing chicken embryos at 37°C and stored at −70°C. These yolk sac-propagated stocks were used for characterization.

In vitro characteristics of mutant strains. (i) Thermosensitivity of chlamydial growth. The influence of temperature on the one-step growth cycle of the *ts* 1B and *ts* 1H mutants was determined by titration of the total infective yield of chlamydia cultures on McCoy monolayers in flat-bottom tubes harvested 1, 2, 3, or 4 days after inoculation. Inoculation was performed with the aid of centrifugation for 1 h at 900 × *g* at room temperature and with a multiplicity of infection (MOI) of 100 PFU per host cell. After removal of the inoculum, the infected cells were washed twice in PBS before incubation in 1 ml of MEM. The incubation was performed in water-jacketed CO_2 incubators (Forma Scientific), the temperature of which was controlled by registering.

Microscopic examination after acridine orange staining (14) was used to monitor cytoplasmic inclusion formation and morphology.

(ii) Thermolability of infectivity. Five tubes with 1 ml of yolk sac-propagated chlamydiae diluted in 10 ml of PBS and containing 10^7 PFU were incubated 0, 10, 20, or 30 min at 51°C. Residual infectivity of the chlamydiae was determined by plaque assay.

Mice. Adult Swiss female OF1 mice (Iffa-Credo) were reared in an air-conditioned building (21°C, 60% relative humidity) on sterilized wood shavings with free access to water and sterilized food. They were divided into groups at random.

In vivo characteristics of mutant strains. (i) Splenic multiplication in nonpregnant mice. A total of 192 mice (6 weeks old; average weight, 20 g) divided into three groups were inoculated in the left hind footpad with 0.05-ml suspension of about 5×10^4 PFU of yolk sac-propagated chlamydiae *ts* 1B, *ts* 1H, or the parent strain AB7 per mouse. Mice were sacrificed in groups of eight per strain daily from 3 to 13 days after inoculation. The spleen and the left popliteal lymph node were removed. Chlamydiae were enumerated by the plaque assay method (17). Briefly, the spleen was weighed and emulsified in 1 ml of PBS in a glass tissue grinder. Then 0.5 ml of the crushed spleen was diluted in 1.5 ml of PBS. From this suspension and from the successive appropriate dilutions, 0.2-ml portions were spread on McCoy cell monolayers in dishes of Disposo-Tray multidishes and then incubated in an atmosphere of CO_2 for 1 h. After removal of inoculum, infected cells were washed once with PBS before incubation with solid overlay medium. Spleens were titrated individually, whereas popliteal lymph nodes were pooled and titrated as a mixture.

(ii) Virulence for pregnant mice after intraperitoneal inoculation. Intraperitoneal inoculation was carried out after an 18-h starvation. A total of 192 mice of the same age (10 to 11 weeks), same weight (30 to 35 g), and same stage of gestation (11 ± 1 days) were divided into 10 groups. Nine groups of 20 mice received 0.2 ml of a suspension of 7×10^3 to 3.8×10^6 PFU of yolk sac-propagated chlamydiae *ts* mutants 1B and 1H per mouse. Ten mice (control group) received 0.2 ml of PBS, since we have previously shown that normal yolk sac suspension does not disturb pregnant mice (16). For monitoring pregnancy, mice were weighed daily after inoculation until they gave birth. The virulence of the three strains was estimated by the number of living offspring per litter, and their survival was monitored daily for 8 days. By convention, abortion was when less than three newborn mice from a mother were alive 24 h after birth. When less than seven newborn mice from a mother were alive, it was considered a pathological parturition.

(iii) Immunogenicity. Mice (6 weeks old; average weight, 20 g) were vaccinated subcutaneously in the back with 0.2 ml of suspension of 10^5 PFU of yolk sac-propagated *ts* mutants. Two months later the vaccinated mice were challenged intraperitoneally as previously described (18) at 11 ± 1 days of pregnancy with 0.2 ml of virulent wild-type strains of *C. psittaci*. The immunity of mice was estimated by the number of living offspring per litter and their survival during week 1 postparturition.

Statistical analysis. Simple *t* tests were used for signification of differences.

RESULTS

Plaque characteristics of *ts* mutants. Only 2 out of 18 potential *ts* mutants were confirmed as being *ts* after two additional plaque purifications. The mutants were designated *ts* 1B and *ts* 1H, and their efficiencies of plating (35°C/39.5°C) were, respectively, 11.4 ± 0.2 and 10^3 ± 0.4. These two *ts* mutants were selected from two separate mutageneses. It was readily apparent that *ts* 1B and *ts* 1H behaved differently under the conditions tested in McCoy cells or mice.

The plaque size and the plaque morphology did not differ between the *ts* mutant and parental strain at growth temperatures ranging from 35 to 38°C. The optimum temperature for the plaque assay was 38°C for all three strains. At 39.5°C, the parental strain AB7 made large clear plaques, whereas *ts* mutant strains 1B and 1H gave tiny dim plaques. Clonal selection did not alter the plaque morphology of the parent strain.

Thermosensitivity of replication. The thermosensitivity of replication was tested for the two *ts* mutant strains 1B and 1H comparatively to the parent strain AB7 at 39.5, 38, 37, and 35°C (Fig. 1). No difference was observed between 37 and 38°C. Strain AB7 grew equally well at 37 and 39.5°C, but at this higher temperature, stability of infective chlamydiae out of the lysed host cells was strongly reduced. At 35°C, growth was slightly reduced.

Strains 1B and 1H grew poorly at 39.5°C (37°C versus 39.5°C; or in comparison with the paren-

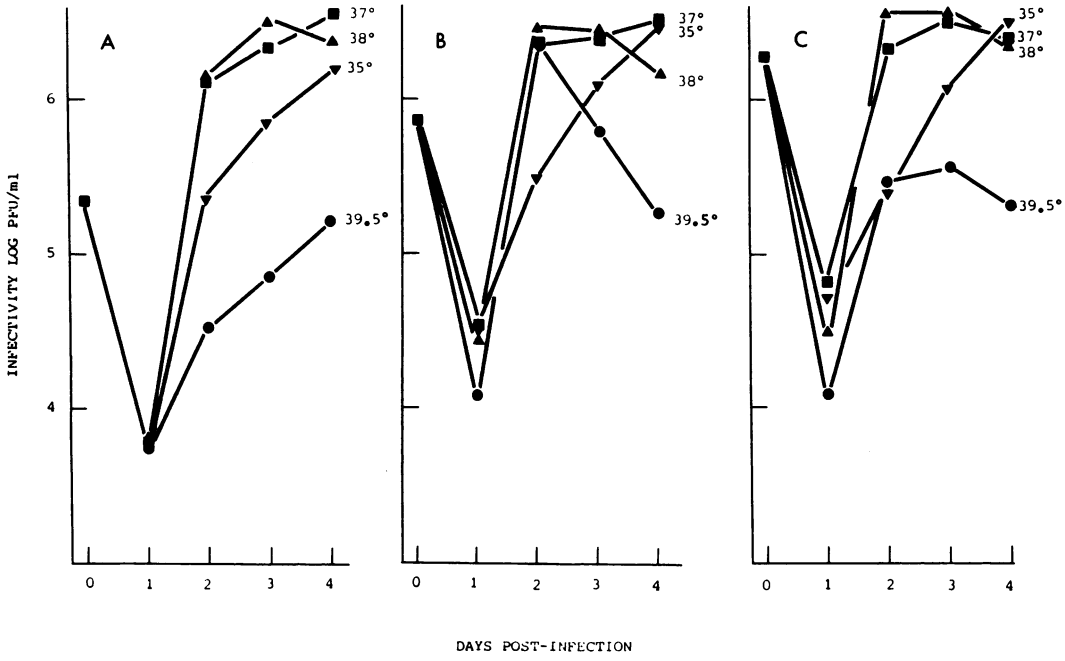

FIG. 1. Developmental cycle of parent strain AB7 (B) and of *ts* mutants 1B (A) and 1H (C). Total infective yield of chlamydiae inoculated at room temperature with the aid of centrifugation on McCoy cells (multiplicity of infection, 100 PFU per cell).

tal strain at 39.5°C [AB7 versus 1H or AB7 versus 1B] at 2 days postinoculation [$P > 0.001$]). They grew slower at 35°C than at 37°C at 2 days ($P > 0.001$), as did strain AB7 ($P > 0.001$). The same results were obtained in a duplicate experiment.

Thermolability of infectivity at 51°C. The chlamydial survival at 51°C was compared for the two *ts* strains and eight wild-type strains. This test of thermolability of infectivity at 51°C allowed us to place the strains into three classes (Fig. 2) and distinguish the mutants from the wild strains with $P > 0.001$ after 30 min at 51°C.

Class 1 contained two intestinal ovine strains (IB1 and IB2), one ovine pneumonitis strain (109-75), and two abortive strains of ovine strain AB9 and caprine strain AC1 origins. After 30 min at 51°C, the infectious titer was approximately 1.6 logs lower.

Class 2 contained five abortive strains: four of ovine AB4, AB6, AB7, and AB10 origins, and one of bovine AV1 origin. The reduction of the infectious titer was about 2.4 logs after 30 min at 51°C.

Class 3 contained the two *ts* mutants. After 30 min at 51°C, the reduction of the infectious titer of strain 1B was greater than 3.5 logs; after 20 min at 51°C, we could not find any infectious particles with strain 1H.

Splenic multiplication in nonpregnant mice. After subcutaneous inoculation of nonpregnant mice, *ts* mutants induced a lower level of splenic infection than did the parental strain ($P > 0.001$) (Fig. 3). With the AB7 strain, all eight spleens were infected up to 10 days postinoculation, and six out of eight remained infected 12 days after inoculation. With the 1B strain, all eight spleens were infected 8 days postinoculation, but only two out of eight remained infected 11 days postinoculation. With the 1H strain, only six out of eight and two out of eight were infected, respectively, at 8 and 11 days postinoculation.

These results showed that the two *ts* strains were able to multiply in the spleens of mice but that they were not able to persist there as long as did the parent strain.

Virulence for pregnant mice. Virulence of the mutant strains, after intraperitoneal inoculation of pregnant mice, was estimated by the number of living offspring per litter and their survival, which was monitored daily for 8 days. The two *ts* strains induced intrauterine mortality but it was necessary to inoculate 10 times more strain 1B and 4 times more strain 1H than strain AB7 to obtain the same number of living offspring per litter. So, among the 19 pregnant mice inoculated with 1.9×10^6 PFU of strain 1B, 12 aborted. There were 13 abortions out of the 19 pregnant mice inoculated with 7.6×10^5 PFU of strain 1H and 1.8×10^5 PFU of strain AB7. With all three strains, the infant mice born alive survived week 1 postparturition (Fig. 4).

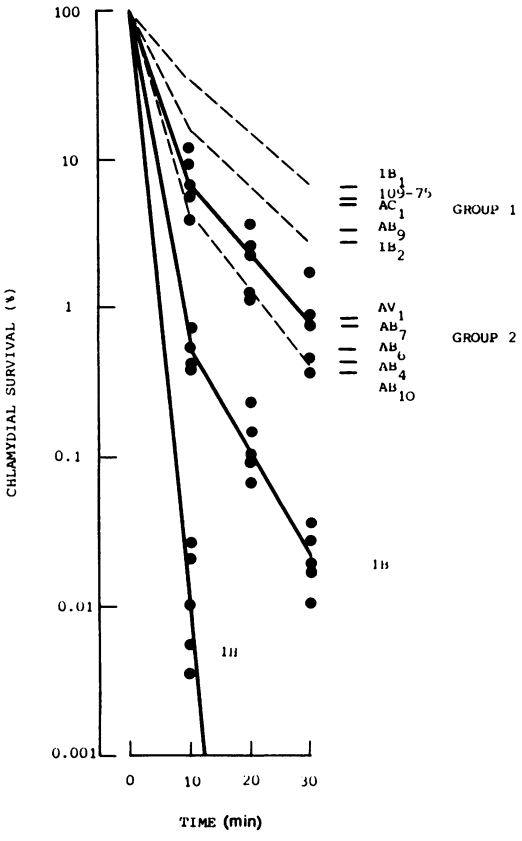

FIG. 2. Inactivation at 51°C of different wild-type strains of *C. psittaci* and the two *ts* mutants 1B and 1H. Three classes can be recognized. The upper broken-line zone included two intestinal ovine strains (IB and IB2), one ovine pneumonitis strain (109-75), and two abortive strains of ovine AB9 and caprine AC1 origins. The lower broken-line zone included five abortive strains, four of ovine AB4, AB6, AB7, and AB10 origins and one of AV1 bovine origin. The lowest zone included the two *ts* mutants only. Results of five different experiments with strains 1H, 1B, and AB7 are shown by dots.

Thus, the two *ts* strains had an attenuated virulence for pregnant mice: strain 1B was 10 times less virulent and strain 1H was 4 times less virulent than the parent strain AB7.

Immunogenicity. Immunizing capacities of the *ts* strains were tested on pregnant mice challenged with virulent wild-type chlamydiae. An inoculum of 10^5 PFU of *ts* strains did not disturb gestation when mice were mated 6 weeks after vaccination.

The number of living offspring per litter of the challenged vaccinated groups was not significantly different from that of the unchallenged control ($P \geq 0.001$) (Table 1).

DISCUSSION

The mechanisms of virulence of *C. psittaci* are not fully understood. Adaptation of *C. psittaci* to cultured cells or chicken embryos involves a reduction in virulence but the virulence is generally restored on the first passage in animals (2). To obtain a faster, more lasting attenuation, we used mutagenesis with NTG, which often induces multiple mutations in closely linked genes (8). Since virulence is obviously a complex function of variables, and since there are still no markers of attenuation, it was not possible to directly select attenuated mutants. Because the *ts* phenotype is consistently associated with attenuation of viruses in animals and humans, this marker was used. As the purpose of this study was to obtain strains which would provide a live vaccine for ewes, a temperature of 39.5°C was chosen as the restrictive temperature. The parental strain AB7 of *C. psittaci* was our refer-

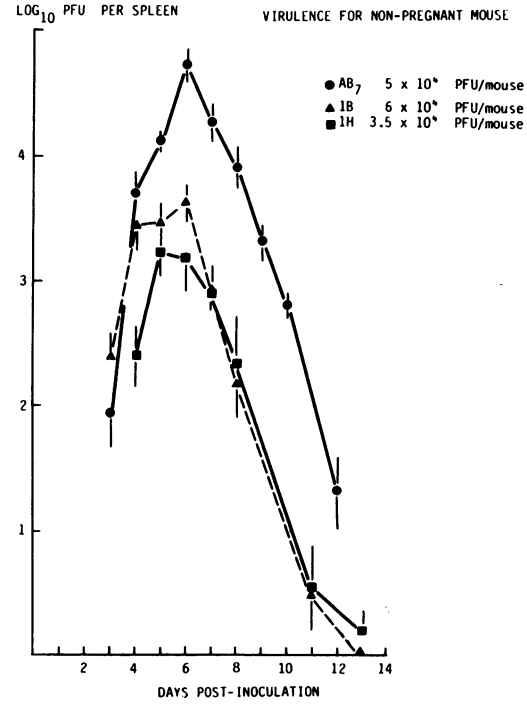

FIG. 3. Kinetics of *C. psittaci* splenic infection. Comparative multiplication of two *ts* mutants (1B and 1H) and the parent strain (AB7). Mice were inoculated subcutaneously in the footpad with 5×10^4 PFU of strain AB7 (●); 6×10^4 PFU of strain 1B (▲); or 3.5×10^4 PFU of strain 1H (■) per mouse. Bars represented the average number of chlamydiae per spleen of eight mice ± standard error. When the eight mice of the group were not all infected, the number of positive spleens was shown near the point. The average number of chlamydiae per spleen took the negative spleens into account. The log of the negative spleens was taken as zero.

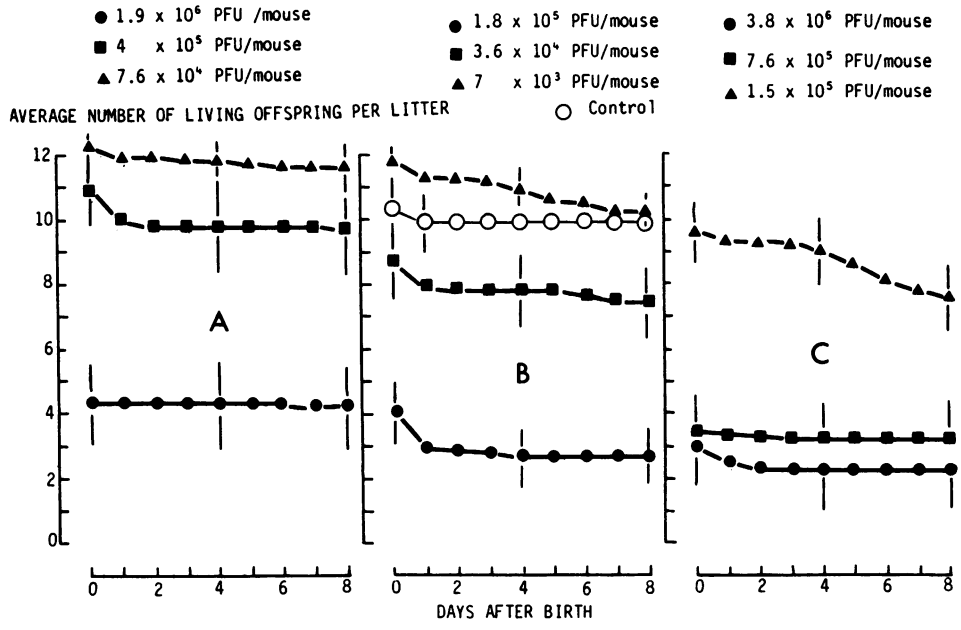

FIG. 4. Virulence of chlamydial strains 1B (A), AB7 (B), and 1H (C) for pregnant mice estimated by survival of infant mice after intraperitoneal inoculation at 11 ± 1 days of pregnancy. Bars represent the group mean ± standard error.

ence strain. It was in its second passage in yolk sacs of embryonated chicken eggs after having been reisolated from the fetus of an aborted ewe. It was fully virulent for ewes and mice (16, 19).

Two mutant strains, 1B and 1H, were isolated on the basis of their efficiency of plating at 35 and 39.5°C (10 and 10^3, respectively). These mutants were capable of normal growth at temperatures ranging from 35 to 38°C, but in contrast to parent strains, which formed large clear plaques at 39.5°C, these mutants formed tiny dim plaques. In liquid medium, the total infec-

TABLE 1. Comparative protection of pregnant mice with *ts* mutants of *C. psittaci* against virulent challenge

Vaccination[a]	No. of pregnant mice	Avg no. ± SD of living offspring per liter	
		At birth	At 8 days after birth
Unchallenged			
	32	11.5 ± 0.4	10.8 ± 0.4
1B	35	11.75 ± 0.4	11.1 ± 0.4
1H	10	11.7 ± 0.5	11.2 ± 0.5
Challenged[b] with:			
AB7 (10^5 PFU per mouse)			
	24	0.5 ± 0.5	0.5 ± 0.5
1B	35	11.75 ± 0.5	11.1 ± 0.5
1H	35	10.3 ± 0.5	10.1 ± 0.5
AC1 (10^5 PFU per mouse)			
	10	0.8 ± 0.7	0.2 ± 0.2
1B	15	11.7 ± 0.6	11.0 ± 0.6
AV1 (7×10^5 PFU per mouse)			
	10	1.4 ± 0.9	1.4 ± 0.9
1B	15	11.3 ± 0.9	10.0 ± 0.7

[a] Nonpregnant mice were vaccinated subcutaneously in the footpad with 10^5 PFU of *ts* strain 1H or 1B per mouse.

[b] Two months later, at 11 ± 1 days of pregnancy, mice were challenged with virulent abortive strains of chlamydiae by intraperitoneal route: parental strain AB7, ovine origin; strain AC1, caprine origin; and strain AV1, bovine origin.

tive yield of chlamydiae was also reduced, more so for strain 1B than for 1H.

The two strains were also less virulent for pregnant and nonpregnant mice. Subcutaneous inoculation in the footpad was used to measure the invasiveness of the strains, whereas intraperitoneal inoculation took into account the capacity of the strains to colonize the placenta (5).

The two mutants were able to colonize the placenta as established by the *Brucella* model (4); this colonization may induce intrauterine mortality. This mortality, however, was moderate compared with the mortality of groups inoculated with strain AB7 in the same condition (Fig. 4).

The 1H strain was less invasive than the parental strain as determined by the restriction of chlamydial growth in the spleen (Fig. 3). During the first days postinoculation, strain 1B was as invasive as the parental strain. The restriction of its virulence seemed to be related to the capacity of the infected mice to eliminate more rapidly the mutant than the parental strain. The attenuation of the virulence of the two *ts* strains was apparently not related to the shut-off temperature of 39.5°C since body temperatures of normal or infected mice remained within the permissive temperature range of the *ts* mutants (38°C). This result is no different from the results obtained with *ts* mutants of virus (9, 10, 12, 15).

Both strains induced a solid immunity to challenge lasting at least two months (Table 1). The number of living offspring per litter was almost equal to that recorded for the unchallenged control group and greater than that of the best commercial vaccine we have tested (18).

These results were confirmed with a sheep model (21). Whereas the parent strain provoked abortion in sheep, mutant strains did not disturb pregnancy and induced immunity lasting at least 1 year. So, after further investigations concerning their safety, efficacy, and stability, these strains could be used as potential live vaccines.

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