Veterinary Microbiology, 28 (1991) 171–188
Elsevier Science Publishers B.V., Amsterdam

Biological properties of RB51; a stable rough strain of *Brucella abortus*

Gerhardt G. Schurig[1], R. Martin Roop II[2], T. Bagchi, S. Boyle, D. Buhrman and N. Sriranganathan

Department of Pathobiology, Veterinary Microbiology Research Laboratories, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

(Accepted 11 January 1991)

ABSTRACT

Schurig, G.G., Roop, R.M. II, Bagchi, T., Boyle, S., Buhrman, D. and Sriranganathan, N., 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. *Vet. Microbiol.*, 28: 171–188.

A rifampin-resistant mutant of *Brucella abortus*, designated RB51, was derived by repeated passage of strain 2308 on Trypticase soy supplemented with 1.5% agar and varying concentrations rifampin or penicillin. The RB51 colonies absorbed crystal violet and RB51 cell suspensions autoagglutinated, indicating a rough type colonial morphology for this strain. No O-chain component was detected in lipopolysaccharide (LPS) extracted from RB51 on SDS-PAGE gels stained with silver. Western blot analysis with the monoclonal antibody BRU 38, which is specific for the perosamine homopolymer O-chain of smooth *Brucella* LPS, indicated that the LPS of RB51 is highly deficient in O-chain when compared with the parenteral smooth strain 2308 or rough strain 45/20. Biochemically, RB51 resembles parental strain 2308 in its ability to utilize erythritol. Intraperitoneal inoculation of RB51 into mice results in a splenic colonization which is cleared within four weeks post infection. RB51 does not revert to smooth colony morphology upon passage in vivo (mice) or in vitro. Mice infected with RB51 produce antibodies against *B. abortus* antigens including class 2 and 3 outer membrane proteins but not against the O-chain. Furthermore, rabbits, goats and cattle hyperimmunized with sonicates of RB51 develop antibodies to *B. abortus* cellular antigens but do not develop antibodies specific for the O-chain. Immunization of mice with 1×10^8 viable RB51 organisms confers significant protection against challenge with virulent *B. abortus* strain 2308.

INTRODUCTION

Brucellosis is a chronic infection which can result in abortion and infertility in animals and undulant fever in humans (Acha and Szyfres, 1980). *Brucella abortus*, *B. melitensis* and *B. suis* occur naturally in the smooth phase and

[1]Author to whom all correspondence should be addressed.
[2]Present address: Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 W. Markham, Little Rock, Arkansas 72205-7199, USA.

0378-1135/91/$03.50

contain a homopolymer of perosamine as the O-chain component of their lipopolysaccharide (LPS) (Bundle et al., 1987). These species are responsible for the naturally occurring cases of brucellosis in cattle, goats and swine, and along with *B. canis* are also responsible for human brucellosis (Acha and Szyfres, 1980). The perosamine O-chain appears to be an immunodominant antigen since a major proportion of the antibody response of animals or humans infected or immunized with smooth *Brucella* species is directed against this antigen (Diaz et al., 1968; G. Schurig and R.M. Roop, unpublished data). Moreover, the agglutinating antibodies detected in the standard serologic tests for brucellosis are almost exclusively specific for the perosamine O-chain (Diaz et al., 1968; Schurig et al., 1981).

This strong humoral immune response to the O-chain can cause problems related to the serologic diagnosis of bovine brucellosis. A proportion of cattle vaccinated with live Strain 19 will maintain agglutinating titers (Nicholletti, 1981; Woodard, 1981), which impedes the clear distinction of naturally exposed from vaccinated animals on serologic examination. The use of a stable, rough variant of smooth *B. abortus* which completely lacks the O-chain, or contains an insufficient amount of this molecule to induce the production of O-chain specific antibodies in immunized animals, could potentially overcome this serologic problem. Moreover, such a strain would be an excellent vaccine candidate if it were able to induce protective immunity in the host, while only replicating for a short period of time.

Rough strains of both *B. abortus* (McEwen, 1940) and *B. melitensis* (Diaz et al., 1979) have been described. Strain 45/20 is probably the best described rough strain of *B. abortus* and this strain has been used as a killed vaccine (McEwen, 1940). Vaccination with killed 45/20 requires the use of an adjuvant and booster immunizations, and overall the results of vaccine trials with this strain in cattle have not been completely satisfactory (McEwen, 1940; Worthington and Horwell, 1974; Woodard and Jasman, 1983). Furthermore, studies in our laboratory indicate that 45/20 is not totally devoid of O-chain (Schurig et al., 1984; Roop et al., 1987) and others have reported reversion of 45/20 when used as a live vaccine (McEwen, 1940; Worthington and Horwell, 1974).

In this report, we describe a rough mutant, strain RB51, which was derived from virulent *B. abortus* strain 2308. This strain was developed in our laboratory and has since been used by a variety of investigators (Santos et al., 1984; Corbeil et al., 1988; Ficht et al., 1988; Smith et al., 1990a,b) due to its stable rough characteristics. The increasing use of this strain in brucellosis research warrants a description of its most outstanding characteristics. RB51 is stable both in vitro and in vivo. RB51 shows reduced virulence in the mouse model, as measured by a short duration of splenic colonization, elicits antibodies specific for *B. abortus* antigens, but not to the O-side chain. Furthermore, infection with RB51 provides mice with significant protection against

subsequent challenge with virulent strain 2308. Therefore, RB51 would appear to be an excellent vaccine candidate.

MATERIALS AND METHODS

Bacterial strains

Brucella abortus strains 2308, 19 and 45/20 as well as *Yersinia enterocolitica* serovar 0:9 were used in this study. *B. abortus* 2308 is a standard challenge strain, which produces abortion in experimentally infected cattle (Verstreate et al., 1982) and goats (Meador and Deyoe, 1986). Strain 19 is a live, attenuated vaccine strain (Verstreate et al., 1982). Strain 45/20 is an unstable, partially rough B. abortus, derived by repeated passages in guinea pigs (McEwen, 1940; Schurig et al., 1984). Working cultures were grown on Trypticase soy broth supplemented with 1.5% agar (TSBA) plates or slants at 37°C under an atmosphere of 5% CO_2 for 48 h and stored at 4°C with monthly transfer. Stock cultures were stored in skim milk at −80°C or under liquid nitrogen. *Y. enterocolitica* serovar 0:9, NCTC 11147 was obtained from the National Collection of Type Cultures, London, UK. This organism contains an O-antigen which is nearly identical to the A antigen of *B. abortus* (Ahvonen et al., 1969; Caroff et al., 1984; Caroff et al., 1984; Meikle et al., 1989).

Derivation of rifampin-resistant mutants of B. abortus *2308*

Figure 1 outlines the derivation of RB51 from *B. abortus* 2308. All cultures were grown on TSBA agar plates or TSBA agar plates supplemented with rifampin or penicillin in the concentrations shown in Fig. 1 at 37°C with 5% CO_2 for 48 h. Single colonies showing the desired characteristics (antibiotic resistance and rough colonial morphology) were transferred to fresh media as outlined in Figure 1. Stock and working cultures of RB51 were maintained as described above for the other *B. abortus* strains used in this study.

Determination of colony type

The colonial type (i.e. smooth or rough) of the bacterial strains was determined by autoagglutination (Braun and Bonestell, 1947), uptake of crystal violet by the colonies (White and Wilson, 1951) and confirmed by a colony blot enzyme-linked immunosorbance assay (ELISA) procedure (Roop et al., 1987) using a monoclonal antibody (mAb) (BRU 38) which recognizes the perosamine O-chain of the *B. abortus* LPS (Schurig et al., 1984). Briefly, the colony blot ELISA was carried out by blotting a sterile nitrocellulose disk (82mm) onto a 48 h culture of the strain to be tested. The disk was removed and the organisms lysed by immersion in chloroform. The disk was then treated with bovine serum albumin (BSA), DNase and lysozyme and an ELISA was performed with the mAb BRU38 (anti O-chain; Schurig et al.,

1984) as the primary antibody. After reaction with secondary antibody conjugated with horseradish peroxidase and exposure to substrate, colonies which possessed the perosamine O-chain as a component of their LPS (smooth strains) were stained purple. Rough colonies were not stained using this procedure. In addition, during the derivation of strain RB51, isolates were tested for their inability to absorb BRU38 antibody activity using a previously described procedure (Schurig et al., 1984).

Preparation of bacterial antigens

Crude bacterial cell wall preparations were obtained in the following manner. Bacterial growth from approximately 30 TSBA plates incubated at 37°C for 48 h was harvested in 60 ml distilled water and autoclaved at 121°C for 15 min. Twenty ml of glass beads (Glasperlen, 0.11 mm) were added and the bacterial cells disrupted in a Braun MSK cell homogenizer (B. Braun Instruments, Burlingame, CA) for 4 minutes. The lysate was filtered through a scintered glass filter to remove the glass beads. The lysate was centrifuged at 9500 *g* for 20 min at 4°C. The supernatant was discarded and the pellet resuspended and washed three times in distilled water by centrifugation. After the third washing, the suspension was centrifuged at 300 *g* for 10 min at 4°C. The supernatant, containing the cell wall preparation was lyophilized and stored at room temperature.

LPS was extracted from the bacterial cells using a modification of previously described procedures (Moreno et al., 1979; Wu et al., 1987). Bacterial growth from approximately 10 TSBA plates incubated as described above was harvested in 25 ml of 10 mM Tris, pH 8.0 and the cells were killed by the addition of an equal volume of acetone with overnight stirring. Cells were centrifuged at 13 000 *g* for 5 min at 4°C, washed once with 100 ml sterile distilled water, and resuspended in 45 mi sterile distilled water. Fifty-five ml of phenol was added and the cells incubated at 68°C for 40 min. The mixture was then centrifuged at 4°C for 10 min at 17 000 *g* and the phenol layer removed. Fifty five ml of fresh phenol was added to the cell suspension and the extraction process repeated. A total of three phenol extractions were performed. The aqueous phase was stored at 4°C. The phenol phases were pooled and washed a total of five times with hot (66°C) distilled water as described previously. LPS was precipitated from the phenol phase by the addition of 5 vol. of cold methanol reagent (99:1 methanol:methanol saturated with sodium acetate) followed by stirring at 4°C for 1 h. The resulting pellet was harvested by centrifugation as described above and dissolved in 10 ml distilled water. The LPS preparations from both aqueous and phenol phases were dialyzed overnight against distilled water and lyophilized.

Sonicates of *B. abortus* organisms were prepared by suspending acetone killed bacteria in saline to 5% transmittance (525 mm). The suspension was sonicated with a Fisher Sonic dismembrator (Fisher Scientific, Norcross, GA,

USA) model 300 set at 35% power for 30 seconds twice on ice, and the preparation was lyophilized.

Outer membrane proteins (OMPs) group 2 and 3 extracted from *B. abortus* strain RB51 (Santos et al., 1984) were obtained from Dr. Alex Winter, N.Y. State College of Veterinary Medicine, Cornell University, Ithaca, N.Y.

Passage of Strain RB51 in vivo and in vitro

Colonies of *B. abortus* Strain RB51 isolated from spleens of experimentally infected BALB/c mice at three weeks post infection were grown on plates as previously described and reinoculated intraperitoneally (i.p.) into two mice (1×10^8 c.f.u. per mouse). Mice were killed two weeks later, colonies isolated from the spleens were pooled and reinoculated into 2 mice. This procedure was repeated until 15 passages were completed.

Strain RB51 was passaged 93 times at 37°C, 5% CO_2 on plates approximately every third day of growth. Following each passage colonies were inspected for roughness by crystal violet staining and autoagglutination. Colonies from the final passage were tested in all the described assays including the colony blot ELISA with BRU38 (Roop et al., 1987) to detect reversion of individual colonies to smooth characteristics.

Immunization of rabbits, goats and cattle with acetone killed strain RB51

One New Zealand white rabbit (No. 3189), two goats (No. 48 and No. 47) and one steer (No. 66) were immunized with sonicated, acetone killed strain RB51 organisms. Animals were immunized three or four times subcutaneously with sonicate containing 250 μg protein in saline emulsified in Freund's complete adjuvant (first immunization) or Freund's incomplete adjuvant (following immunizations) 14 to 21 days apart. Animals were bled to obtain sera before immunizations and after the last immunization. Another steer (No. 121) was similarly immunized with sonicated, acetone killed strain 2308 organisms.

Clearance of strain RB51 from mice

Six groups of 5 BALB/c mice each were inoculated with 1×10^8 colony forming units (c.f.u.) of RB51 organisms i.p. (this does was selected because preliminary experiments with lower doses did not consistently infect mice). One group of mice was killed by CO_2 asphyxiation at 1, 2, 3, 4, 5 and 6 weeks post-innoculation and spleens were cultured for the presence of *B. abortus* strain RB51 organisms utilizing methods described by Montaraz and Winter (1986). Serum was obtained before infection by retroorbital bleeding and at termination by cardiac puncture.

Protection of mice against challenge with B. abortus *strain 2308*

Two groups of 10 BALB/c mice each were inoculated with 1×10^8 viable c.f.u. RB51 organisms in saline or with saline alone (control). Both groups of mice were challenged with 1×10^5 *B. abortus* strain 2308 organisms seven weeks later. Half of the mice in each group were killed one week postchallenge and the other half four weeks after challenge. Spleens were cultured at necropsy and the number of c.f.u. per spleen were determined as described above. Statistical analysis of the number of c.f.u.s was performed using one way analysis of variance followed by Duncan's multiple range test (Snedecor, 1956). Serology was not performed on these groups.

SDS–PAGE and western blot analysis

Crude cell wall preparations and sonicates (2.5 and 1.5 mg dry weight respectively) were suspended in 0.95 ml of 10 mM Tris–HCl, pH 8. Fifty μl of a freshly made solution of 1 mg/ml lysozyme (Sigma, St. Louis, MO, USA) in 10 mM Tris–HCl, pH 8.0 was added to the cell wall suspension and sonicates and the mixture incubated at 37°C for 2 h. Two mg of lyophilized LPS was suspended in 1 ml of 10 mM Tris–HCl, pH 8.0. Fifty μl each DNase (Sigma, St. Louis, MO, USA) and RNase (Sigma, St. Louis, MO, USA) were added to the crude cell wall, sonicate and LPS preparations and the mixtures incubated at 37°C for 1 h. Following DNase and RNase digestion, 50 μl of proteinase K (Sigma, St. Louis, MO, USA) was added only to the LPS preparation and incubation continued for 3 h at 56°C. Stock solutions of RNase, DNase and proteinase K were prepared at 1 mg/ml in 10 mM Tris–HCl, pH 8.0. An equal volume of double strength Laemmli sample buffer (Laemmli, 1970) was then added and the mixture heated at 100°C for 5 min. Electrophoresis was performed with a Hoefer Mighty Small II unit using the discontinuous SDS–PAGE system of Laemmli (1970) with a resolving gel containing 12.5% acrylamide. Twenty-five μl of each sample was applied per lane and 25 mA current was used per gel. Cooling was provided by circulating water at 4°C through the apparatus for the duration of the run. Electrophoresis was terminated when the dye front reached approximately 0.5 cm from the bottom of the gel. Pre-stained molecular weight markers (BioRad, Richmond, CA, USA) were used as standards.

Following electrophoresis, preparations were stained with silver (Hitchcock and Brown, 1983), Coomassie Blue (Hames and Rickwood, 1981), or transferred to nitrocellulose membranes using the procedure of Towbin et al. (1979). Transfer was performed for 2 h with 125 V at 4°C in a Transphor unit (Hoefer Scientific Instruments, San Francisco, CA). The nitrocellulose membrane was incubated in Tris buffered saline (TBS- 0.02 M Tris, 0.5 M NaCl, pH 7.5) containing 2% BSA for 1 h and then placed into an appropriate dilution of mAb BRU38 or a 1:50 to 1:100 dilution of sera in TBS and incubated overnight at room temperature. The nitrocellulose membrane was

washed by serial passage through five Petri plates each containing 25 ml of TBS with 0.5% Tween-20. The nitrocellulose was then incubated in a 1:800 dilution in TBS of the appropriate anti IgG for 1 h, followed by washing as described above. All secondary antibodies were heavy and light chain specific, conjugated with horseradish peroxidase and were obtained from Organon Teknika-Cappell, Malvern, PA. Following incubation with the secondary antibody and washing, the nitrocellulose was developed in 4-chloro-1-naphthol as described earlier for the RB51 whole cell ELISA.

Tube agglutination test

The tube agglutination test was performed according to methods described by Alton et al. (1975).

Biochemical analysis of strains

B. abortus strains 2308, RB51, 19, and 45/20 were streaked onto TSBA plates and TSBA plates supplemented with 10% defibrinated sheep blood and incubated under 5% CO_2 or under atmospheric conditions for 48 h at 37°C to assess the effect of blood and CO_2 supplementation on the growth characteristics of these strains. Growth from each plate was used to make a Gram stain, and the relative size differences of strains estimated by microscopic examination.

The above strains were tested for their urease and nitrate reductase activity and ability to utilize erythritol and glucose using conventional biochemical techniques (McFaddin, 1980). Unless otherwise indicated, inoculated media were incubated at 37°C for 24 to 48 h. In addition, these four strains were tested in the API Rapid NFT test system (Analytab Products, Plainview, NY, USA). The tests were performed according to the manufacturer's instructions and each strain was tested at least three times.

The susceptibilities of the four strains to a selection of antibiotics were determined using a modification of the agar diffusion method of Bauer et al. (1966). Briefly, Mueller Hinton agar supplemented with 5% defibrinated bovine blood served as a basal medium and zones of inhibition were measured after 36 h growth at 37°C. The following antibiotics were tested using this procedure, the disk concentration is given in parentheses: ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), oxytetracycline (30 μg), penicillin (10 U), streptomycin (10 μg), triple sulfa (1 mg), carbenicillin (100 μg), cephalothin (30 μg), cloxacillin (5 μg), furazolidone (100 μg), methicillin (5 μg), nitrofurantoin (300 μg), tetracycline (30 μg), neomycin (30 μg), trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg), oxacillin (1 μg) and rifampin (2.5, 5.0 and 10 μg). Susceptibility to rifampin was also measured by growing the strains for 48 h on TSBA at 37°C with 5% CO_2 and using a single colony to streak TSBA plates containing 50, 100, 200 and 400 μg/ml of rifampin (Sigma Chemical

Co., St. Louis, MO, USA). The plates were examined for growth after 48 h under the above described conditions.

RESULTS

After three passages of *B. abortus* 2308 on varying concentrations of rifampin, colonies arose which demonstrated rough colonial morphology, as measured by crystal violet uptake and autoagglutination in acriflavine. One of these colonies, designated RB, was passed further on various concentrations of rifampin and penicillin, resulting in a clone, designated RB19 which was unable to absorb BRU38, a monoclonal antibody specific for the perosamine O-chain of *B. abortus*. This clone was passed several times on unsupplemented TSBA plates, in order to stabilize the putative mutation, resulting in the strain designated RB51 (Fig. 1).

Colonies of 48 h old cultures of RB51 and 45/20 were stained with crystal violet and suspensions of bacterial cells from these cultures autoagglutinated. In contrast, bacterial colonies of strains 2308 and 19 did not take up crystal violet, nor did suspensions of strains 2308 and 19 autoagglutinate. Silver stained SDS–PAGE profiles of LPS preparations revealed the presence of O-chain like material in LPS extracted from strains 2308 and 19, but not in LPS

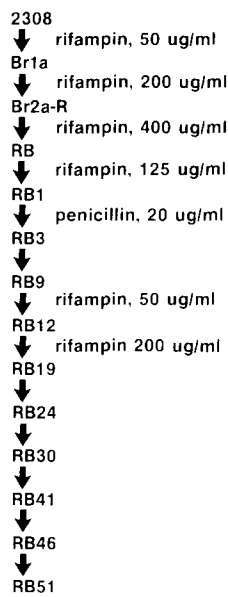

Fig. 1. Derivation of *B. abortus* strain RB51. All incubations were carried out at 37°C for 48 h with 5% CO_2 on unsupplemented TSBA plates or TSBA agar plates supplemented with the antibiotic at the concentration shown.

extracted from RB51 or 45/20 (Fig. 2). As reported by others (Moreno et al., 1979; Corbeil et al., 1988) the O-chain material of the smooth *B. abortus* strains appeared as a diffuse smear ranging from 30 to over 100 kDa on the gel, rather than the clear ladder type of appearance commonly associated with members of the Enterobacteriaceae. This latter profile was observed with the commercial LPS preparation extracted from *Escherichia coli* 0111:B4 (Fig. 2). The mAb BRU 38 reacted well and consistently with the LPS prepared from strains 2308, 19 and 45/20 by western blots, but gave only a very faint and narrow reaction with the LPS isolated from RB51 (Fig. 3). BRU 38 clearly detected O-chain antigen in cell wall preparations from strains 2308 and 19 and detected traces in 45/20 by western blot analysis. No O-side chain was detected by BRU38 in cell wall preparations from RB51 or the commercial preparation of *E. coli* LPS (Fig. 3). Coomassie Blue stained SDS–PAGE profiles of cell wall preparations were similar for all four Brucella strains tested (Fig. 4).

Strain RB51 did not require CO_2 or blood in the medium for growth. Biochemical characteristics of strains RB51, 45/20, and 19 are summarized in (Table 1). When tested in the API NFT system, 2308 produced the profile number 1 241 044, while the profile number 0 240 004 was obtained for the other three strains. All four strains were susceptible to ampicillin, chloramphenicol, gentamicin, kanamycin, neomycin, oxytetracycline, carbenicillin,

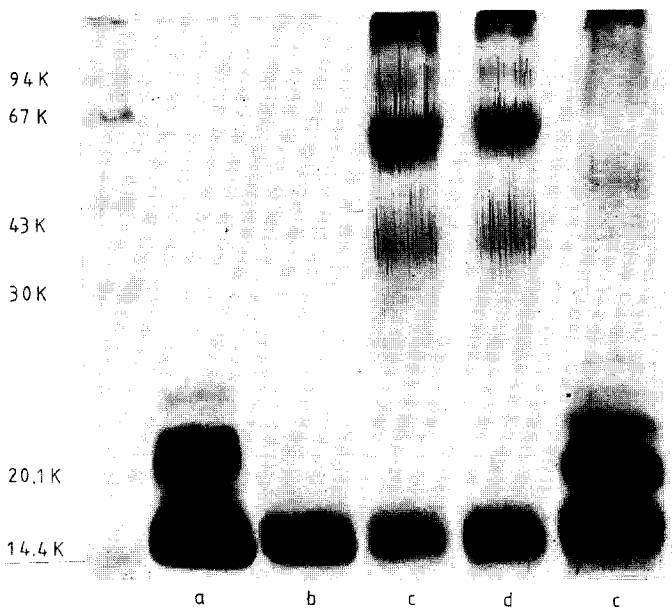

Fig. 2. Silver stained SDS–PAGE profiles of LPS preparations from the *B. abortus* strains used in this study. (a) RB51, (b) 45/20, (c) 19, (d) 2308, (e) *E. coli* 0111:B4.

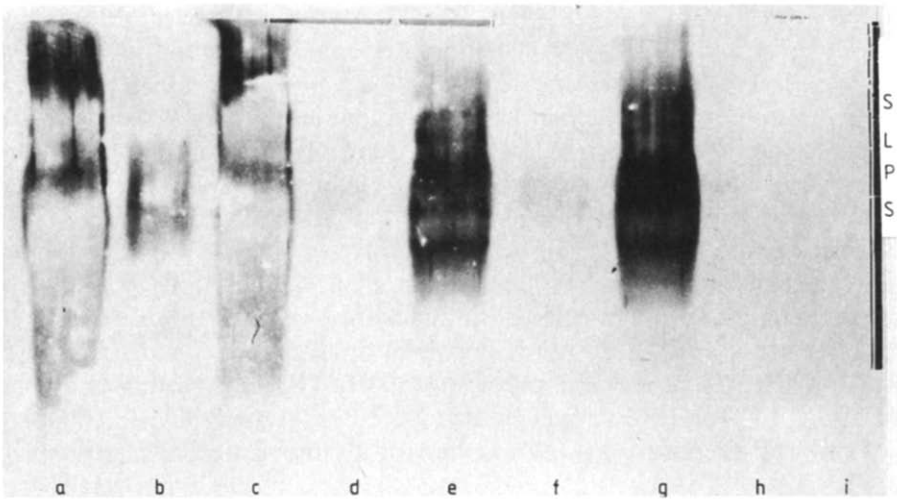

Fig. 3. Immunoblot using mAb BRU38 with LPS and cell wall preparations from the *B. abortus* strains used in this study. (a) 2308 LPS, (b) 45/20 LPS, (c) 19 LPS, (d) RB51 LPS, (e) 2308 cell wall, (f) 45/20 cell wall, (g) 19 cell wall, (h) RB51 cell wall, (i) *E. coli* 0111:B4LPS. S-LPS: Region corresponding to typical O-chain reaction of smooth *B. abortus* strains.

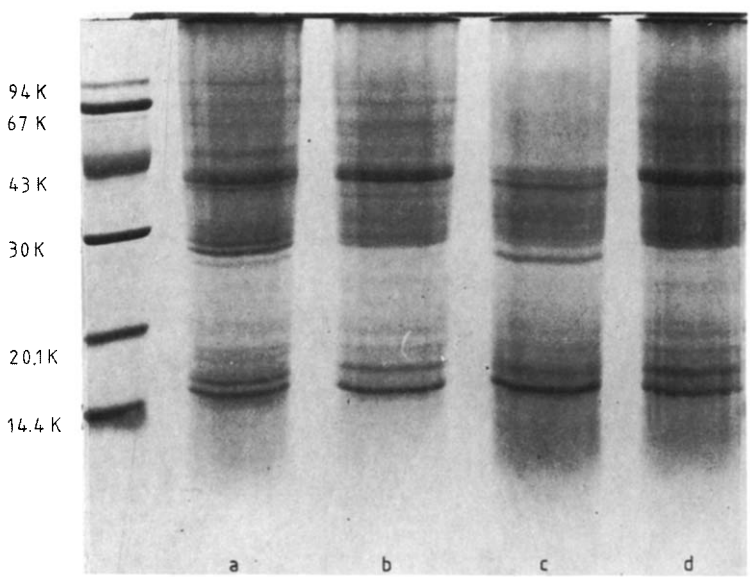

Fig. 4. Coomassie Blue stained SDS–PAGE profiles of crude cell wall preparations from the *B. abortus* strains used in this study. (a) 2308, (b) 19, (c) 45/20, (d) RB51.

TABLE 1

Selected biochemical reactions and antibiotic susceptibility of *B. abortus* strains RB51, 2308, 19 and 45/20.

Item	RB51	2308	19	45/20
Urease	+	+	+	+
Oxidase	+	+	+	+
Nitrate reduction	−	+	−	−
Auxanographic growth with:				
glucose	+	+	+	+
arabinose	−	+	−	−
malate	−	+	−	−
Growth in the presence of				
0.5% erythritol	+	+	−	+
Susceptibility to				
rifampin	−	+	+	+

TABLE 2

Isolation of *B. abortus* strain RB51 from spleens of mice injected i.p. with 1×10^8 c.f.u. of RB51 at 1 through 6 weeks postinoculation

Group	Week after inoculation	Log_{10} c.f.u. Brucellae in spleen (mean ± SD)
1	1	4.09 ± 3.03
2	2	3.19 ± 2.13
3	3	2.35–1.39
4	4	0
5	5	0
6	6	0

Groups of 5 mice

TABLE 3

Protection of mice against challenge[1] with *B. abortus* strains 2308 after immunization with 1×10^8 cfu of strain RB51 i.p.

Group	Vaccination	Log_{10} c.f.u. of *B. abortus* strain 2308 in spleens (mean ± SD)	
		1 week postchallenge	4 weeks postchallenge
1	RB51	3.62 ± 3.34*	4.54 ± 4.27*
2	Saline	5.38 ± 5.07	5.78 ± 4.73*

[1]Mice were challenged ip with 3.3×10^4 strain 2308 organisms seven weeks post vaccination.
*Value significantly different ($P < 0.05$).

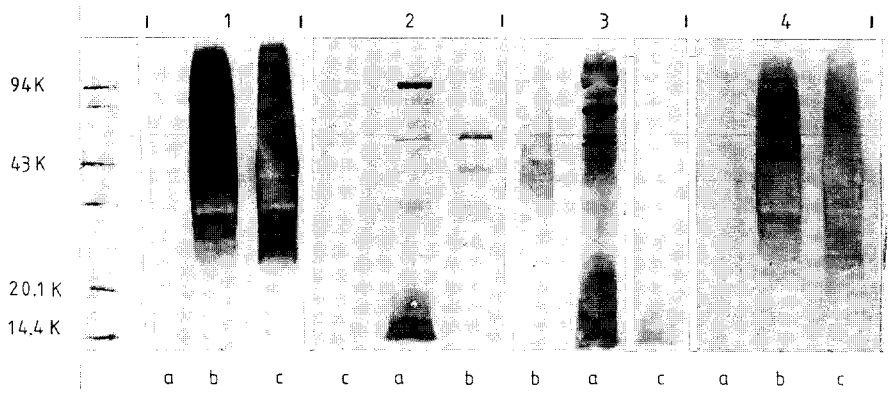

Fig. 5. Western blot analysis of mAb BRU38, cattle and mice sera. Molecular weight standards to the left; a: *B. abortus* strain RB51 sonicate; b: *B. abortus* strain 2308 sonicate; c: *Y. enterocolitica* 0:9 LPS; 1: Reacted with BRU38; 2: Reacted with mice sera obtained 4 weeks after infection with RB51 organisms; 3: Reacted with steer No. 66 (immunized with sonicated RB51); 4: Reacted with steer No. 121 (immunized with sonicated 2308). Note that absence of reactivity with *Y. enterocolitica* 0:9 LPS indicates absence of anti-O-side chain antibodies.

cephalothin, streptomycin, nitrofurantoin, tetracycline, and trimethoprim/sulfamethoxazole. Strains 19 and 45/20 were susceptible to triple sulfa, while strains 2308 and RB51 were resistant. All strains showed intermediate susceptibility to penicillin except RB51, which was resistant. In addition, strains 2308 and 45/20 were intermediately susceptible to furazolidone and erythromycin. Strain 19 was susceptible to erythromycin and furazolidone. RB51 was susceptible to erythromycin and resistant to furazolidone. RB51 was the only strain which was resistant to rifampin while strains 2308, 19 and 45/20 showed zones of inhibition ranging from 12 to 22 mm in diameter. Strain RB51 grew as well on TSBA plates supplemented with 400 μg/ml rifampin after 48 h as it did on the unsupplemented TSBA control plates. The other Brucella strains produced only a few colonies on each of the concentrations of Rifampin tested, but grew well within 48 h on the TSBA control plates.

RB51 organisms isolated from mice after in vivo passage as well as organisms passaged in vitro retained all the original characteristics of RB51 described above indicating antigenic and biochemical stability. Rough, *B. abortus* strain RB51 organisms were recovered from the spleen of BALB/c mice in diminishing numbers through the third week post inoculation (Table 2). No organisms were detected 4 weeks post inoculation. Mice sera did not react against the O-chain as demonstrated by a negative reaction against *Y. enterocolitica* 0:9 LPS in western blots (Fig. 5) and no reactivity with LPS from strain 2308. The sera did react against a variety of antigens present in strains RB51 and 2308 (Fig. 5) including group 2 and 3 OMPs (data not

shown). Agglutinating antibody titers as measured by the tube agglutination test from pre- and postinfection sera did not change (all $< 1:25$).

Post immunization sera from the rabbit, steer, and goats immunized with the strain RB51 sonicate in adjuvant did not react with *Y. enterocolitica* 0:9 crude LPS in western blots (Fig. 5) and agglutination titers among pre and post inoculative serum samples did not change remaining below 1:25.

One i.p. inoculation of 1×10^8 viable c.f.u. RB51 organisms 7 weeks prior to challenge significantly reduced the spleen colony counts at 1 and 4 weeks post challenge ($P < 0.05$).

DISCUSSION

Passage of *B. abortus* 2308 on TSBA plates supplemented with various concentrations of rifampin resulted in the appearance of colonies which exhibited characteristics associated with "rough" *Brucella* colonies, i.e. uptake of crystal violet (White and Wilson, 1951) and autoagglutination (Braun and Bonestell, 1947) when suspended in a neutral solution of acriflavine. These organisms were also unable to absorb the mAb, BRU 38, which is specific for the perosamine O-chain of the LPS of smooth *Brucella* species (Schurig et al., 1984). Rifampin was utilized because it had been observed previously in our laboratory that the addition of rifampicin to the media tended to turn *B. abortus* cultures rough. Also, reports in the literature indicated that organisms resistant to rifampicin are less virulent than rifampicin susceptible strains (Moorman and Mandell, 1981).

After several subcultures on unsupplemented TSBA plates the strain did not revert to the smooth form; this mutant was given the designation RB51. The strain has been used extensively in our laboratory over the last 9 years and distributed to others upon request (Santos et al., 1984; Corbeil et al., 1988; Ficht et al., 1988; Smith et al., 1990a,b). Strain RB51 is stable in vitro and no reversion to smooth form has been observed since its derivation. No reversion was observed in this controlled experiment consisting of 93 in vitro passages. Fifteen passages in mice indicate that RB51 is stable in vivo. Recent observations using RB51 isolates obtained from experimentally infected cattle (Enright et al., 1990) also confirm this stability.

When tested in the colony blot ELISA with BRU38 RB51 whole cells did not react suggesting complete absence of O-chain. The absence of an O-chain reaction on silver stained SDS–PAGE profiles of LPS from strains 45/20 and RB51 agrees with the findings of others (Corbeil et al., 1988). Nevertheless, strain 45/20 clearly demonstrated the presence of the O-chain in western blot analysis with BRU38 confirming previous reports of Schurig et al. (1984). LPS extracted from RB51 (aqueous phase) demonstrated a very faint reaction with BRU38 suggesting that strain RB51 could possess trace amounts of O-chain. The absence of such reactions with the crude cell wall preparations

of RB51 suggests that these trace amounts are not found in this preparation or are washed away during processing. The presence of a clear and consistent O-chain reaction in western blots with "rough" strain 45/20 in contrast to the lack of a visible reaction in silver stained SDS–PAGE advises against the use of this staining method as a sole indicator of the presence or absence of O-chain in *Brucella* spp.

The virtual lack of O-chain in strain RB51 makes this strain useful as a source of cell component preparations for assaying the immune response of infected animals to *B. abortus* antigens other than those associated with the O-chain. Although RB51 is highly deficient in LPS O-chain content as demonstrated in this study most, if not all of the potentially important cell wall associated antigens of *B. abortus* appear to be present. Electrophoretic separation of crude cell wall preparations from strains RB51, 45/20, 19 and 2308 results in almost identical profiles (Fig. 4) and Santos et al. (1984) have shown that RB51 possesses the Groups 1, 2 and 3 outer membrane proteins described for *B. abortus*.

The growth requirements of *B. abortus* RB51 are similar to those of the other laboratory strains of *B. abortus*, 2308, 19 and 45/20 used in this study. All four strains grew on TSBA without blood or supplemental CO_2, but all four grew better with both blood and CO_2 present. This is in contrast to a group of field isolates of *B. abortus* representing biovars 1, 2 and 4, plus an untyped strain, which would not grow under the same conditions without the presence of blood and additional CO_2 (data not shown). Microscopic examination of Gram-stained preparations revealed that RB51 was more similar in size and cellular morphology to the parental strain 2308 and the field strains that it was to strain 45/20 or 19. On the other hand, RB51 was more similar in its carbohydrate utilization profile and failure to reduce nitrate to strains 19 and 45/20, than it was to 2308. It is possible that in the course of mutation to rough morphology, RB51 also underwent mutations affecting its metabolic capabilities. It is noteworthy, however, that RB51 retained its ability to grow in the presence of erythritol. This sugar alcohol is present in large quantities in the gravid uterus (Keppie et al., 1965), and wild type strains of *B. abortus* appear to use this compound in preference to glucose as an energy source. For this reason, the ability to utilize erythritol has been proposed by some as an important factor in the pathogenesis of Brucella-associated abortion in cattle (Keppie et al., 1985). Strain 19, the currently used live, attenuated *B. abortus* vaccine strain, will not grow in the presence of erythritol (McFaddin, 1980), it will, however, in some cases induce abortion (Subcommittee on Brucellosis Research, 1977). The antibiotic susceptibility profiles obtained for strains 2308, 45/20 and 19 are very similar to those reported by others (Hall and Manion, 1970).

Mice are currently used as a model for studying some aspects of bovine brucellosis (Montaraz and Winter, 1986). BALB/c mice infected with RB51 by the ip route cleared the organism from their spleens within 4 weeks. This

is in distinct contrast with strain 2308 which establish a chronic infection that may last as long as 24 weeks (Montaraz and Winter, 1986). At present, it can not be established if strain RB51 is able to replicate within mice due to the relatively low number of c.f.u. observed 1 week after the inoculation of 1×10^8 strain RB51 organisms as well as its gradual elimination over the next 2 to 3 weeks. This question can only be resolved by determining the c.f.u. obtained at shorter time intervals after RB51 inoculation. Nevertheless, the presence of the viable organisms in the spleen for at least 3 weeks post inoculation leads to a strong antibody response to *B. abortus* cellular antigens other than the LPS O-chain (Fig. 5). This suggestion is sustained by the absence of serological reactions against *Y. enterocolitica* 0:9 crude LPS as demonstrated by western blot analysis (Fig. 5). *Y. enterocolitica* 0:9 contains an O-antigen which is nearly identical to the A antigen (O-chain) of *B. abortus* (Ahvonen et al., 1969; Caroff et al., 1984a,b; Meikle et al., 1989) and therefore antibodies to the O-chain of *B. abortus* will react with *Y. enterocolitica* 0:9. This characteristic is useful to establish if sera of animals immunized with *B. abortus* contain antibodies to the O-chain. The absence of agglutinating antibodies detectable in the agglutination test also suggests that anti-O antibodies were not found in the RB51 infected mice. These observations, coupled to the absence of detectable anti O-side chain antibodies in sera of rabbits, goats and cattle immunized with killed RB51 organisms indicates that RB51 is unable to elicit a humoral immune response to the O-chain at least over a period of seven weeks post infection or immunization in a number of animal species.

One inoculation of viable RB51 i.p. conferred significant protection at one and four weeks post strain 2308 challenge. This observation is in agreement with recent adoptive transfer experiments with nylon wool purified spleen lymphocytes obtained from RB51 immunized mice which are capable of transferring protection (Bagchi and Schurig, 1990) at levels comparable to that obtained with lymphocytes from strain 19 immunized mice (Araya and Winter, 1989). Also, preliminary experiments with adult cattle indicate that RB51 immunization can protect this species against abortion induced by strain 2308 and this protection was better than the one induced by Strain 19 (Enright et al., 1990). Hence, the observations described here with the commonly used mouse model for bovine brucellosis suggest that RB51 has the potential of being used as a protective, live vaccine which would not elicit the O-chain specific humoral immune response which interferes with standard serological tests for diagnosing brucellosis. An additional attractive feature of RB51 is its high level of resistance to rifampin which would serve as a useful marker for the identification of this strain.

ACKNOWLEDGEMENTS

The authors thank Jan Simmers and Betty Davis for excellent technical assistance. This report is based on work supported by grant nos. 84-CRSR-2-

2421 and 85-CRCR-1-1848 from the United States Department of Agriculture. Any opinions, findings, conclusions and recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S.D.A.

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