

THE BEHAVIOR AND EFFECTS OF BRUCELLA ABORTUS  
ROUGH STRAIN RB51 IN MICE AND CATTLE

by

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(ABSTRACT)

Brucella abortus st. RB51 is a rough mutant of smooth st. 2308 devoid of O-side chain and resistant to rifampin. The purpose of this investigation was to study the behavior and effects of viable st. RB51 organisms in inoculated mice and cattle and to further substantiate the lack of O-side chain antigens in this strain.

A single injection of live st. RB51 persisted in BALB/C mice up to 28 days. A secondary exposure was cleared in 7-21 days. One or 2 injections of st. RB51 did not induce detectable titers of anti-O-side chain antibodies, although antibody titers to st. RB51 whole cell and cytoplasmic antigens were detected. Mice infected with st. RB51 alone or followed by infection with st. 2308, demonstrated a very strong reaction to a 14-18 Kd antigen which was believed to be the core of the LPS complex. When st. RB51 was administered after injection of st. 2308 the response to the core determinants were inhibited. One vaccination with st. RB51 was able to significantly protect mice against challenge with st. 2308 at one and four weeks post challenge. Two st. RB51

vaccinations were able to protect mice as well as one vaccination at one week post challenge but protection increased by four weeks post challenge.

Strain RB51 was able to survive in cattle a for at least twenty-two days. The organism remained stable, rifampin resistant, and may have induced minor amounts of transient anti-O-side chain antibodies in some cows late in the experiments.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS . . . . . p. iv

LIST OF TABLES . . . . . p. vi

LIST OF FIGURES . . . . . p. viii

INTRODUCTION . . . . . p. 1

LITERATURE REVIEW . . . . . p. 3

MATERIALS & METHODS . . . . . p. 21

CHAPTER I: Experimental infection of mice  
with B. abortus strain RB51:  
clearance of the organism, humoral  
immune response elicited and  
protective effects

RATIONALE . . . . . p. 35

EXPERIMENTAL DESIGN . . . . . p. 36

RESULTS . . . . . p. 41

DISCUSSION . . . . . p. 61

CONCLUSIONS . . . . . p. 80

CHAPTER II: Experimental infection of cattle  
with B. abortus strain RB51.

RATIONALE . . . . . p. 82

EXPERIMENTAL DESIGN . . . . . p. 83

RESULTS . . . . . p. 88

DISCUSSION . . . . . p. 106

CONCLUSIONS . . . . . p. 117

LITERATURE CITED . . . . . p. 119

VITA . . . . . p. 130

## LIST OF TABLES

### Table

I	<u>B. abortus</u> rough strain RB51 clearance experimental design . . . . .	p. 37
II	Humoral immune response of mice infected with different combinations of rough and smooth strains of <u>B. abortus</u> experimental design . . . . .	p. 39
III	Extension of the humoral immune response experimental design . . . . .	p. 40
IV	Protection against challenge with 2308 in RB51 vaccinated mice experimental design . . . . .	p. 42
V	Splenic clearance rates of single and double injections of RB51 . . . . .	p. 43
VI	Average antibody titers of individual mouse sera to RB51 whole cells (W.C.) after one or two exposures to live RB51 . . . . .	p. 45
VII	Clean vs. infected spleens and cfu/spleen of mice infected with different combinations of rough and smooth strains of <u>B. abortus</u> . . . . .	p. 46
VIII	SAT titers of pooled sera following different combinations of rough and smooth strains of <u>B. abortus</u> . . . . .	p. 48
IX	Clean vs. infected spleens and average cfu/spleen . . . . .	p. 52
X	RB51 W.C. antibody titers and SAT titers of pooled mice sera . . . . .	p. 53
XI	Clean vs. infected spleens and average organisms/spleen for RB51 protection study . . . . .	p. 57
XII	RB51 W.C. antibody titers and SAT titers of pooled mice sera . . . . .	p. 59
XIII	Effect to subcutaneous (SC) injections of live RB51 in cattle experimental design . . . . .	p. 84

XIV	Effect of one subcutaneous (SC) injection of live RB51 in cattle experimental design . . . . .	p. 86
XV	Effect of one conjunctivally administered dose of live RB51 in cattle experimental design . . . . .	p. 87
XVI	Lymph node biopsy results from S.C. infected cattle . . . . .	p. 89
XVII	Acridine agglutination (AGG), crystal violet uptake (C.V.), and rifampin resistance (R.R.) of lymph node isolates . . . . .	p. 90
XVIII	Reaction of lymph node isolates in the rapid identification assay with monoclonal antibodies BRU 38 and BRU 48 . . . . .	p. 91
XIX	Reactions of calf tissue culture isolates in the acridine, crystal violet, rifampin resistance and rapid identification assay . . . . .	p. 93
XX	Clearance of isolated RB51 in mice . . . . .	p. 95
XXI	RB51 W.C. antibody titers and SAT results . . . . .	p. 96
XXII	State laboratory SAT results and BAPA results . . . . .	p. 97
XXIII	Acridine agglutination (AGG), crystal violet uptake (C.V.), rifampin resistance (R.R.) and rapid identification tests of 22 dpi lymph node biopsies . . . . .	p. 104
XXIV	RB51 W.C. antibody titers and SAT results . . . . .	p. 105
XXV	RB51 W.C. antibody titers and SAT results . . . . .	p. 107

## LIST OF FIGURES

### Figures

- I Western blot analysis of pooled mice sera infected with different combinations of rough and smooth strains of B. abortus against Y. enterocolitica 0:9 LPS antigen . . . . . p. 49
- II Western blot analysis of pooled mice sera infected with different combinations of rough and smooth strains of B. abortus against RB51 W.C. antigen . . . . . p. 50
- III Western blot analysis of pooled mice sera against RB51 W.C. antigen . . . . . p. 55
- IV Western blot analysis of pooled mice sera against Y. enterocolitica 0:9 LPS antigen . . . . . p. 56
- V Western blot analysis of pooled mice sera against RB51 W.C. antigen . . . . . p. 60
- VI Western blot analysis of pooled mice sera against Y. enterocolitica 0:9 LPS antigen . . . . . p. 62
- VII Rapid identification assay of bovine lymph node isolate utilizing monoclonal antibodies BRU 38 (anti-O-side chain) and BRU 48 (anti-core determinants) . . . . . p. 92
- VIII Western blot analysis of N0133 against RB51 and W.C. and Y. enterocolitica 0:9 LPS antigens . . . . . p. 99
- IX Western blot analysis of Y141 against RB51 W.C. and Y. enterocolitica 0:9 LPS antigens . . . . . p. 100
- X Western blot analysis of N0132 against RB51 W.C. and Y. enterocolitica 0:9 LPS antigens . . . . . p. 101
- XI Western blot analysis of positive control animals against RB51 LPS, Y. enterocolitica 0:9 LPS, and RB51 W.C. antigens . . . . . p. 102



## INTRODUCTION

Bovine brucellosis is a zoonotic disease characterized by abortion, decreased fertility and decreased milk production in cattle herds (1,2). Because of severe economic losses to cattlemen caused by these characteristics and the health hazards associated with the disease in people, the Department of Agriculture has conducted a nationwide Brucellosis Eradication Program based on vaccination, serological testing and slaughter of serologically positive animals since 1940 (1). Although the program has greatly reduced the incidence of the disease in the U.S.A., bovine brucellosis continues to be a costly problem to cattle producers as well as to the federal government.

The accepted method of calfhood vaccination in the U.S.A. is with attenuated Brucella abortus st. 19. This strain can cause abortions in cattle (3) and vaccination may lead to persistent antibody titers which complicates accurate diagnosis of the disease by serological assays. Antibody titers resulting from vaccination cannot be distinguished easily from those caused by natural exposure. In addition, st. 19 has a reported efficacy of only 65-75% in calfhood vaccinates. For these reasons a new vaccine would be desirable which would protect a higher percentage of animals, which would not cause disease because of vaccination, which would not induce a humoral immune response complicating serologic diagnosis but would establish an adequate protective immune response.

Vaccinating with st. 19 induces antibodies directed against the O-side chain of the lipopolysaccharide (LPS) molecule. The resulting antibodies are indistinguishable from the antibodies due to an infection. Development of B. abortus st. RB51 lacking the O-side chain has created an interest in the immune response post-infection with this strain and its capability to induce protective immunity against virulent field strains. Previous attempts to confer protective immunity with nonviable Brucella have not been successful (4, 5), therefore the in vivo survivability and stability of st. RB51 must be evaluated if this strain can be considered as a vaccine candidate.

The objectives of this research were:

1. To determine if st. RB51 is capable of establishing an infection in mice and cattle. If so, to study the humoral immune response in those animals.
2. To study the stability and survivability of st. RB51 by analyzing isolates obtained from st. RB51 infected mice and cattle.
3. To determine if mice can be protected from field strain infections after vaccination with st. RB51.
4. To determine if infection with virulent smooth B. abortus (st. 2308) affects the humoral immune response to rough B. abortus (st. RB51) antigens and vice versa.

## Literature Review

### HISTORY AND PATHOGENESIS OF BRUCELLA

Brucella abortus is a small gram negative coccobacilli belonging to the genus Brucella. The genus consists of six species including B. abortus, B. melitensis, B. suis, B. ovis, B. canis and B. neotomae (6). The primary reservoirs of these bacteria are cattle, goats, sheep, dogs and the desert wood rat, respectively, although cross infection can occur and infections of species not named may also occur.

Although the disease in humans was described by Marston in 1861, Sir David Bruce isolated B. melitensis in 1887 and designated it Micrococcus melitensis (6). In 1862, the presence of the bacteria between fetal membranes and the uterine wall of a pregnant cow was observed. However, not until 1897 did Bang, a Danish veterinarian, isolate B. abortus, and he linked the bacteria to infectious abortions. B. suis was isolated from a premature pig in 1914 (6). The genus was recognized after the close bacteriologic and serologic relationships had been elucidated by Evans. The organism was named Brucella in honor of Sir David Bruce (6).

B. abortus, B. melitensis, B. suis and B. neotomae naturally are smooth organisms with a LPS molecule containing an O-side chain (6). B. canis and B. ovis are naturally occurring rough organisms with a LPS molecule containing no O-side chain. Truly rough strains do not possess the O antigen.

All of the species of Brucella except B. ovis and B. neotomae are capable of causing human brucellosis. The disease in humans is commonly called Malta or undulant fever. The disease is characterized by intermittant fever, fatigue and myalgia and often leads to long periods of invalidism (1).

In contrast, bovine brucellosis is characterized by abortion, decreased milk production, decreased fertility in the cow, and orchitis in the bull. The primary modes of transmission in cattle are the oral route, conjunctiva and respiratory mucosa. The urogenital tract, skin, teat canals and parenteral route can also serve as the site of entry (1,2). The disease is primarily caused by infection with B. abortus, but B. suis and B. melitensis are occasionally implicated (2).

Caprine brucellosis is similar to bovine brucellosis. In addition, arthritis, and chronic bronchitis are observed. The infection is caused by B. melitensis and occasionally by B. abortus (2). Exposure is similar to bovine brucellosis.

Porcine brucellosis is characterized by manifestations similar to bovine and caprine infections. Although the causative agent is almost exclusively B. suis, but B. abortus is occasionally isolated. Transmission occurs primarily by animal-to-animal contact usually by ingestion of infected material or venereally (2).

Canine brucellosis is characterized by abortions, stillbirths, and conception failures (2). Transmission can occur congenitally, venereally or by ingestion of infected material (2). B. canis is the

predominant causative agent of canine brucellosis although B. abortus, B. melitensis, and B. suis have been isolated from dogs. These latter species of Brucella do not cause the typical disease (induced by B. canis) in dogs.

Ovine brucellosis is characterized by epididymitis, orchitis, impaired fertility, abortion, placentitis and perinatal mortality (2). B. ovis is not known to cause natural infection in other animals, species or in people.

#### VACCINATION AGAINST B. ABORTUS

In an attempt to control and eventually eradicate outbreaks of brucellosis in beef and dairy herds U.S.D.A. established a nationwide Brucellosis Eradication Program in 1940. The methods and rules of the program were under continual revision until 1947. At that time the U.S.D.A. established "Recommended Uniform Methods and Rules." These rules state the minimum standards for states to achieve eradication.

Before 1932 the U.S. Bureau of Animal Industry (BAI) allowed vaccination of cattle with live, virulent B. abortus strains to protect against the uncontrollable spread of the disease. Several investigators (7-10) identified problems with the use of such vaccines, including contamination of marketed vaccines with B. suis. Upon this revelation, the BAI banned the use of these virulent vaccines and only permitted the production and use of BAI st. 19 or other strains of apparent low virulence (1).

Encouraging results from many studies (11-16) prompted the BAI in 1936 to carry out a large scale field study utilizing st. 19 as a calfhood vaccine. Of 8,182 heifer calves vaccinated 96.2 % did not abort and had normal deliveries (1). As a result of these statistics, calfhood vaccination with st. 19 was adopted as an adjunct to the official "Test and Slaughter" policy (1).

Since its inclusion into the federal-state brucellosis eradication program in 1941 (1), st. 19 vaccination has revealed some inherent problems associated with the use of this vaccine. Strain 19 was determined by Manthei (17) using data compiled at the Animal Disease Laboratory that only 65-75% of calfhood vaccinates were adequately protected against most kinds of exposure (1). How long resistance lasts is unknown, although McDiarmid (18) concluded that one dose of st. 19 conferred immunity for at least five pregnancies and probably for the normal milking life of the cow. These conclusions supported the work done by Goode et al. (19). These studies were corroborated by Manthei's work in 1959 (17).

The persistence of st. 19 in some vaccinated cattle has also been well documented since the widespread use of the vaccine (3, 20-25). The dangers associated with persistent infection with the vaccine strain are colonization of the udder and subsequent shedding of virulent organisms in the milk, shedding from vaginal secretions, venereal transmission and infection of fetuses and neonates. The strain can cause abortions in cattle particularly if administered during pregnancy.

A primary problem hindering total eradication is the difficulty in accurate diagnosis of infected cattle (1). To add to the disadvantages of using st. 19 for vaccination, this strain may induce antibody titers that may persist for years in some individuals. From the National Research Council, 1977, "In all available literature and reports of diagnostic tests for bovine brucellosis, there were no statistically valid data to indicate any serological test(s) available that are capable of distinguishing between vaccinal titers and titers due to field strain infection" (1).

Smooth field strains as well as the attenuated, smooth st. 19 produce similar humoral immune responses, therefore, antibodies induced by st. 19 are indistinguishable from those induced during an actual infection. The humoral immune response is primarily directed toward the O-side chain component of the LPS molecule which appears to be the immunodominant antigen in smooth brucella strains (26). The O-side chain possessed by smooth brucella strains is known to be a homopolymer of perosamine (27).

Another vaccine, st. 45/20, has been studied extensively in vitro and in vivo. Much of the early work with st. 45/20 was performed by McEwen and Roberts in 1936 (28). Upon passing st. 45 in guinea pigs a rough variant was isolated and designated st. 45/20. The strain appeared promising because it was rough as judged by colony morphology. Because of the difficulty with serologic diagnosis due to indistinguishable anti-O-side chain antibodies, researchers felt the

lack of O-side chain would be advantageous for the vaccination program. But, results from various studies suggest that rough st. 45/20 is unstable, virulent (causes abortions), and not totally devoid of the "O" component of the LPS molecule (29-34).

Although existing rough variants of *B. abortus* are not stable or as protective as st. 19 after one dose (35), they possess characteristics desirable in a vaccine. A rough variant would allow the induction of a humoral immune response to other potentially important antigens without O-chain antibody interference. The lack of anti-perosamine induced antibodies would alleviate the difficulties with serological diagnosis. Therefore development of an effective subcellular vaccine devoid of O-side chain contamination may be advantageous.

*B. abortus* rough st. RB51 was derived by in vitro repeated passage of field st. 2308 on TSBA plates containing rifampin in the laboratory of G. Schurig (26). This rifampin resistant rough mutant is stable in vitro and in vivo (26). No reversion to smooth form has been observed in this strain for over seven years. Electrophoretic separation of cell wall preparations from strains RB51, 45/20, 19, and 2308 show almost identical protein profiles (26) and Groups 1, 2, and 3 outer membrane proteins (OMP) do exist in st. RB51 as demonstrated by Santos et al. (36). The lack of the O-side chain on this strain has been demonstrated (26). Strain RB51 autoagglutinates when suspended in acriflavine (37) and individual colonies take up crystal violet (38). The organism is unable to absorb out the activity of monoclonal antibodies (Bru 38)



specific for the O-side chain of smooth Brucella species (26, 39). Silver-stained electrophoretic profiles of LPS preparations from st. RB51 show no indication of the presence of O-side chain (26, 40). Strain RB51 whole cells, LPS, and cell wall preparations fail to react with Bru 38 in Western Blot analysis and the Rapid Identification colony blot ELISA. All of these findings indicate st. RB51 is a truly rough strain of B. abortus.

#### OUTER MEMBRANE PROTEINS (OMP) OF B. ABORTUS

OMP of Brucella have been isolated and studied by several investigators (41-48). The principle OMP's of Brucella are the Group 2 and Group 3 proteins and the lipoprotein (44,48). Douglas et al. (43) has demonstrated that Group 2 proteins are porins of Brucella. The porins of Brucella were designated Group 2 proteins by Verstrete et al. (48) before their identification and function as porins by Douglas et al. (43).

These porins exist as trimers in their native state (48), are comparable in size to the OmpF of E. coli (43) and occur in B. abortus smooth and rough strains in a one to one ratio with Group 3 proteins (36). The molecular weight of Group 2 proteins ranges from 35-40 kilodaltons (Kd) (41).

Group 3 proteins are less well defined although they are believed to exist as dimers and constitute the major OMP in strains of B. melitensis, B. ovis and B. canis (41). The proportion of porins in

these species is substantially lower than in B. abortus. Group 3 proteins may be the counterpart to the E. coli OmpA (48). The apparent molecular weight of Group 3 proteins is 25-30 Kd.

Group 1 proteins range in size from 88 to 94 Kd, are minor components, and are physically associated with the Group 3 OMP (48).

Verstrete et al. (44) tested 49 strains of B. abortus to determine if antigenic relationship between Groups 2 and 3 proteins is species wide and to investigate the possibility of using sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the OMP to distinguish between strains of B. abortus. The investigators determined that Groups 1, 2, and 3 proteins existed in all 49 strains tested. It was also determined that Group 2 proteins have a specific determinant, designated antigen [b] that was present on all 49 strains tested and that Group 3 proteins possessed three determinants (antigen's [c], [d], and [e]) (47). These antigens were shared by Group 2 and 3 OMP of B. melitensis, B. ovis, and B. canis. Verstrete et al. (44) raised the possibility of incorporating these OMP from a single strain for species wide protection due to the demonstrated cross reactivity. Groups 1, 2, and 3 proteins are closely associated with the LPS of Brucella which complicates isolation, purification, and characterization of them.

Another major OMP of *Brucella* is a lipoprotein covalently bound to the peptidoglycan (41). *Brucella* outer membrane is similar to the outer membranes of *E. coli* and other gram negative bacteria with respect to the presence of LPS porins, and OmpA-like proteins as major components (49). When Verstrete et al. (48) isolated and characterized OMP from *B. abortus* in 1982, they found no evidence of a low molecular weight lipoprotein as was found in *E. coli* and other gram-negative bacteria but did not exclude the possibility of its occurrence. In 1986 Gomez-Miguel and Moriyon (50) demonstrated peptidoglycan-linked lipoprotein. Modifying the sodium dodecyl sulfate (SDS) extraction-trypsin digestion protocol used by Braun and Sieglin, the lipoprotein from *B. abortus* was isolated and characterized. Gomez-Miguel and Moriyon (50) found the lipoprotein to be similar to *E. coli* lipoprotein in its behavior in SDS-PAGE gels, isoelectric point in urea, molecular weight (8,000), presence of both ester- and amide-linked fatty acids and amino acid composition. A hypothesis was postulated that it is the only major protein covalently linked to the peptidoglycan (50). The investigators purified the lipoprotein and hyperimmunized rabbits as well. When tested for reaction with rough-LPS (R-LPS), the antisera did not react in enzyme-linked immunosorbent assays (ELISA) or Western Blots. Conversely, anti-R-LPS antiserum did not react with the lipoprotein either, demonstrating the preparation to be free of any LPS.

Schurig et al. (46,47) found that infected cattle develop precipitins to seven distinct antigens of rough st. 45/20. The exact location of these antigens in the organism is unknown (46), although the investigators determined that these antigens, which are distinct from the smooth lipopolysaccharide (S-LPS) complex, were not detected on the surface of smooth strains. Up to 20 distinct precipitin lines have been demonstrated with rabbit antisera using antigen mixtures from B. suis (51).

#### LPS OF B. ABORTUS

The LPS of B. abortus consists of three components which are common constituents of smooth gram-negative bacteria. The three components are the O-side chain, the core polysaccharide, and the lipid A.

The chemical composition of smooth and rough B. abortus LPS was elucidated in 1979 by Moreno et al. (52). Using a phenol-water method for extraction, they harvested the S-LPS from the phenol phase and the R-LPS from the aqueous phase. Further purification was accomplished by various treatments (52). The chemical composition of S-LPS was determined to be (in percentages of total dry weight of the fractions): 26.4% fatty acids, 11.6% total carbohydrates, 0.62% 2-keto-3-deoxyoctonate (KDO), 6.3% protein, and less than 1% nucleic acids. R-LPS: 27% fatty acids, 6.5% total carbohydrates, 0.74% KDO, 1.5%

protein, and less than 1% nucleic acids. The absence of heptose from B. abortus LPS is not unique to this organism, it occurs commonly in other bacterial LPS's.

The exact composition of the O polysaccharide was determined by Caroff et al. (53) in 1984. B. abortus 1119-3 was used for extraction of LPS by a hot phenol-water procedure. The LPS was cleaved by acid hydrolysis to yield lipid A and the O-chain polysaccharide. After exhaustive assaying Caroff et al. (53) identified the polysaccharide as an unbranched linear homopolymer of 1,2-linked 4,6-dideoxy-4-formamido- $\alpha$ -D-mannopyranosyl (perosamine) residues. In concurrent studies by Caroff et al. (54) the investigators determined that Yersinia enterocolitica serotype O:9 had an identical O-chain polysaccharide. These discoveries explained the cross-reactivity of serological reactions between Yersinia enterocolitica O:9 and B. abortus. These revelations could assist in the possible development of protective vaccines, specific diagnostic reagents and particularly the production of monoclonal antibodies. Caroff et al. (53) also determined the only differences in the two O-chain polysaccharide's (Y. enterocolitica and B. abortus) was in the minor reducing end terminal core regions. This difference makes it possible to determine if antibodies are directed toward B. abortus O chain or core polysaccharide.

Bundle et al. (55) developed B. abortus and Y. enterocolitica O:9 O-polysaccharide specific monoclonal antibodies at about the same time the chemical structure of the O-chain was elucidated. Ten hybridomas

induced by immunization with B. abortus, and seven hybridomas induced by immunization with Y. enterocolitica 0:9 were studied. The serological cross-reactivity observed among the monoclonals was consistent with the chemical analysis of the B. abortus and Y. enterocolitica 0:9 O-chain polysaccharide.

At the same time Schurig et al. (39) developed two monoclonal antibodies with specificity toward the O polysaccharide. Bru 38 and Bru 28 are rat monoclonals of the IgG2a and IgG2b subclasses, respectively, induced by immunization with B. abortus 1119. Schurig et al. (39) demonstrated the specificity of the antibodies with absorption studies using purified S-LPS and acid degraded polysaccharide which removed antibody activity. Absorptions with purified R-LPS, dominant Brucella fatty acids, and E. coli KDO failed to remove activity, supporting the conclusion that the antibodies were not specific for the lipid A or core components of the B. abortus LPS. Bru 38 was used in the research presented in this thesis. Another extensively used monoclonal antibody was Bru 48. The antigen recognized by Bru 48 is associated with the core region of B. abortus LPS (26). Bru 48 was developed in mice immunized with B. abortus rough st. RB51.

#### HUMORAL PROTECTION AGAINST B. ABORTUS INFECTION:

Most experimentation dealing with immune mechanisms in Brucella infections have dealt with the dual role of anti-O antibodies and cell mediated immunity.

The relative contribution of anti-O-side chain antibodies to protection has been established by many authors (4,5,45,56-60). Montarez et al. (5) has shown passive transfer of O-side chain monoclonal antibodies and subsequent challenge with st. 2308 could reduce the number of bacteria in spleen and liver significantly at one and four weeks post infection (challenge). This protective phenomenon has also been demonstrated in other bacterial infections by monoclonal antibodies directed toward the LPS's of P. aeruginosa, E. coli 0111:B4 and S. typhimurium (5). Montarez et al. (5) failed to demonstrate passive protection in mice with porin (Group 2 proteins)-specific monoclonal antibodies.

In a paper comparing living and nonliving vaccines for B. abortus, Montarez and Winter (4) demonstrated protection in mice at one and four weeks post challenge with st. 2308 after vaccinating with live st. 19 four weeks before to challenge. The magnitude of the protection increased at the four-week observation. If challenged with st. 2308 six weeks after vaccination with live st. 19, instead of four-weeks, the magnitude of protection diminished at the four week observation. When mice were vaccinated with nonliving antigens from st. 2308 in mineral oil with trehalose dimycolate (TDM) and muramyl dipeptide (MDP) as adjuvants, they found protection to be similar to that induced with live st. 19 vaccination, and protection also diminished after the four-week observation. In the same study Montarez and Winter (4) also demonstrated substantial protection in adoptive (spleen cells) and

passive (immune sera) transfer experiments at one-week post challenge. Protection was found to be greater in the recipients of immune cells than in the recipients of immune sera. Passive protection was not demonstrable four weeks post challenge, in contrast to their other work using passively transferred LPS-specific monoclonal antibodies (5). Significant protection was observed in mice one week after receiving antiserum from mice vaccinated with st. 2308 cell envelopes in TDM-MDP adjuvant or st. 2308 cell envelopes in incomplete Freund's adjuvant. The greatest passive protection was achieved in mice receiving antiserum from live st. 19 vaccinated donors. They concluded, based on the adoptive and passive transfer studies and the effectiveness of the nonliving vaccines with or without adjuvants that vaccinal immunity at one-week post infection was due to anti-O-antibodies with a minor role for cell-mediated immunity and a contribution from other nonspecific effects.

Before these works Bascoul et al. (58) demonstrated the positive role of immune sera in murine brucellosis. Fractions extracted from B. melitensis were used to vaccinate mice. These fractions were designated PI, the phenol-insoluble fraction obtained by the Westphol Method; 4A, obtained by treating PI with DNase, RNase, pepsin, papain and pronase; and fraction 5, obtained by treating PI with DNase, RNase, papain and pronase. All three fractions contained proteins, lipoproteins, sugars, amino-sugars, and trace nucleic acids. Peptidoglycan was linked to the lipoproteins in all three fractions. At vaccination mice received one



of the three fractions in saline. At fourteen days post vaccination mice immunized with 4A had the best blood clearance of intravenously (i.v.) inoculated Brucella. At thirty and forty-five days post vaccination the PI immunized mice had attained the best clearance of i.v. inoculated Brucella. The effect of fraction 5 was similar to that of PI. All three groups showed an accelerated clearance compared to non-immunized mice (controls). All three immunized groups also demonstrated a diminished rate of multiplication in the spleen after the i.v. inoculum seven days post challenge, with the best splenic infection index achieved by the PI immunized mice. In their passive transfer experiment Bascoul et al. (58) found that blood clearance of the challenge Brucella strain was highly accelerated compared to the controls, but clearance was not as rapid as that observed in actively immunized mice. After fractionating immune serum they found that the protection conferred by different IgG fractions was repeatedly lower than that conferred by whole serum (58). These experiments show not only that antibodies but other serum constituents with molecular weights lower than those of intact immunoglobulins play a positive role in immune protection to Brucella infection conferred by immunization with phenol insoluble Brucella fractions.

In a study conducted by Winter et al. (56) porin complexed to S-LPS was as protective as vaccination with live st. 19. The investigators also found that one vaccination of porin complexed with R-LPS provided no protection, but two vaccinations of this complex led

to a low level of protection. Also, one vaccination with rough strain porin complexed with purified O-polysaccharide provided protection equivalent to that of complexes of porin-S-LPS or vaccination with live st. 19. These studies and others provide evidence that O-side chain antibodies play an important role in protective immunity.

Riezu-Boj et al. (61) demonstrated that sheep infected with B. ovis produced an antibody response to the rough LPS and to proteins present in hot saline extracts.

The analysis of rough B. ovis protein antigens was investigated by Gamazo et al. (45). Using forty-one strains of B. ovis, a naturally occurring rough Brucella, the distribution and antigenic relatedness of proteins extracted by hot saline of whole cells and those in outer membrane blebs was investigated. The proteins were identified as Group A (25.5-32 Kd), Group B (21.5-22.5 Kd), Group C (18-19.5 Kd), Group D (13-15.5 Kd) and proteins of 43 Kd, all of which had been freed from the R-LPS. The SDS-PAGE and Western Blot profiles for these proteins were similar for the forty-one strains of B. ovis and twenty-six strains of B. melitensis tested. An antigenic relationship was shown between Group 3 OMP of Brucella and Groups A, B, and C proteins by immunoblots with Group 3 specific antiserum. Interestingly, the specificity of this antiserum was attested by its nonreactivity with purified R-LPS and its failure to react with Group 3 proteins unless the proteins had been freed from the R-LPS. Gamazo et al. (45) suggests that because B. ovis occurs rough naturally, and a humoral immune response is generated to

these OMP, antibodies have ready access to these antigens and are able to interact with them and mediate various protective functions. Many B. abortus researchers have attempted to induce immunity in animals against the OMP and other cell-wall antigens without inducing O-side chain antibodies.

#### CELL-MEDIATED IMMUNITY AGAINST B. ABORTUS:

The relative role of cell-mediated immunity in brucellosis is mentioned in most of the work done on humoral protective immunity. In the adoptive transfer study done by Montarez et al. (4) spleen counts were decreased in mouse groups receiving immune cells, but this protection was not as significant as the protection achieved in mice that were passively transferred with immune serum (4,5).

Cell-mediated responses are important in the development of immunity to facultative intracellular pathogens such as Brucella (62), and cell-mediated responses are necessary for the development of a protective immune response to Brucella infection (63). The entire array of protein components needed for effective stimulation of cellular responses has yet to be identified. Brooks-Alder and Splitter (62) extracted various proteins from st. 19 and transferred them onto nitrocellulose. Nitrocellulose sections were cultured with bovine peripheral blood mononuclear (PBM) cells. They found that the primary and secondary stimulation responses of the PBM cells were kinetically similar to the responses of PBM cells stimulated with whole irradiated

B. abortus st. 19 or whole irradiated B. abortus st. 19 blotted onto nitrocellulose. The PBM cells were stimulated with portions of the blot containing high (>45,000), medium (25,000-45,000), or low (25,000) molecular weight proteins. The responses generated against the separated proteins attached to the nitrocellulose closely parallel the responses generated to the whole bacteria (62).

Cheers (57) investigated the importance of cell-mediated immunity and antibodies in murine brucellosis and the possible reasons for the persistence of the infection in the face of an immune response. She found that resistance could be conferred in CBA mice by adoptively transferring T cells, but not B cells. The T-cell subset conferring resistance to brucellosis in mice was the Ly 1+2+ subset.

It has been demonstrated that live, attenuated vaccines provide better protection in mice than killed or adjuvant based preparations (4). With the use of live vaccines as well as with passive transfer of antibodies it has been demonstrated that O-side chain antibodies provide significant but not complete protection in mice against B. abortus (4,5). Furthermore, cell-mediated immune mechanisms involving T lymphocytes have been shown to provide some protection by adoptive transfer of these cells (57). Nevertheless, the relative importance and exact role and interaction of each of these immune components remains unknown.

## MATERIALS AND METHODS

### ANIMALS

Female BALB/c mice between the ages of 4-6 weeks were purchased from Dominion Labs, Dublin, Va. and housed in the animal holding facility at the Veterinary Medical Research Center (VMRC), Virginia Tech.

Cattle used in this project were Holstein cows between the ages of 4 and 7 years. They were housed at the large animal isolation facility at Louisiana State University.

### ORGANISMS

All organisms used in this project originated from stock cultures held in the P3 facility at the VMRC. Trypticase soy broth supplemented with 1.5% agar (TSBA) plates were streaked with organisms from a stock slant, incubated at 37°C for 48 hr in an air atmosphere supplemented with 5% CO<sub>2</sub> and harvested according to different protocols depending on their intended use. B. abortus st. 2308 used in these studies was obtained from Dr. A. Winter, N.Y. State College of Veterinary Medicine, Ithaca, N.Y.

**LIVE INOCULATIONS**

Bacteria were harvested from plates in 0.85% NaCl solution (saline) and brought to the appropriate % transmittance in a Bausch & Lomb Spectronic 20 spectrophotometer. *B. abortus* st. RB51 was suspended to 10% transmittance at 525 nm and a series of ten-fold dilutions were carried out. Viable counts were performed on all inoculum by plating 50 microliters (ul) of each ten-fold dilution on TSBA plates in 10 us spots. Plates were incubated for 72 hr at 37°C with 5% CO<sub>2</sub>. Colonies in each 10 ul spot were counted and number of viable bacteria the mice received was determined. A 1:10 dilution was used to inoculate mice with 0.25 milliliters (ml) of suspension. This inoculum contained approximately  $1 \times 10^8$  colony forming units (cfu). The same procedure was followed for st. 2308 except that 0.1 ml of the 1:10,000 dilution of the 10% suspension was used to inoculate mice. This inoculum contained approximately  $5 \times 10^4$  cfu. All injections were administered intraperitoneally with a tuberculin syringe and a 27 gauge needle.

Bacteria used for inoculation of the cattle were prepared the same way except the harvested st. RB51 were lyophilized and shipped to L.S.U. The organisms were reconstituted in an equal volume of sterile saline and dilutions prepared as above. Cattle received subcutaneous injections of either 1 ml or 0.1 ml of cell suspensions in the neck region leading to an infection dose of either  $1 \times 10^{10}$  or  $1 \times 10^9$  bacteria, respectively.

**BLEEDINGS, SPLEEN CULTURES AND LYMPH NODE BIOPSIES**

During the course of each mouse experiment blood was collected by the intraorbital route. Mice were euthanized in a 4 liter beaker flooded with CO<sub>2</sub>. Blood was collected from the heart after death, and spleen cultures performed. Spleens were aseptically removed from each mouse and thoroughly ground with a 5 ml pipet in a sterile 10 ml test tube containing a small amount of sterile sand and 2 ml sterile saline. Ten-fold dilutions were carried out in a series of sterile tubes with saline and 50 ul of each dilution was plated on trypticase plates in 10 ul spots and incubated for 72 hr at 37°C with 5% CO<sub>2</sub>. Cfu were then counted and the number of bacteria per spleen was determined.

Cattle were bled at weekly intervals from the caudal vein. Prescapular lymph node biopsies were performed aseptically at 7, 15 and 22 days post first inoculation. Node samples were weighed and ground in 10 ml of phosphate buffered saline (PBS). Ten-fold serial dilutions were performed and the dilutions were plated on TSBA plates, incubated 72 hr, and counted. After counting, the plates were tested in the Rapid Identification Test (RIT) to identify rough or smooth colonies (64).

**ANTIGEN PREPARATION**

1. Cell Walls - B. abortus st. RB51 cell walls were prepared as follows. Organisms were grown as above and rough colony morphology was confirmed using the acriflavine and crystal violet tests (37,38). Ten

plates were harvested in 20 ml sterile distilled water and autoclaved for 20 min at 121°C. Twenty ml 1,00-1,05 mm of Glasperlen Beads and the cell suspension were mixed and homogenized in a Braun homogenizer (B. Braun, Melsungen, West Germany) for 4 min. Sample and beads were then filtered through a sintered glass filter and washed with distilled water until the sample was washed off the beads. The sonicate was then pelleted by centrifugation at 10,000 x g for 20 min at 4°C and the pellet was washed three times with distilled water. Then the pellet was resuspended and centrifuged for 10 min at 400 x g. Supernatant was collected, frozen and lyophilized. Two and half mg of this material was reconstituted in 950 ul 10 millimolar (mM) Tris pH 8.0 buffer. Fifty ul of lysozyme (1mg/ml 10 mM Tris) was added, the mixture was incubated at 37°C for 2 hr and refrigerated until used.

2. Purified LPS - LPS was isolated from B. abortus st. RB51 and Yersinia enterocolitica serotype 0:9 by modifications of procedures of Wu et al. (65) and Moreno et al. (52). Bacteria were grown as above and harvested in a volume of sterile distilled water equal to twice the number of plates being harvested. Cells were heat killed in an agitating water bath for 30 minutes at 60°C. The suspension was centrifuged for 10 min at 10,000 x g at 4°C. The resulting pellet was frozen until further processing. The pellet was thawed and resuspended in 55 parts of sterile distilled water and 45 parts of 90% phenol in distilled water. This mixture was agitated in a 68°C water bath for 40



min and centrifuged as above. For st. RB51 extractions the water phase was removed and 55 ml water added. For the Y. enterocolitica extraction, the phenol phase was removed and 45 ml phenol added. This was repeated three times and the separate phases pooled. The pooled phenol fractions were washed 10 times in an equal volume of 66°C distilled water by shaking and centrifugation. The aqueous or phenol fractions were then precipitated by adding 5 volumes of cold methanol reagent (99 parts methanol: 1 part methanol saturated with sodium acetate) stirred at 4°C for 1 hr. The precipitate was collected after centrifugation, redissolved in distilled water, and dialyzed overnight (Spectra/Por 3 Dialysis membrane Tubing, m.w.cutoff 3,500) against various changes of distilled water and then lyophilized with a Labconco Lyophilizer. One milligram of this material was reconstituted in 1 ml 10 mM Tris pH 8.0 buffer, digested with 10 ul Proteinase K (1mg/ml 10 mM Tris) for 3 hr at 56°C, and refrigerated until used in various assays.

3. Crude LPS - Organisms were grown as above and harvested in 0.1 M magnesium chloride in distilled water. The solution was autoclaved for 20 minutes at 121°C, then centrifuged 8,000 x g for 10 min and supernatant saved. The pellet was reconstituted in 0.1 M magnesium chloride, autoclaved and centrifuged again. The supernatants were combined and dialyzed (Spectra/Por 3 Dialysis membrane Tubing m.w. cutoff: 12,000-14,000) for 48 hr against running tap water. The harvest

was collected and lyophilized with a Labconco Lyophilizer. Material was reconstituted and stored as the purified LPS.

4. Whole Cells - Bacteria were harvested in distilled water from 48 hr old culture plates, pooled and centrifuged for 20 min at 12,000 x g at 4°C. The pellet was resuspended in acetone (50 ml per 10 plates harvested) and stirred for 3 hr at room temperature in order to kill the organisms. The cells were centrifuged at 12,000 x g for 10 min and the acetone discarded. The pellet was resuspended in 10 mM Tris pH 8.0 buffer (8 ml buffer per 10 plates harvested). One mg lysozyme (per 8 ml Tris) was added to the resuspended cells and stirred overnight at room temperature, and centrifuged as above. The pellet was discarded and aliquots of the supernatant were frozen at -70°C until use. Upon thawing, an equal volume of SDS 2x sample buffer was added to the antigen and boiled for 5 min before loading gels.

#### POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

One volume of antigen was combined with an equal volume of SDS 2x sample buffer containing 0.0625 M Tris buffer pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol (2-ME), and 0.001% bromphenol blue as the tracking dye. The antigen was further solubilized by immersion in boiling water for 5 min. Electrophoresis was performed using a discontinuous buffer system described by Laemmli (66). Resolving gels and stacking gels contained 12.5% acrylamide. These gels were prepared

by polymerizing a solution of acrylamide and N,N'-methylene -bis-acrylamide at a ratio of 30:0.8 with N,N,N',N'-tetramethylenediamine and ammonium persulfate. A Hoeffer Scientific Mighty Small II mini gel apparatus was used to run the 1.5 mm gels. Fifteen ul samples of antigen were loaded per well. A 10 ul sample of Pharmacia low molecular weight standards was also loaded on every gel to determine the molecular weight of individual antigens (67). Molecular weight standards consisted of phosphorylase b (m.w. 94,000), albumin (m.w. 67,000), ovalbumin (m.w. 43,000), carbonic anhydrase (m.w. 30,000) trypsin inhibitor (m.w. 20,1000), and  $\alpha$ -lactalbumin (m.w. 14,400). Gels were run at 20-30 milliamps until the tracking dye was approximately 1 centimeter (cm) from the bottom of the gel. They were either stained with Coomassie Brilliant Blue R or silver stained, or transferred onto nitrocellulose membrane via Western Blot technique. Coomassie blue stains were performed by staining gels overnight in a solution containing 3.0 g Coomassie Brilliant Blue R, 10% acetic acid and 45% ethanol. The gels were detained the following day in several changes of a solution of 10% acetic and 25% ethanol. Silver staining was performed using a modified version of Tsai et al. technique (68). Gels were fixed overnight in 50% methanol, washed 30 min in distilled water, 30 min 50% methanol and again for 1 hr in distilled water. After washing, they were agitated in a solution containing 2.5 ml isopropanol, 0.7 ml acetic acid 1.05 g periodic acid, and 160 ml distilled water for 5 min. Gels

were washed for 2 hr in distilled water. The staining reagent consisted of 28 mls 0.1M sodium hydroxide, 2 ml ammonium hydroxide, and 115 ml distilled water. To this solution, 5 ml of 20% silver nitrate was added drop wise and agitated for 10 min. Gels were then washed for 40 min in distilled water. Developing the gels took place in a solution of 1 liter distilled water, 50 mg citric acid, and 0.5 ml 37% formaldehyde. The reaction was stopped by agitating the gels in a mixture of 190 ml distilled water and 10 ml 7% acetic acid for 10 min. Gels were stored in a closed container containing distilled water until dried, photographed or discarded.

#### WESTERN BLOTS

A "sandwich" was assembled which consisted of a Scotch Bright pad, 2 pieces of Whatman #1 filter paper, the gel, nitrocellulose membrane, 2 pieces of filter paper and another pad pressed between 2 cassettes. The sandwich was placed in a Hoeffer Scientific Instruments TE Series Transphor Electrophoresis Unit containing transfer buffer consisting of 25mM Tris, 192 mM glycine, and 20% methanol. The cassette was positioned in such a way that the gel was closest to the cathode and the nitrocellulose closest to the anode. Transfer took place over 2 hr at 125 volts. Upon completion the nitrocellulose was cut into strips corresponding to the gel lanes, and blocked in 0.25% gelatin in TBS for 1 hr. The molecular weight standards were immediately stained in

Ponceau S stain, detained in water, and the resulting bands were marked over with pencil to obtain a permanent record. Molecular weight determinations were made by following the method of plotting  $\log_{10}$  molecular weight of the standards versus distance of migration. After blocking, the strips were incubated in a 1:100 dilution of anti-serum in TBS (Tris buffered saline) overnight. They were washed in TBST (Tris buffered saline plus Tween) for 10 min and incubated in the appropriate secondary antibody (peroxidase conjugated rabbit IgG fraction anti-bovine at 1:1000, goat anti-mouse at 1:800, or goat anti-rat at 1:500 in TBS) for 1 hr. Strips were then washed for 10 min in TBST and developed in 100 ml TBS, 10 ml methanol, 60 mg 4-chloro-1-naphthol, and 0.6 ml 30% hydrogen peroxide. The reaction was stopped by submerging the strips in a large volume of distilled water.

#### COLONY BLOTS

The Colony blot enzyme-linked immunosorbent assay (RIT) procedure developed by Roop and coworkers was utilized to identify bacterial isolates from mice and cattle (64). Isolates were plated and grown as described above then blotted onto sterile nitrocellulose membrane disks. The disks were then submerged in 25 ml chloroform for 10 minutes, removed and allowed to dry 15 min at room temperature under an activated charcoal hood. They were placed in 25 ml of a blocking-digestion buffer consisting of 0.15 M sodium chloride, 0.02 M Tris, 0.005 M magnesium

chloride pH 7.5. The buffer was supplemented with 2 ug/ml DNase I, 80 ug/ml lysozyme, and 3% bovine serum albumin. Disks were agitated in this buffer for 45 min, washed in TBST and incubated with an appropriate monoclonal antibody diluted 1:100 in TBS for 1 hr. Disks were washed again and incubated in an appropriate secondary antibody (peroxidase conjugated goat IgG fraction anti-mouse at 1:800 or goat anti-rat at 1:500 in TBS) for 1 hour. After washing in TBST the disks were developed in 100 ml TBS, 10 ml methanol, 60 mg 4-chloro-1-naphthol, and 0.6 ml 30% hydrogen peroxide. To stop the reaction the disks were submerged in distilled water.

#### SERUM AGGLUTINATION TEST (SAT)

Brucella st. 19 Diagnostic Antigen was received from U.S.D.A. and diluted to the working dilution by adding 1 ml of stock to 99 ml 0.5% phenolized saline for a working O.D. of 0.25 at 525 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. A 1:25 dilution of antisera were made for each sera sample in phenolized saline and two-fold dilutions carried out to a given end point. The tubes were incubated at 37°C for at least 24 hr before reading.

#### CRYSTAL VIOLET STAINING

The method of White and Wilson (38) was used for differentiation of smooth and rough colonies. A stock solution of crystal violet was prepared by dissolving 2.0 g crystal violet and 0.8 g ammonium oxalate in a solution of 20% ethanol. This was further diluted to a working solution by diluting it to 1:40 in distilled water. Colonies on plates were flooded with working solution for 20 sec then observed for staining. Stained colonies are considered of rough morphology.

#### ACRIFLAVINE AGGLUTINATION

This assay was performed according to a modified method of Braun and Bonestell (37). One mg acriflavine was dissolved in 1 ml distilled water. Approximately 50 ul of this solution was placed on a slide and a small amount of bacterial growth was mixed into it with a loop. The slide was then gently rocked back and forth for 2 min and checked for agglutination. Rough organisms will agglutinate.

#### ABSORPTION OF SERA

Selected samples of sera from vaccinated cows were absorbed with either live bacteria or bacterial cell walls preparations. Viable bacteria were harvested in saline and brought to 5%, 10%, 30% and 60% transmittance at 525 nm in a Bausch and Lomb Spectronic 20 spectrophotometer. One ml of the suspension was centrifuged in a Fisher Scientific Micro-Centrifuge for 2 min and the supernatant discarded.

Bacteria were resuspended in 1 ml of sera diluted 1:30 in TBS. The samples were rotated at 150 r.p.m. in a Queue Radial Shaker for 1 hour. Samples were centrifuged again for 2 min, the absorbed sera collected, and the pellet discarded. Sera was further diluted to 1:100 in TBS for Western Blot analysis. Similarly, 1 ml aliquots of sera diluted 1:30 in TBS were mixed with 1 mg of cell wall preparation and processed as described above.

#### BIO DOTS (TITER ANALYSIS)

Titer analysis was performed using the BioRad, BioDot chamber. Nitrocellulose membrane prewetted in TBS was placed in the chamber, closed and tightly sealed. Autoclaved whole bacteria cells (W.C.) were used as (antigen suspended to 30% transmittance at 525 nm in TBS in a Bausch and Lomb Spestronic 20). Thirty  $\mu$ l of antigen was added to the wells and incubated at room temperature for 30 min before applying vacuum. The vacuum was released and a 0.25% solution of gelatin in TBS was added to each well and incubated for 30 min. The nitrocellulose membrane was washed one time with TBST and dried by applying vacuum. Serial dilutions of sera in TBS were added to the wells and incubated for 30 min. The wells were washed 5 times with TBST and dried under vacuum. The appropriate secondary antibody (peroxidase conjugated rabbit IgG fraction anti-bovine at the dilutions stated above) was added and incubated for 30 min, washed five times with TBST then dried



for several min. The apparatus was disassembled and the nitrocellulose membrane removed and developed in the same reagents as the Western strips. The color reaction was stopped by submerging the sheet in a large volume of distilled water. The nitrocellulose membrane was then left to dry on a paper towel in the dark before titers were scored. Two systems were used to score titers based on the degree of staining of dots on the nitrocellulose. The systems used were visual scoring and reading on a densitometer. For the visual scoring each assay included a negative and a positive serum sample. The dilution of the negative serum showing no reaction was selected as a cut off point. Color reactions stronger than the cut off point were scored as positive. Scores were assigned by two different individuals. Scores obtained from densitometer readings were inconclusive, therefore the results were not used to report antibody titers.

#### ANTIBIOTIC SUSCEPTIBILITY

Several isolates from lymph node and spleens were tested for rifampin susceptibility. One hundred mg rifampin was solubilized in 1 ml methanol, and an appropriate volume of this was added to liquid TSBA to arrive at the desired concentration of rifampin. Plates were poured and allowed to solidify and dried overnight. Plates were streaked the following day with isolants and rifampin susceptible controls and

incubated as described above. If no growth occurred after 48 hr the plates were allowed to stay incubated for up to 1 week before scoring.

## CHAPTER 1

EXPERIMENTAL INFECTION OF MICE WITH B. ABORTUS ST. RB51:  
CLEARANCE OF THE ORGANISM, HUMORAL IMMUNE RESPONSE ELICITED AND  
PROTECTIVE EFFECTS.

### RATIONALE

Because st. RB51 is described as a completely rough strain (no O-side chain) of B. abortus it was important to determine if the organism can survive within animals and if so, to determine how long it survives before elimination. Smooth B. abortus st. 19 is able to infect mice and persist for 6 weeks (56). Smooth st. 2308 is able to persist in mice for more than 24 weeks (56,69). Preliminary experiments indicated that i.p. injections of st. RB51 in numbers less than  $10^7$  did not result in spleen colonization 5 days post infection. For this reason, doses of organisms greater than  $10^7$  were used in the following clearance experiments. Also, it was considered important to determine the clearance rate of st. RB51 in order to challenge mice at a time in which st. RB51 had been eliminated to better evaluate changes in the humoral immune response and their protective effects.

A second question was can st. RB51 influence the humoral immune response to st. 2308 or vice versa? More precisely, did st. RB51 influence the response to the O-side chain, the immunodominant antigen present on smooth strains of B. abortus (26,33,70)? For this purpose experiments were carried out in which animals were injected with both

rough and smooth strains in different combinations. Immunization with st. RB51 leads to a response which is not dominated by the O-side chain because st. RB51 is devoid of this antigen (71,72). It is therefore conceivable that priming animals with st. RB51 may change the O-side chain response if animals are later exposed to a smooth strain. It was important to assess if mice infected with st. RB51 were able to produce antibodies to the O-side chain, since the true roughness of st. RB51 is still being debated.

A third question being posed was: can st. RB51 protect mice against a challenge with the standard virulent strain (st. 2308)? Recent work using vaccines devoid of O-side chain components suggest that protection with out this component could not be achieved (56).

#### EXPERIMENTAL DESIGN

Murine Experiment I: B. abortus rough st. RB51 clearance.

In murine experiment I (outlined in Table I) twenty-four female BALB/c mice were infected on day 0 with live st. RB51 then euthanized on predetermined days post infection to obtain serum and cfu/spleen according to the following schedule. On day 14 post infection 3 mice were euthanized and on day 28, 2 mice were euthanized. Also, on day 28, 17 of the remaining 19 mice received a second inoculum of live st. RB51, thus, leaving 2 mice with a single st. RB51 injection. On days 29 and

TABLE I

## Murine Experiment I

B. ABORTUS ROUGH STRAIN RB51 CLEARANCE EXPERIMENTAL DESIGN

Mouse	Day 0	Day 14	Day 28	Day 29	Day 31	Day 33	Day 35	Day 49
1,2,3	RB51 <sup>a</sup>	bleed, SC <sup>c</sup>						
4,5	RB51		bleed, SC					
6,7,8,9	RB51		RB51 <sup>b</sup>	bleed, SC				
10,22,12,13	RB51		RB51		bleed, SC			
14,15,16	RB51		RB51			bleed, SC		
17,18,19	RB51		RB51				bleed, SC	
20,21,22	RB51		RB51					bleed, SC
23,24	RB51							bleed, SC

- 
- a. Day 0 all mice received  $1.8 \times 10^8$  organisms/mouse i.p.
- b. Day 28, 17 mice received  $8.5 \times 10^7$  organisms/mouse i.p.
- c. Spleen cultures (SC) performed.

31 four mice were euthanized on each day, and on days 33 and 35 three mice were euthanized on each day. On day 49 the five remaining mice (two of which received only one st. RB51 injection) were euthanized.

Persistence of st. RB51 was determined by culturing spleens and determining cfu in all experiments. Antibody titers were measured using the BioRad Biodot System in this experiment as well as in experiments II, III and IV.

**Murine Experiment II:** Humoral immune response of mice infected with different combinations of rough and smooth strains of B. abortus.

In experiment II (outlined in Table II) blood was collected on days 14, 28, 44 and 65 for Western Blot analysis. On days 44 and 65 one-half of each group of mice were euthanized and spleens were cultured to determine persistence of infection and level of protection.

**Murine Experiment III:** Extension of the humoral immune response of mice infected with different combinations of rough and smooth strains of B. abortus.

In experiment III (outlined in Table III) blood was collected on days 28, 42, 77, and 84 for determination of antibody titer and Western Blot analysis. All mice were euthanized on day 84 and spleen cultured to determine level of infection.

TABLE II

## Murine Experiment II

HUMORAL IMMUNE RESPONSE OF MICE INFECTED WITH DIFFERENT  
COMBINATIONS OF ROUGH AND SMOOTH STRAINS OF B. ABORTUS

## EXPERIMENTAL DESIGN

<u>GROUP<sup>a</sup></u>	<u>DAY 0</u>	<u>DAY 14</u>	<u>DAY 28</u>	<u>DAY 37</u>	<u>DAY 44<sup>b</sup></u>	<u>DAY 65<sup>b</sup></u>
1	RB51 <sup>d</sup>	bleed	bleed	Saline <sup>j</sup>	bleed, SC <sup>c</sup>	bleed, SC
2	RB51 <sup>d</sup>	bleed	bleed	RB51 <sup>f</sup>	bleed, SC	bleed, SC
3	RB51 <sup>d</sup>	bleed	bleed	2308 <sup>g</sup>	bleed, SC	bleed, SC
4	Saline <sup>j</sup>	bleed	bleed	RB51 <sup>f</sup>	bleed, SC	bleed, SC
5	2308 <sup>e</sup>	bleed	bleed	Saline <sup>j</sup>	bleed, SC	bleed, SC
6	2308 <sup>e</sup>	bleed	bleed	2308 <sup>i</sup>	bleed, SC	bleed, SC
7	2308 <sup>e</sup>	bleed	bleed	RB51 <sup>h</sup>	bleed, SC	bleed, SC
8	Saline <sup>j</sup>	bleed	bleed	2308 <sup>i</sup>	bleed, SC	bleed, SC

a. Each group consisted of 6 mice.

b. One-half of each group euthanized.

c. Spleen cultures (SC) performed.

d. Mice infected with RB51 day 0 received  $1 \times 10^8$  organisms/mouse i.p.

e. Mice infected with 2308 day 0 received  $5.4 \times 10^4$  organisms/mouse i.p.

f. Groups 2 and 4 day 37 received  $1 \times 10^8$  organisms/mouse i.p.

g. Group 3 day 37 received  $4.6 \times 10^4$  organisms/mouse i.p.

h. Group 7 day 37 received  $8 \times 10^7$  organisms/mouse i.p.

i. Groups 6 and 8 day 37 received  $2.5 \times 10^4$  organisms/mouse i.p.

j. Groups inoculated with saline received 0.25 ml saline i.p.

TABLE III

## Murine Experiment III

## EXTENSION OF THE HUMORAL IMMUNE RESPONSE

## EXPERIMENTAL DESIGN

<u>GROUP</u> <sup>a</sup>	<u>DAY 0</u>	<u>DAY 28</u>	<u>DAY 35</u>	<u>DAY 42</u>	<u>DAY 77</u>	<u>DAY 84</u>
1	2308 <sup>c</sup>	bleed	RB51 <sup>e</sup>	bleed	bleed	bleed,SC <sup>b</sup>
2	Saline <sup>d</sup>	bleed	RB51 <sup>e</sup>	bleed	bleed	bleed,SC

a. Each group consisted of 8 mice, all euthanized day 84.

b. Spleen cultures (SC) performed.

c. Group 1 day 0 received  $3.4 \times 10^4$  organisms/mouse i.p.

d. Group 2 received 0.25 ml Saline i.p.

e. Group 1 and 2 received  $9.6 \times 10^7$  organisms/mouse i.p.



**Murine Experiment IV:** Protection against challenge with st. 2308 in st. RB51 vaccinated mice.

In experiment IV (outlined in Table IV) blood was collected on days 28, 45, 56 and 77 for antibody titer determination and Western Blot analysis. On days 56 and 77 one-half of the mice in each group were euthanized and spleens cultured to determine if infection was persistent and the level of protection attained. Statistical analysis on the number of cfu was performed using the Fisher's Exact Test.

## RESULTS

**Murine Experiment I:**

### Spleen Cultures

The splenic average cfu/spleen are reported in Table V. The limit of the spleen cultures does not allow detection of less than 40 organisms per spleen, therefore less than 40 organisms/spleen was reported as negative in all experiments.

BALB/c mice infected with  $10^8$  st. RB51 still had approximately  $10^3$  bacteria in their spleens 14 days after exposure, and were able to completely eliminate the infection by 4 weeks post infection (wpi), as demonstrated by mouse 4 and 5. Mice receiving only one inoculation of st. RB51 were still negative 7 weeks after infection (mouse 23 and 24).

TABLE IV

## Murine Experiment IV

## PROTECTION AGAINST CHALLENGE WITH 2308 IN RB51 VACCINATED MICE

## EXPERIMENTAL DESIGN

GROUP <sup>a</sup>	DAY 0	DAY 28	DAY 35	DAY 45	DAY 49	DAY 56 <sup>b</sup>	DAY 77 <sup>b</sup>
1	RB51 <sup>d</sup>	bleed	Saline <sup>e</sup>	bleed	2308 <sup>g</sup>	bleed,SC <sup>c</sup>	bleed,SC
2	RB51 <sup>d</sup>	bleed	RB51 <sup>f</sup>	bleed	2308 <sup>g</sup>	bleed,SC	bleed,SC
3	Saline <sup>e</sup>	bleed	Saline <sup>e</sup>	bleed	2308 <sup>g</sup>	bleed,SC	bleed,SC

- 
- a. Each group consisted of 10 mice.
- b. One-half of each group euthanized.
- c. Spleen cultures (SC) performed.
- d. Groups 1 and 2 day 0 received  $1.1 \times 10^8$  organisms/mouse i.p.
- e. Group 3 day 0 and Groups 1 and 3 day 35 received 0.25 ml Saline i.p.
- f. Group 2 day 35 received  $9 \times 10^7$  organisms/mouse i.p.
- g. Group 1, 2 and 3 day 49 received  $3.3 \times 10^4$  organisms/mouse i.p.

TABLE V

## Murine Experiment I

## SPLENIC CLEARANCE RATES OF SINGLE AND DOUBLE INJECTIONS OF RB51

<u>MOUSE<sup>a</sup></u>	<u>14 dpi</u>	<u>28 dpi<sup>b</sup></u>	<u>29 dpi</u>	<u>31 dpi</u>	<u>33 dpi</u>	<u>35 dpi</u>	<u>49 dpi</u>
1,2,3 <sup>c</sup>	7.9x10 <sup>2</sup>						
4,5 <sup>c</sup>		neg.					
6,7,8,9			5.3x10 <sup>4</sup>				
10,11,12,13				2.5x10 <sup>3</sup>			
14,15,16					4x10 <sup>2</sup>		
17,18,19						40	
20,21,22							neg.
23,24 <sup>c</sup>							neg.

- 
- a. Day 0 all mice received  $1.8 \times 10^8$  organisms/mouse i.p.
- b. Day 28, 17 mice received  $8.5 \times 10^7$  organisms/mouse i.p.
- c. Mice 1, 2, 3, 4, 5, 23 and 24 did not receive secondary exposure.

Seven days following second exposure mouse 17, 18 and 19 had only the minimum detectable organisms present. The infection was cleared by 22 days post secondary exposure (mouse 20, 21 and 22).

### Serology

In Table VI a three-fold increase in titer is seen between 14 and 28 days following the first exposure. One day following the second inoculation a dramatic decrease in titer is observed as expected. Mice 23 and 24 not receiving the second exposure to st. RB51 maintained antibody titers to the antigen throughout the 49 days almost equivalent to those attained by mice before the second inoculation.

### Murine Experiment II:

#### Spleen Cultures

Clean vs. infected spleens and average cfu/spleen are summarized in Table VII. Group 1 having received st. RB51 five weeks before spleen culture had one mouse with the minimum detectable number of organisms present. The spleen culture results of all other groups were expected in that the respective infections were not cleared. At 4 weeks wpc groups 1, 2 and 4 had completely eliminated the st. RB51 infection. At that time no mice in groups 5, 6 and 8 had eliminated the st. 2308 infection. In group 3, 4 wpc, two of three mice had cleared both vaccination and challenge strains and in Group 7, one of three mice had cleared both vaccination and challenge strains. The groups that

TABLE VI

## Murine Experiment I

AVERAGE ANTIBODY TITERS OF INDIVIDUAL MOUSE SERA TO RB51 WHOLE  
CELLS (W.C.) AFTER ONE OR TWO EXPOSURES TO LIVE RB51

<u>MOUSE</u>	<u>DAY OF BLEEDING</u>	<u>AVG. ANTI-RB51 W.C. TITER</u>
1, 2, 3 <sup>a</sup>	14 dpi	1:40
4, 5 <sup>a</sup>	28 dpi	1:320
6, 7, 8, 9	29 dpi	1:60
10, 11, 12, 13	31 dpi	1:140
14, 15, 16	33 dpi	1:640
17, 18, 19	35 dpi	1:426
20, 21, 22	49 dpi	1:920
23, 24 <sup>a</sup>	49 dpi	1:200

---

a. Mice 1, 2, 3, 4, 5, 23, 24 did not receive secondary exposure.

TABLE VII

## Murine Experiment II

CLEAN VS. INFECTED SPLEENS AND CFU/SPLEEN OF MICE INFECTED WITH DIFFERENT COMBINATIONS OF  
ROUGH AND SMOOTH STRAINS OF B. ABORTUS

GROUP <sup>a</sup>	DAY 0	DAY 37	Clean/Infected Spleens		Average cfu/spleen <sup>d</sup>	
			1wpc <sup>b</sup>	4wpc	1 wpc	4 wpc
1	RB51 <sup>c</sup>	Saline	2/1	3/0	40 ± 23.1	0
2	RB51	RB51	1/2	3/0	80 ± 80	0
3	RB51	2308	0/3	2/1	1.5x10 <sup>4</sup> ± 1.1x10 <sup>4</sup>	1.6x10 <sup>4</sup> ± 2.8x10 <sup>4</sup>
4	Saline	RB51	0/3	3/0	2.3x10 <sup>4</sup> ± 8.8x10 <sup>3</sup>	0
5	2308	Saline	0/3	0/3	6.1x10 <sup>5</sup> ± 8x10 <sup>5</sup>	1.2x10 <sup>5</sup> ± 1.5x10 <sup>5</sup>
6	2308	2308	0/3	0/3	1.3x10 <sup>5</sup> ± 6x10 <sup>5</sup>	1.8x10 <sup>4</sup> ± 2.8x10 <sup>4</sup>
7	2308	RB51	0/3	1/2	1.2x10 <sup>5</sup> ± 2x10 <sup>4</sup>	1.6x10 <sup>5</sup> ± 2.7x10 <sup>5</sup>
8	Saline	2308	0/3	0/3	2.5x10 <sup>5</sup> ± 1.1x10 <sup>5</sup>	1.6x10 <sup>5</sup> ± 4.1x10 <sup>4</sup>

a. Six mice 1 group.

b. wpc = weeks post challenge

c. See Table I for doses

d. Average cfu ± S.D.

received one or two injections of st. 2308 and no st. RB51 were unable to clear the infections.

### Serology

Results of the SAT are reported in Table VIII. Groups 1, 2 and 4 fail to agglutinate the smooth antigen throughout the experiment. Group 3 reacts 4 weeks after receiving st. 2308. Groups 5, 6 and 7 react 14 days after receiving st. 2308. Group 8 reacts one week after st. 2308.

### Western Blot Analysis

Western blot analysis was performed using Y. enterocolitica 0:9 LPS and RB51 W.C. as antigens. These are presented in Figures I and II respectively. Groups 1, 2 and 4 show no reaction with the 0:9 LPS for the duration of the experiment (Figure I). Group 3 responds to the 0:9 LPS 4 wpc with st. 2308. Groups 5, 6 and 7 begin responding to the 0:9 LPS by 28 dpi and continue throughout the experiment. Group 8 responds by 4 wpc which corresponds to the onset of the reactivity of Groups 5, 6 and 7. Groups of mice receiving only st. RB51 in any combination did not respond to the Y. enterocolitica 0:9 LPS.

Groups 1, 2 and 3 demonstrate a progressively stronger response to st. RB51 throughout the experiment (Figure II). Group 4 begins to react with the antigen by 4 wpc. Groups 5, 6 and 7 react mildly with RB51 W.C. by 28 dpi. The response becomes progressively stronger with the antigen by 1 and 4 wpc. The lower number of organisms/spleen in group 3 prompted the design of experiment IV. With two injections of st. RB51 and a larger number of animals experiment IV was designed to investigate

TABLE VIII

## Murine Experiment II

SAT TITERS OF POOLED SERA FOLLOWING DIFFERENT COMBINATIONS OF ROUGH AND  
SMOOTH STRAINS OF B. ABORTUS

<u>DAY</u>	<u>GROUP<sup>c</sup></u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>
14		<25 <sup>a</sup>	<25	<25	<25	50	50	25	<25
28		<25	<25	<25	<25	400	<25	200	<25
1 wpc		<25	<25	<25	<25	800	400	200	50I <sup>b</sup>
4 wpc		<25	<25	200	<25	400	400	800	200

- 
- a. All numerical values expressed as reciprocals.  
 b. Incomplete agglutination at a dilution of 1:50.  
 c. Sera from mice in each group were pooled.



FIGURE I

Murine Experiment II

WESTERN BLOT ANALYSIS OF POOLED MICE SERA INFECTED WITH DIFFERENT COMBINATIONS OF ROUGH AND SMOOTH STRAINS OF B. ABORTUS AGAINST Y. ENTEROCOLITICA 0:9 LPS ANTIGEN

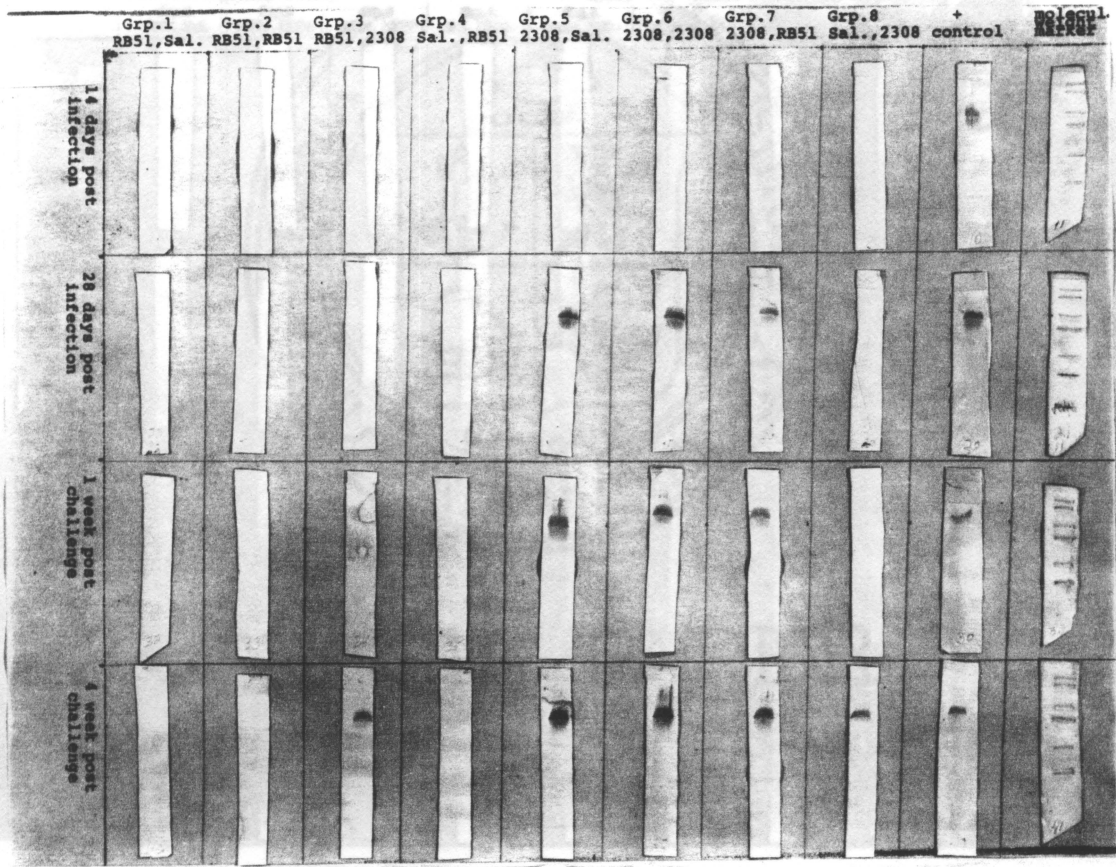


FIGURE II

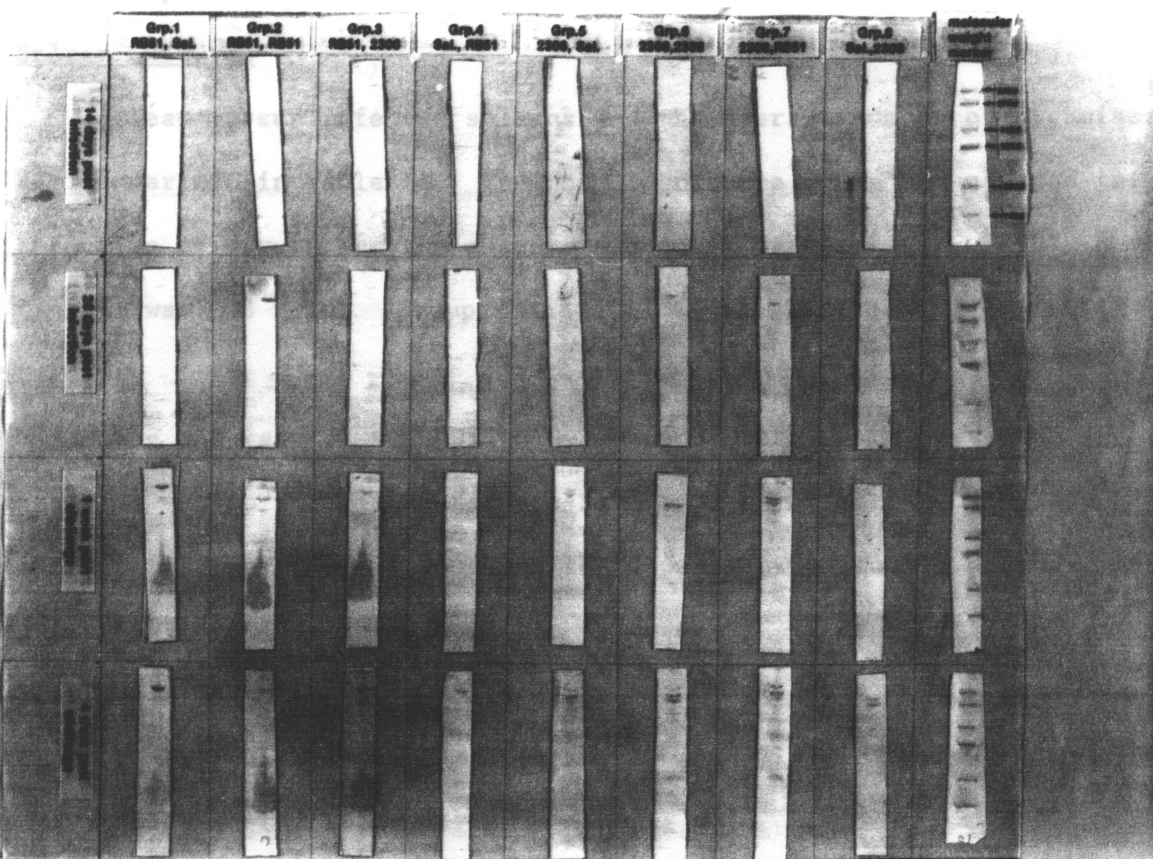
Murine Experiment II

WESTERN BLOT ANALYSIS OF POOLED MICE SERA INFECTED WITH DIFFERENT

COMBINATIONS OF ROUGH AND SMOOTH STRAINS OF B. ABORTUS AGAINST

RB51 W.C. ANTIGEN

Murine Experiment III:



Western Blot Analysis

Western blot analysis was performed on the same sera. Antigens used for the assay were RB51 W.C. and Y. enterocolitica 0:9 LPS. Group 1 mice recognized an antigen present in the RB51 W.C. preparation in the

protection with numbers of animals large enough to achieve statistical significance. The humoral immune response of group 7 at the termination of the experiment indicated that a time extension of this group would allow a better analysis of the response. For this reason experiment III was designed.

#### Murine Experiment III:

##### Spleen Cultures

Clean versus infected spleens and the average number of organisms are summarized in Table IX. In Group 1 only one mouse had cleared the infection by day 84 and the average number of bacteria (st. 2308) present was  $2.3 \times 10^4$ . Group 2 had cleared all infection by day 84.

##### Serology

The SAT results and RB51 W.C. antibody titers are presented in Table X. Group 1 agglutinated the SAT at 28 days post st. 2308 and these titers persisted throughout the experiment. Group 2 had no agglutination titer throughout the experiment. Group 1 attained a relatively high titer to RB51 W.C. of 1:640 by day 77 of the experiment. Group 2 never attained such a high titer and 77 dpi the titer was 1:160. One week later the titer began to decline.

##### Western Blot Analysis

Western blot analysis was performed on the same sera. Antigens used for the assay were RB51 W.C. and Y. enterocolitica 0:9 LPS. Group 1 mice recognized an antigen present in the RB51 W.C. preparation in the

TABLE IX

## Murine Experiment III

CLEAN VS. INFECTED SPLEENS AND AVERAGE CFU/SPLEEN

Group <sup>a</sup>	<u>Inoculum</u> <u>day</u>		<u>Clean/infected</u> <u>Spleens</u>	<u>Avg.</u> <u>cfu/spleen</u>
	0	35	day 84	day 84
1	2308 <sup>b</sup>	RB51	1/7	$2.3 \times 10^4 \pm 5.4 \times 10^4$
2	Saline	RB51	8/0	0

- 
- a. 8 mice/group.
- b. For doses see Table II.

TABLE X

## Murine Experiment III

## RB51 W.C. ANTIBODY TITERS AND SAT TITERS OF POOLED MICE SERA

	RB51 WC TITER				SAT TEST TITER					
	day	28	42	77	84	day	28	42	77	84
Group 1		<20 <sup>a</sup>	80	640	640		200	400I <sup>b</sup>	400	400
Group 2		<20	<20	160	80		<25	<25	<25	<25

- 
- a. All numerical values expressed as reciprocals.
- b. Incomplete agglutination at 1:400 dilution.

67-70 Kd range by 28 dpi. (Figure III). One wpc this groups' reaction intensified toward antigens in that range, but no core response was seen. Eleven weeks (77 days) post challenge, group 1 mildly reacted with core determinants. The reaction did not increase in intensity by week 12.

Group 2 did not recognize the 67 Kd antigen 1 week after receiving st. RB51, or the core antigen (Figure III). Eleven weeks after receiving st. RB51 Group 2 reacts with the high molecular weight antigen stronger than with the core determinants. Both responses were decreasing by week 12.

Group 1 responded to the Y. enterocolitica 0:9 LPS from the first bleeding 28 dpi throughout the experiment (Figure IV). Group 2 did not recognize any antigenic determinants present in the LPS preparation throughout the experiment (Figure IV).

#### **Murine Experiment IV:**

##### Spleen Cultures

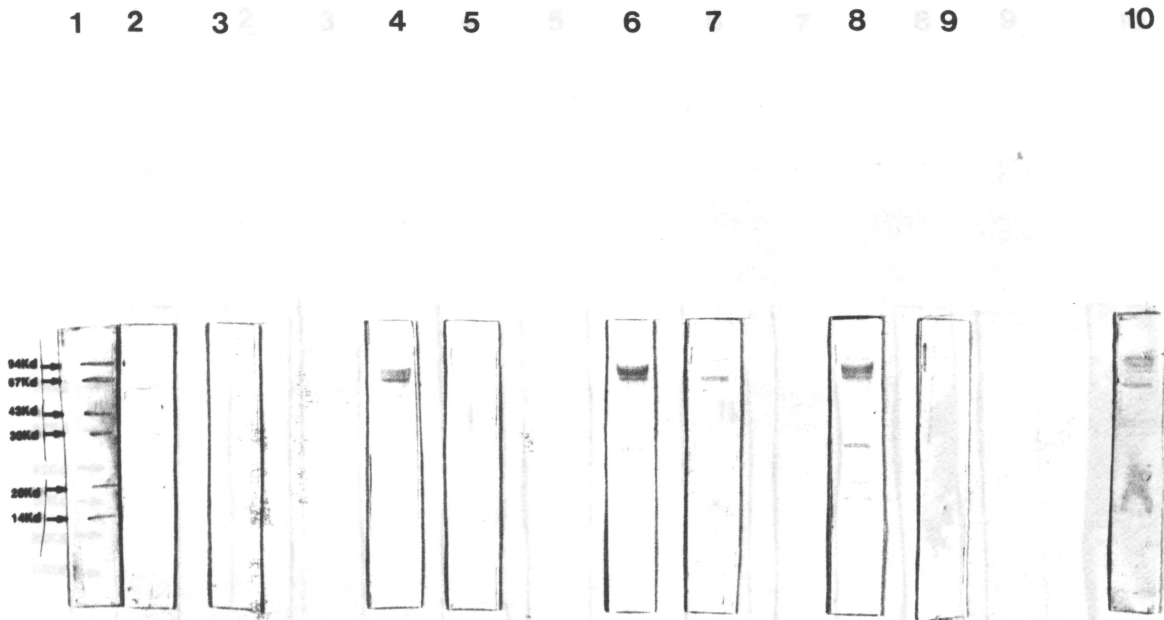
The spleen conditions and average cfu/spleen, for Experiment IV are reported in Table XI. At 1 wpc, one mouse in Group 1 and 3 mice in Group 2 had cleared the st. 2308 challenge, in contrast to the control group in which none had cleared the infection. The infected mice of Groups 1 and 2 having received initial exposure to st. RB51 also demonstrated 2 logs of protection at 1 wpc when compared to controls. A significant difference of  $p < .047$  derived from the Fisher's Exact Test

## Figure III

## Murine Experiment III

## WESTERN BLOT ANALYSIS OF POOLED MICE SERA AGAINST RB51 W.C. ANTIGEN

O-9 LPS ANTIGEN



Lane 1 Molecular Weight

Lane 2 Group 1, day 28

Lane 3 Group 2, day 28

Lane 4 Group 1, day 42

Lane 5 Group 2, day 42

Lane 6 Group 1, day 77

Lane 7 Group 2, day 77

Lane 8 Group 1, day 84

Lane 9 Group 2, day 84

Lane 10 Steer 66, positive control

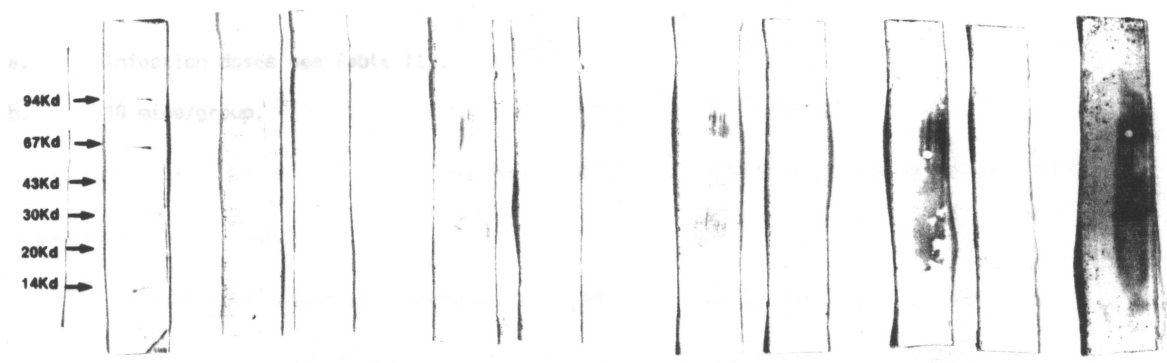
Figure IV

Murine Experiment III

WESTERN BLOT ANALYSIS OF POOLED MICE SERA AGAINST Y. ENTEROCOLITICA

0:9 LPS ANTIGEN

GROUP	1	2	3	4	5	6	7	8	9	10
	DAY 7	DAY 35	DAY 49	1 spc	4 spc	1 spc	4 spc	1 spc	4 spc	
1	R851 <sup>0</sup>	Saline	2308	1/4	0/5	$4.4 \times 10^3 \pm 9 \times 10^3$	$3.4 \times 10^4 \pm 1.1 \times 10^4$			
2	R851	R851	2308	3/2	2/3	$4.8 \times 10^3 \pm 1 \times 10^4$	$7.7 \times 10^2 \pm 1.4 \times 10^3$			
3	Saline	Saline	2308	0/5	1/4	$2.5 \times 10^5 \pm 1.5 \times 10^5$	$6.1 \times 10^4 \pm 4 \times 10^4$			



Lane 1 Molecular Weight

Lane 2 Group 1, day 28

Lane 3 Group 2, day 28

Lane 4 Group 1, day 42

Lane 5 Group 2, day 42

Lane 6 Group 1, day 77

Lane 7 Group 2, day 77

Lane 8 Group 1, day 84

Lane 9 Group 2, day 84

Lane 10 Steer 121, positive control



TABLE XI

## Murine Experiment IV

## CLEAN VS. INFECTED SPLEENS AND AVERAGE CFU/SPLEEN FOR RB51 PROTECTION STUDY

GROUP <sup>b</sup>	DAY 0	INOCULUM		DAY 49	Clean/Infected Spleens		Average Organisms Spleen	
		DAY 35			1 wpc	4 wpc	1 wpc	4 wpc
1	RB51 <sup>a</sup>	Saline		2308	1/4	0/5	$4.4 \times 10^3 \pm 9 \times 10^3$	$3.4 \times 10^4 \pm 3.1 \times 10^4$
2	RB51	RB51		2308	3/2	2/3	$4.8 \times 10^3 \pm 1 \times 10^4$	$7.7 \times 10^2 \pm 1.4 \times 10^3$
3	Saline	Saline		2308	0/5	1/4	$2.8 \times 10^5 \pm 1.5 \times 10^5$	$6.1 \times 10^5 \pm 5.4 \times 10^5$

a. Infection doses see Table III.

b. 10 mice/group.

was found in the severity of infection between Group 1 and 3 and 2 and 3 but not between 1 and 2 at 1 wpc. At 4 wpc Group 2 had 2 mice cleared of infection. The control, Group 3, had one mouse cleared of infection. Group 2, at 3 wpc, demonstrated 3 logs of protection over controls, and Group 1, one log. A significant difference in the severity of infection of  $p < .047$  was found between Groups 1 and 2, Groups 1 and 3 and Groups 2 and 3.

### Serology

The SAT test results and RB51 W.C. antibody titers are presented in Table XII. After challenge with st. 2308 Groups 1 and 3 began reacting in the SAT 4 wpc. Group 2 did not demonstrate an agglutination reaction at 4 wpc.

After the second inoculation of st. RB51, Group 2 shows the highest titer against RB51 W.C. Four weeks after challenge with st. 2308, Groups 1 and 3 showed increased titers against RB51 W.C. Group 2 maintained it's titer to the antigen.

### Western Blot Analysis

Western blot analysis was performed utilizing RB51 W.C. and Y. enterocolitica 0:9 LPS as antigens. Groups 1 and 2 showed reaction to two high molecular weight antigens (93 Kd and 67 Kd) by 28 days post first injection and reaction to the core antigen (14-18 Kd) (Figure V). Group 3 did not react 28 days post first injection. This response increased in intensity by 10 days post second inoculation in Groups 1 and 2, with no reaction in Group 3. At 1 wpc Groups 1 and 2 continue

TABLE XII

## Murine Experiment IV

## RB51 W.C. ANTIBODY TITERS AND SAT TITERS OF POOLED MICE SERA

Group	RB51 W.C. Antibody Titer				STA TEST			
	Day 28	Day 45	Day 56	Day 77	Day 28	Day 45	Day 56	Day 77
1	<20 <sup>a</sup>	80	20	320	<25	<25	<25	400
2	<20	320	160	160	<25	<25	<25	<25
3	<20	<20	20	40	<25	<25	<25	200

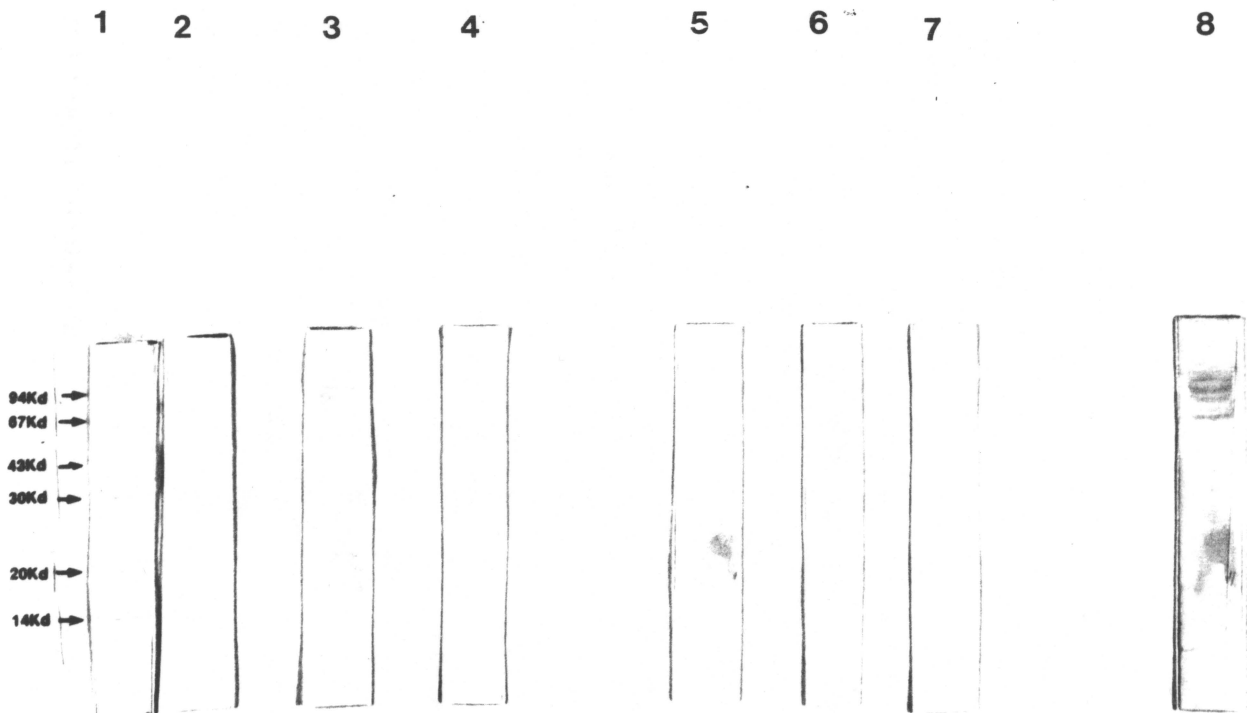
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a. All numerical values expressed as reciprocals.

## Figure V

## Murine Experiment IV

## WESTERN BLOT ANALYSIS OF POOLED MICE SERA AGAINST RB51 W.C. ANTIGEN



Lane 1 Molecular Weight

Lane 2 Group 1, 1 wpc

Lane 3 Group 2, 1 wpc

Lane 4 Group 3, 1 wpc

Lane 5 Group 1, 4 wpc

Lane 6 Group 2, 4 wpc

Lane 7 Group 3, 4 wpc

Lane 8 Steer 66, positive control

the strong response to the antigens, but group 3 did not react (Figure V). At 4 wpc there was little difference in the Group 1 and 2 response, but Group 3 began to recognize the 67-70 Kd antigen group. The 0:9 LPS Westerns blots revealed no reaction to the antigen at any of the four bleedings in Group 2 (Figure VI). Groups 1 and 3 began reacting with the LPS by 4 wpc.

## DISCUSSION

### Murine Experiment I:

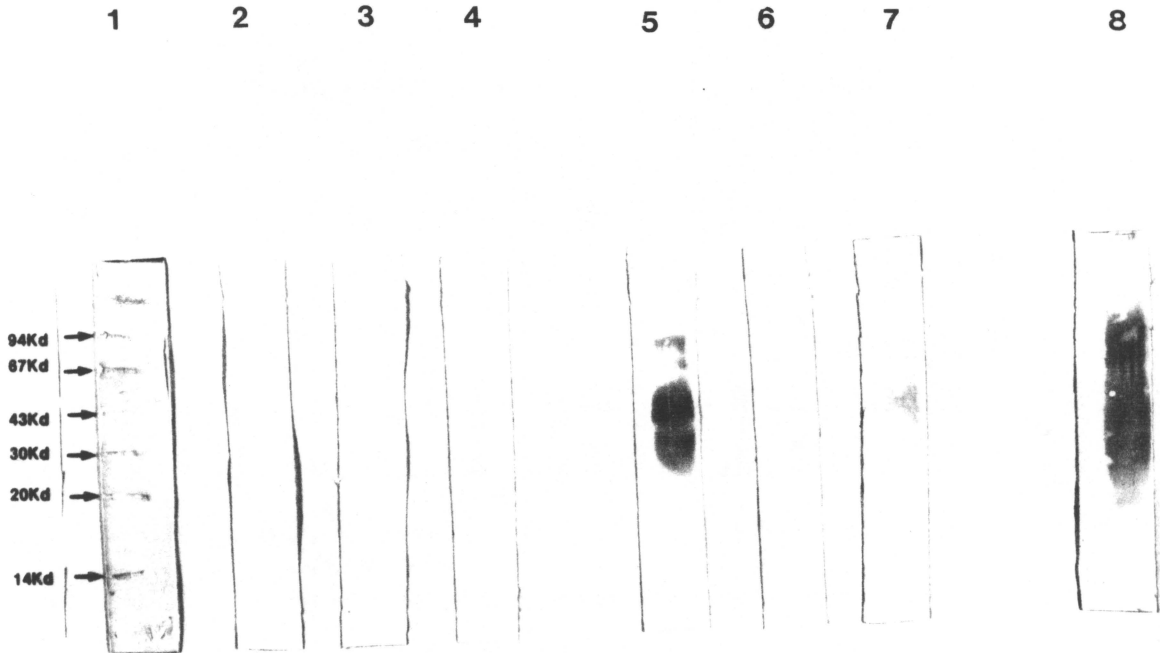
In previous work done in this lab by Ward et al. (73) the investigators determined that C3H/He mice infected with  $10^7$  to  $10^8$  live st. RB51 were still infected with approximately  $10^2$  organisms 14 days after exposure and were able to almost completely clear the infection by 3 weeks post exposure. The data here and from Experiment II, group 4, were consistent with this observation. These experiments suggest that after a single exposure to  $10^8$  organisms of st. RB51, mice were able to clear the infection by 21-28 days post infection with some exceptions (Experiment II, Group 1).

Upon secondary exposure the mice were able to clear the infection rapidly. There was a steady decline in the number of organisms per spleen over the seven days following second inoculation suggesting that

Figure VI

Murine Experiment IV

WESTERN BLOT ANALYSIS OF POOLED MICE SERA AGAINST Y. ENTEROCOLITICA 0:9  
LPS ANTIGEN



Lane 1 Molecular Weight  
Lane 2 Group 1, 1 wpc  
Lane 3 Group 2, 1 wpc  
Lane 4 Group 3, 1 wpc

Lane 5 Group 1, 4 wpc  
Lane 6 Group 2, 4 wpc  
Lane 7 Group 3, 4 wpc  
Lane 8 Steer 121, positive control

no bacterial replication occurred. The mice completely eliminated the Brucella organisms after the second infection between seven and twenty days.

The antibody titers against RB51 W.C. are listed in Table VI. The decrease observed one day following secondary exposure could be due to exposure of new antigen to existing serum antibodies which led to absorbance of these antibodies by antigen and resulting in a temporary decrease of specific serum immunoglobulins. The data were consistent with the kinetics of a secondary antibody response after being reexposed to st. RB51. At 28 dpi the antibody response to one exposure of st. RB51 was increasing but was decreasing by 49 dpi as seen in mice 23 and 24. These results were in contrast mice receiving two inoculations of st. RB51 which were showing an increase of titers at 49 dpi. This suggests that two inoculations of st. RB51 enabled the mice to mount a higher antibody titer to the antigen, perhaps enabling them to clear the antigen more rapidly.

#### **Murine Experiment II:**

The Group 1 mouse that had not cleared infection at 1 wpc with saline had received st. RB51 approximately 5 weeks before spleen culture. Experiment I and the Ward et al. data (73) demonstrated that st. RB51 is cleared by BALB/c and C3H/He mice by 3 to 4 weeks after initial exposure. One mouse was still infected with the minimum number of detectable organisms at 5 wpi. This may be explained by the

individual genetic variability that existed between the mice. Another explanation may be that the mouse was unable to clear the infection due to the presence of another subclinical disease condition. The lack of clearance may also be due to the inoculation of a larger infecting dose of st. RB51. Error may be due to st. RB51's autoagglutin ability.

Group 4 corroborated and strengthened previous data. The group was positive 7 days after initial exposure to st. RB51, but had cleared the infection by 28 days. The clearance results from this group suggested that the one positive mouse at 1 wpc in Group 1 was exceptional. Earlier work with st. RB51 (73) showed that at 7 days after exposure there was an average of  $5 \times 10^4$  bacteria present in the spleen. In this experiment approximately the same number of organisms were present in Group 4 at the same time.

Group 3 demonstrated an interesting effect at 4 wpc. Two of the 3 remaining mice cleared the st. 2308 challenge. According to Montarez and Winter's data (4) exposure to st. 2308 leads to a chronic infection lasting greater than 24 weeks. At 4 weeks after exposure they found the number of bacteria in the spleen to have increased by approximately 2 logs above the infecting dose. The infection had plateaued by 8 weeks and then declined gradually, with a substantial number of st. 2308 present at 24 weeks ( $>1 \times 10^4$ ). In another study, Phillips et al. (69) monitored splenic replication of st. 2308 in vaccinated and nonvaccinated strains of mice 1 to 5 wpc. The vaccinated mice were inoculated with proteinase K digested LPS. In all of the nonvaccinated



mice a 2 log increase in the number of organisms took place over five weeks compared to vaccinated mice. The number of organisms remained high in the nonvaccinated mice until the experiment ended at 12 wpc. Conversely, my data showed that in the remaining infected mouse the number of st. 2308 organisms did not increase over the 4 weeks, suggesting that vaccination with st. RB51 may be as protective as vaccination with st. 19 or proteinase K digested LPS during the first 4 wpc.

Group 7 at 4 wpc showed one mouse cleared of infection, after receiving st. 2308 and then st. RB51. The spleen isolates were tested with acriflavine and crystal violet and were found to be smooth, indicating the absence of st. RB51. This is consistent with the data that st. RB51 was cleared 3 to 4 weeks after initial exposure. My data was unusual and inconsistent with other investigators work (4,57,69) that one mouse was not infected with st. 2308 only 9 weeks after having been exposed to it. Serum agglutination test (SAT) was performed using the pooled sera from each group. The results are presented in Table VIII. Groups 1, 2, and 4 were negative in the SAT test as expected because these groups were not exposed to smooth st. 2308. These results suggest that st. RB51 did not possess the O-side chain at least at levels necessary to induce antibodies. Group 3 was challenged with st. 2308 and began producing detectable levels of anti-O-side chain antibodies by 4 wpc. This observation was in agreement with the Western Blot analysis. Group 3 began reacting with the LPS antigen at 4 wpc

demonstrating the presence of O-antibodies. Groups 5, 6 and 7 reacted as expected in the SAT. At the onset of a primary antibody response the predominant antibody being produced was IgM. This initial IgM production was switched to IgG, a highly specific immunoglobulin. The SAT detected serum IgG and IgM where as the Western Blot detects mainly IgG. The antibodies present in Groups 5, 6 and 7 were able to agglutinate the cells in the SAT, but did not react on Western Blot by day 14 with the Y. enterocolitica 0:9 LPS. By day 28 serum antibodies in these groups developed high enough levels of IgG to be detected by Western Blot analysis. Also, paralleling the initiation of positive Western Blots, there was an increase in titer demonstrated in the SAT for Groups 5 and 7. Group 6 did not react in the SAT but did react on Western Blot suggesting that this nonreactivity experienced by Group 6 on day 28 in the SAT was due to technical error. Because of lack of serum, the test could not be repeated.

Group 8 began to demonstrate a low titer in the SAT only 7 days after smooth exposure. This reaction was not detected on Western Blot analysis suggesting low levels of IgG antibodies. The results from Group 8, 1 wpc were in contrast to Group 3 at that time. After having been challenged with st. 2308 seven days prior, Group 3 demonstrated no reaction in the SAT. These results may suggest that by priming with st. RB51 the anti-O-side chain antibody response was inhibited. My data can also be used to support the mounting evidence that st. RB51 has no O-side chain since priming with st. RB51 did not lead to a rapid anti-O

chain response when the animal was exposed to that antigen. By 4 wpc Group 8's antibody titer had increased in the SAT and reacted with LPS on Western Blot. Also, by 4 wpc this group demonstrated anti-O-side chain antibody titers on the SAT similar to Groups 5 and 7 four weeks after exposure to st. 2308.

To investigate the qualitative differences in the humoral immune responses of the different mouse groups Western Blot analysis's are compared in Figures I and II. At 14 and 28 days post st. RB51 exposure Groups 1, 2 and 3 responded to three antigens (Figure II). The higher molecular weight antigens are approximately 93 and 67-70 Kd's. The third antigen which all three groups recognized, although mildly, was approximately 14-18 Kd's. The antigen was believed to be the core of the LPS molecule because proteinase digestion of the antigen did not affect st. RB51 immune sera reaction with the 14-18 Kd antigen (data not shown) and the antigen migrates in approximately the same region as the core of E. coli. No reaction is apparent in Groups 4 or 8, 14 or 28 days post saline.

Fourteen days post st. 2308, Groups 5, 6 and 7 did not respond to any antigens present in the RB51 W.C. preparation. At 28 days after exposure these groups recognized an antigen in the 67-70 Kd range. There was no response in the core region at 28 days, though st. 2308 appeared to possess the same core determinants as st. RB51 (Schurig, unpublished data).

At 1 wpc, Group 1 exhibits a much stronger response to the core antigen and the 93 Kd antigen. The core response became less well pronounced by 4 wpc suggesting a decrease in titer to that antigen. Group 2 at 1 and 4 wpc with st. RB51 exhibits a strong response to the core and antigens in the 93 and 63 Kd range. Group 3 exhibited a very similar response to that of Group 2 after being challenged with st. 2308. After being primed with st. RB51, Group 3 may have experienced a secondary antibody response to the core due to the exposure to the core of st. 2308 which appeared to have the same determinants as st. RB51 (Schurig, unpublished data). A secondary response was a likely explanation because of the very rapid recognition of the antigen only 7 days after having received st. 2308. Group 4 showed a very mild reaction to the core and high molecular weight antigen 4 weeks after having received st. RB51.

Groups 5, 6 and 7 began to show a more pronounced reaction to the 67-70 Kd antigen and various other high molecular weight antigens present in RB51 W.C. at 1 wpc. Also at that time, the only group to exhibit a mild response to the core antigen was Group 7, having received st. RB51 at challenge. Group 8 demonstrated no reaction at that time. At 4 wpc Groups 6 and 7 continued to develop strong reactions to a variety of antigens. At that time Group 6, having received st. 2308 at challenge, began to recognize the core antigen. Groups 5 and 8 having received only one st. 2308, and no st. RB51, did not recognize this antigen. At 4 wpc a stronger response to the core antigen was expected

in Group 7. Having been exposed to st. 2308 eight weeks beforehand, a priming to the core antigen should have taken place. Upon challenge with st. RB51 a rapid response was expected to take place against the core as seen in Group 3 and 1 and 4 wpc. Instead, a weak response to the core antigen is seen, similar to that of Group 4 at 4 wpc. This data suggests if one assumes that st. 2308 shared core determinants with st. RB51, that challenge with st. 2308 either did not prime the animals to the core determinants or a reaction to the core is inhibited. It appeared that because of the lack of O-side chain the core antigen becomes one of the immunodominant antigens. In latter experiment III, Group 7 and 4 were carried out further to determine if this phenomenon was inhibition or just a delay in the primary and secondary immune response.

To further demonstrate the lack of O-side chain on st. RB51, another Western Blot analysis was performed. Because of the identical O-side chains existing on B. abortus and Y. enterocolitica serotype 0:9 (54), the latter purified LPS preparation was used as antigen. The 0:9 LPS was used to prevent confusion with cross-reacting core antibodies (generated by exposure to st. RB51) from reacting with st. 2308 LPS preparations in which the core is present. Only antibodies induced by the O-side chain will react with Y. enterocolitica 0:9 LPS because this LPS has been determined by Caroff et al. (53) that the core regions of B. abortus and Y. enterocolitica 0:9 do not share antigenic determinants. These Western Blots are compared in Figure I.

Groups 1, 2 and 4 demonstrated no reaction with the LPS antigen throughout the experiment. These groups further corroborated the work done throughout showing the absence of O-side chain in st. RB51. Group 3 reacted with the antigen 4 weeks after having been exposed to st. 2308 as well as Groups 5, 6 and 7 which began to react with the LPS by 28 days post st. 2308 exposure and continued to react throughout the duration of the experiment, clearly demonstrating anti-O-side chain antibodies present in the serum. Group 8 having received saline then st. 2308 began reacting with the LPS 28 days post challenge which was in agreement with the onset of reactivity of Groups 5, 6 and 7.

#### **Murine Experiment III:**

The SAT results were in agreement with my previous results. Group 1 having received st. 2308 showed an agglutination reaction by 28 dpi (No earlier serum samples had been collected in this experiment to determine the onset of agglutination reactions). This titer persisted throughout the duration of the experiment. Challenge with st. RB51 did not affect this response. Group 2 having received only st. RB51 did not demonstrate an agglutination titer at any point during the experiment, further demonstrating the lack of induction of anti-O-side chain antibodies by st. RB51.

By one week after receiving st. RB51 Group 1 responded to the RB51 W.C. antigen as seen by the increase in titer. They demonstrated a three-fold increase by six weeks following the st. RB51 injection. The

immediate (one week) and rapid increase in titer was characteristic of an anamnestic response. Strain 2308 and st. RB51 share common antigens (except the O-side chain). For this reason it was possible that the mice recognized antigens in st. RB51 to which they were primed. By 7 weeks after st. RB51 exposure the animals' response appeared to have plateaued. Group 2, having received one st. RB51, at one week post exposure was not yet responding to the RB51 W.C. antigen. By 6 weeks after exposure a titer of 1:160 was attained. The titer was two-fold lower than Group 1. By 7 weeks after exposure the titer began to drop.

Twelve weeks after receiving st. 2308 and 7 weeks after st. RB51, Group 1 still maintained an average of approximately 4 logs of bacteria per spleen. This number of bacteria still present in the spleen is almost 2 logs lower than what Montarez and Winter (4) demonstrates at the same time period with the same infection dose. But, in their experiment no subsequent challenge followed exposure to st. 2308. Also, Phillips et al. (69) showed that 5 strains of unvaccinated mice maintained a population of st. 2308 between  $10^5$  and  $10^6$  out to 12 weeks post exposure. Because of Montarez and Winter (4) and Phillips et al. (69) data, my results may suggest that st. RB51 has some type of action on the usual course of st. 2308 infection if administered after st. 2308 infection. Unfortunately this suggestion cannot be stated conclusively because this experiment did not include a st. 2308 alone control group

and the number of cfu found at 4 wpi in this system (Experiment II, Group 8) appeared to be in general lower than the number found by Montarez and Winter (4).

Groups 1 and 2 of this experiment reacted as expected on Western Blot analysis against Y. enterocolitica 0:9 LPS. Group 1 responded to the LPS by 28 dpi. This reaction endured throughout the experiment. This reaction was in agreement with the results of Experiment II. These results were also in accordance with the high titers seen in the SAT (Table X) of this experiment and the previous experiment. Group 2 failed to react with the LPS throughout the experiment suggesting the absence of O-side chain antibodies produced by this group.

The Western Blot analysis against RB51 W.C. revealed several interesting observations. The purpose of this experiment was to extend Group 7 of Experiment II. This group was repeated in the present experiment as Group 1. Group 1 received st. 2308 and was challenged with st. RB51 five weeks later. In Experiment II the question was posed that by priming with st. 2308, was the strong core response to st. RB51 observed in st. RB51 injected animals inhibited when later challenged with the rough strain? Four weeks after receiving st. 2308 Group 1 reacted with an antigen in the 67-70 Kd range, the same response seen in the previous experiment. One week after receiving st. RB51 (6 weeks post st. 2308) the reaction in this molecular weight region intensified as seen in the recognition of slightly larger antigens within close proximity of the 67-70 Kd antigen. At that time no reaction with the



core was seen. This observation was in agreement with Experiment II. In Experiment II, a slight core reaction was observed by 4 wpc in the group. We wished to see if a stronger response would occur as the response occurred in other groups (Groups 2 and 3, Experiment II). At 6 wpc in the present experiment, day 77, I observed that a more intense core reaction did not develop in Group 1. There was recognition of higher molecular weight antigens, and weak recognition of the core. This reactivity did not change by 7 wpc, (day 84). These observations suggested that by priming with st. 2308 an inhibition mechanism is turned on that prevents the animal from displaying a full blown core response upon receiving st. RB51 at a later date. A full blown core response was depicted in Groups 2 and 3 at 1 and 4 wpc Experiment II (Figure II). There may be two possible explanations for this response or lack thereof. Because of the lack of response, it may be possible that st. RB51 is not able to survive long enough (in st. 2308 primed animals) to establish an active infection and expose the animal long enough to the antigen. Experiment I and II and other laboratory data (73) demonstrated that one inoculation of st. RB51 is cleared in 3 weeks. Animals primed with st. 2308 may have an ability to clear the organism faster. A faster clearance is logical because the two strains apparently share the same antigenic make up (26) with the exception of the O-side chain. If faster clearance is the case, st. RB51 following st. 2308 would be reminiscent to the animal of a secondary exposure to st. RB51 which was cleared in 7 to 10 days. Another explanation for the

lack of a full blown core response may be an inhibition mechanism turned on by priming with st. 2308. This explanation may be more logical because animals receiving secondary exposure to st. RB51 were still able to respond strongly to the core (Figure II, Experiment II), where animals primed with st. 2308 than st. RB51 were not. Also strengthening this explanation was the fact that animals which were primed with st. RB51 then st. 2308 were not inhibited from responding strongly to the core.

Group 2 of the present experiment did not react with RB51 W.C. until 5 weeks after having received it. The core response at that time was weak, and all responses weaken further by 6 weeks. These results were in agreement with Group 1 of Experiment II between 1 and 4 wpc.

This data suggested that some animals receiving one injection of st. RB51 were able to develop a strong response to the core antigen . (Group 1, 1 wpc, Experiment II). The data also suggested that other animals do not (Group 4, 4 wpc, Experiment II and Group 2, 5 wpc, Experiment III). Also, animals primed with st. 2308, then later exposed to st. RB51, did not develop the strong response (Group 7, 4 wpc, Experiment II, Group 1, 6 and 7 wpc, Experiment III). Animals primed with st. RB51, then later exposed to st. 2308 (Group 3, Experiment II) are able to develop a strong core response.

**Murine Experiment IV.:**

One week post challenge the splenic infection of mice who had not cleared the infection (4 mice) demonstrated 2 logs of protection over controls. There was a significant difference ( $p < .047$ ) between Groups 1 and 3. The protection decreased by 4 wpc, with only one log of protection existing. This protection was significantly different ( $p < .047$ ) from Groups 2 and 3 at 4 wpc. Group 2 contained three mice which had cleared the infection. The control group had cleared none. Group 2 demonstrated two logs of protection at that time over controls, and three logs of protection at 4 wpc. Group 2 was significantly different ( $p < .047$ ) from Group 3 at 1 and 4 wpc, as was Group 1. To summarize the differences, Group 1 and 2 were significantly different from Group 3 at 1 wpc ( $p < .047$ ), but not significantly different from each other. At 4 wpc, Group 2 was significantly different from 1 and 3, and group 1 was significantly different from 2 and 3 ( $p < .047$ ). It can be concluded that significant protection was achieved following one st. RB51 injection. This protection decreased by 4 wpc but was still significant. These results also suggest that 2 injections of st. RB51 were even more protective against st. 2308 challenge. At 1 wpc protection was equivalent to that of one st. RB51 injection (no significant difference), but this protection increased by 4 wpc and became significantly different from 1 injection. Because st. RB51 was devoid of the O-side chain, these results contrast Winter et al. (56) conclusions that O polysaccharide has to be an essential component of

an effective vaccine and that some level of O-side chain antibodies has to be present to provide protective immunity in brucellosis. Also, according to Montarez and Winter's data (14) mice vaccinated with st. 19 two weeks before challenge with st. 2308 and euthanized 1 wpc (two week interval) demonstrated approximately one log of protection. If vaccinated at a four-week interval these animals demonstrated approximately two logs of protection. At 4 wpc, the 2 week interval between vaccination and challenge group demonstrated an increase in splenic bacteria of approximately one log when compared to the 1 wpc, whereas the 4 week interval between vaccination and challenge group demonstrated a log decrease in splenic bacteria when compared to the 1 wpc. In a group that sustained a 6-week interval between vaccination and challenge there was approximately 2 logs of protection seen at 1 wpc and 1 log protection seen at 4 wpc. Although none of the groups in this experiment can be directly compared with Montarez and Winter's (4), Group 1 sustained a 7 week interval and Group 2 received two inoculations of st. RB51, Group 2 splenic bacterial counts follow a very similar pattern to those of Montarez and Winter's (4) 4 week vaccination-challenge interval group. These comparisons may suggest that 2 vaccinations with live st. RB51 may be as protective in mice as one st. 19 vaccination.

Forty-five days after receiving st. RB51, Group 1 showed a moderate antibody response st. RB51. At one wpc with st. 2308 a decrease in titer was observed. By four weeks post challenge, the group

demonstrated a four fold increase in titer. Group 2 mounted a much higher response to RB51 W.C. after two injections of st. RB51. Challenge with st. 2308 did not increase this titer, unlike the other two groups. At 1 and 4 wpc, Group 2 demonstrated a plateau effect. Group 3 showed the lowest antibody titer to RB51 W.C. antigen.

For the first three serum collections in this experiment, the SAT results were close in agreement with experiments II and III. Mice in all three groups showed no agglutination reaction up to 1 wpc with st. 2308. Group 1, having received only one st. RB51 inoculation before challenge with st. 2308 demonstrated a high titer to the smooth tube antigen 4 wpc, as did Group 3 in experiment II. Group 3 of this experiment having received only saline before to challenge did not show a response at 1 wpc, unlike Group 8 of experiment II. These results did not pose an unusual problem because at 1 wpc an antibody response against the O-side chain was only beginning to develop, as can be seen by the very low titer (1:25) of Group 8 in the experiment II. Apparently, Group 3 in the present experiment had a titer of less than 1:25. At 4 wpc, Groups 1 and 3 of this experiment exhibited an antibody titer very similar to the comparable groups in experiment II, Groups 3 and 8. An interesting result was seen at 4 wpc in Group 2. This group still did not agglutinate the smooth antigen 4 weeks after having been exposed to st. 2308, a point in time where all other groups regardless of previous inoculums, did agglutinate the antigen (experiments II and III). Western Blot analysis against Y. enterocolitica 0:9 LPS confirmed

this lack of reaction. The question was raised of the possibility of st. RB51 inhibiting/delaying an anti-O side chain antibody response. These results clearly demonstrated the possibility of the phenomenon. These results also raised the possibility pooling the serums after euthanasia, the two clean mice serums decreased the titers of the three infected mice, therefore lowering the group titer to less than 1:25. This explanation was unlikely because the negative sera could have diluted the antibodies from positive sera only by a factor of 1:0.4.

Western Blot analysis against RB51 W.C. follow the patterns observed in Experiments II and III. Groups 1 and 2 recognized antigens in the 93 and 67-70 Kd range as well as core determinants in the 14-18 Kd range 28 days after receiving st. RB51. These observations are in agreement with my previous experiments. Group 3 recognized no antigens at that time. In Group 2 the reaction to these antigens became enhanced 10 days after the second inoculation of st. RB51. At that time Group 1's reaction did not change quantitatively, and Group 3 still did not react. At 1 wpc, Group 2 demonstrated an even stronger response to the core, as was seen in Group 3 of Experiment II. Another high molecular weight antigen was also recognized between the 93 and 70 Kd antigens. Group 1's core response strengthened, but no increase in the intensity of reaction with high molecular weight antigens was seen. At this time, 1 wpc, Group 3 still exhibited no response to RB51 W.C. These results were in agreement with Group 8 Experiment II. By 4 wpc, Group 1's reaction to the high molecular weight antigen was seen. At this time, 1

wpc, Group 3 still exhibited no response to RB51 W.C. These results were in agreement with Group 8 Experiment II. By 4 wpc, Group 1's reaction to the high molecular weight antigens and the core antigen had strengthened. Group 2 remained the same as at 1 wpc, except that this group was only recognizing one high molecular weight antigen of approximately 67-70 Kd. Also at that time, 4 wpc, Group 3 began to react with the same antigen but failed to react with the core antigen 4 wpc, which was in agreement with Group 8 of Experiment II.

The Western blot analysis, using Y. enterocolitica 0:9 LPS gave typical results at 28 days post first inoculation and 10 days post second inoculation in that no groups responded to the LPS. Furthermore, none of the three groups responded at 1 wpc. These results were typical when compared to the onset of reaction with LPS in Experiment II. By 4 wpc, Groups 1 and 3 responded to the antigens as seen in previous experiments. Group 2, having received two st. RB51 injections before challenge with st. 2308 showed no reaction to the LPS antigen 4 wpc. These results were in accordance with the results of the SAT. The question was posed in Experiments II and III that by priming with st. 2308 was the response to the core antigen inhibited when later exposed to st. RB51? Experiment II and III suggested response is inhibited, but for reasons unknown as of yet, although logical explanations were proposed. Conversely, in this experiment, the question arose that by priming with 2 injections of st. RB51, is the response to the O-side chain being inhibited when later exposed to st. 2308? The data here

suggest that inhibition was the case as seen in the results of the SAT and Y. enterocolitica 0:9 LPS Western Blot. One injection of st. RB51 had not led to this effect, as seen in Group 1, Experiment IV and Group 3, Experiment II. Experiment IV indicated that two injections of st. RB51 are protective and able to inhibit the production of anti-O-side chain antibodies for at least 4 weeks following challenge with st. 2308.

#### CONCLUSIONS

The results from murine Experiments I, II, III and IV led to the following conclusions:

1. An initial injection of st. RB51 in mice was cleared by 4 weeks post inoculation.
2. A second st. RB51 injection in mice is cleared in 1 to 3 weeks.
3. Primary exposure to st. RB51 then secondary exposure to st. 2308 resulted in a very strong humoral response to core determinants by 1 week post secondary exposure.
4. Primary exposure to st. 2308 then secondary exposure to st. RB51 did not result in this strong response which suggested the animals had not been primed to the antigen(s) or that the response was inhibited.



5. One or two injections of st. RB51 were protective in mice against challenge with virulent st. 2308. Two injections provided more significant protection 4 weeks after challenge.
6. Two injections of st. RB51 resulted in the failure in mice to produce anti-O-side chain antibodies after challenge with st. 2308 for at least 4 weeks.

If these observations could be repeated in cattle, that is protection, no induction of anti-O-side chain antibodies by the vaccine or after exposure to a virulent strain, st. RB51 would be the ideal bovine vaccine strain.

## CHAPTER 2

### EXPERIMENTAL INFECTION OF CATTLE WITH ST. RB51

#### RATIONALE

The previous murine experiments (I,II,III,IV) demonstrated that st. RB51 was a rough mutant of B. abortus able to induce a humoral immune response directed toward antigens distinct from the persoamine O-side chain. Murine Experiment IV demonstrated that st. RB51 induced a protective immune response. Because of these results investigations into the biological behavior of st. RB51 in cattle (the primary reservoir of B. abortus) to begin assessing st. RB51's potential as a vaccine strain became of interest.

The experiments in Chapter 2 were designed to evaluate several parameters: 1) the survival capabilities of st. RB51 2) the organism's stability in cattle and 3) the humoral immune response of st. RB51 infected cattle. Survival of the organisms should be long enough to induce an immune response in cattle. The stability of st. RB51 in cattle is important because reversion to smooth form is not desirable. The vaccine currently used, st. 19, a smooth B. abortus strain, produces antibody titers indistinguishable from titers induced by natural exposure (1). These titers are the primary problem hindering the eradication of the disease (48). These titers are the result of the

animal's response to the O-side chain of smooth strains. My data demonstrated (Chapter 1, Experiments II, III and IV) that st. RB51 does not induce O-side chain antibody titers (in the SAT and on Western Blot analysis) in the mouse. I therefore wanted to determine the ability of st. RB51 to maintain its roughness colony morphology when introduced into cattle.

#### EXPERIMENTAL DESIGN

Bovine Experiment I: Effect of two subcutaneous injections of live st. RB51 in cattle.

The experimental design is outlined in Table XIII. Four cows, N0132, N0124, N0133 and Y141, were inoculated subcutaneously on each side of the neck with live st. RB51. Two cows, N0133 and Y141, received a total of  $2 \times 10^9$  organisms (low dose) and 2 cows, N0124 and N0132, received a total of  $2 \times 10^{10}$  organisms (high dose) for the first inoculation. A pre-inoculation blood sample was obtained, and weekly blood samples thereafter for the duration of the experiment. Seven and 15 days post first inoculation prescapular lymph node biopsies were obtained and cultured for the presence of st. RB51. The cattle were inoculated with a second dose of st. RB51 approximately 3 months after the primary inoculation. The booster dose was similar to the initial dose.

## TABLE XIII

## Bovine Experiment I

EFFECT OF TWO SUBCUTANEOUS (SC) INJECTIONS OF LIVE RB51 IN CATTLE

## EXPERIMENTAL DESIGN

2 COWS (N0133 and Y141) INOCULATED WITH  $10^9$  LIVE RB51 S.C. IN THE NECK REGION.

2 COWS (N0124 AND N0132) INOCULATED WITH  $10^{10}$  LIVE RB51 S.C. IN THE NECK REGION.

weeks<sup>c</sup> 0<sup>a</sup> 1<sup>b</sup> 2<sup>b</sup> 3 4 5 6 7 8 9<sup>a</sup> 10 11 12 13 . . . . . 56

- 
- a. RB51 inoculated week 0 and 9.
- b. Lymph node biopsies obtained at 7 and 15 days post inoculation.
- c. Weekly bleedings obtained throughout the experiment.

One low dose cow, Y141, was approximately 2 months pregnant at the initiation of the study. This cow delivered a full term calf that died at birth. One high dose cow was bred two months after the second inoculation.

**Bovine Experiment II:** Effect of one subcutaneous injection of live st. RB51 in cattle.

The experimental design is outlined in Table XIV. The design was similar to that of Experiment I except only one injection was administered subcutaneously and lymph node biopsies were obtained 22 days after inoculation.

**Bovine Experiment III:** Effect of one conjunctival inoculation of live st. RB51 in cattle.

The experimental design is outlined in Table XV. The design was similar to that of Experiments I and II except that cows were inoculated once intraconjunctivally and no lymph node biopsies were obtained.

## TABLE XIV

## Bovine Experiment II

EFFECT OF ONE SUBCUTANEOUS (SC) INJECTION OF LIVE RB51 IN CATTLE

## EXPERIMENTAL DESIGN

4 COWS (Y148, Y150, Y151, Y154) INOCULATED WITH  $10^9$  LIVE  
RB51 S.C. IN THE NECK REGION.

weeks<sup>c</sup>    0<sup>a</sup> 1 2 3<sup>b</sup> 4 5 6 7 8 9 10 11 12 13 . . . . 18

---

- a. RB51 inoculated week 0.
- b. Lymph node biopsies obtained 22 days post inoculation
- c. Weekly bleedings obtained throughout the experiment.

## TABLE XV

## Bovine Experiment III

THE EFFECT OF ONE CONJUNCTIVALLY ADMINISTERED DOSE OF LIVE RB51 IN  
CATTLE  
EXPERIMENTAL DESIGN

4 COWS (Y145, Y146, Y147, Y149) INOCULATED WITH  $10^9$  LIVE RB51  
CONJUNCTIVALLY.

weeks<sup>b</sup>    0<sup>a</sup>  1  2  3  4  5  6  7  8  9  10  11  12  13  . . . . . 18

---

a.    RB51 inoculated week 0.

b.    Weekly bleedings obtained throughout the experiment

\*\*NOTE: No lymph node biopsies obtained.

## RESULTS

### Bovine Experiment I

#### Lymph Node Biopsies

Data on lymph node biopsies and the number of organisms recovered are summarized in Table XVI. All four cows biopsied were positive for st. RB51 7 dpi. Three of four cows were positive for st. RB51 15 dpi.

The lymph node isolates from the animals were tested for acriflavine agglutination, crystal violet staining and rifampin resistance. The results are summarized in Table XVII. All 7 and 15 day isolates agglutinated in acriflavine, picked up crystal violet stain and were resistant to rifampin at 400 ug/ml.

These isolates and the rifampin resistant growth were tested in the RIT (64) against monoclonal antibodies Bru 38 and Bru 48. The results are listed in Table XVIII and the representative reaction is shown in Figure VII.

Six isolates obtained from various bodily locations of a full-term calf that died were tested in all of the above assays as well. These results are summarized in Table XIX.

#### Isolate Testing

One isolate obtained 15 dpi was selected to be cultured and used to reinfect mice. Upon primary inoculation, st. RB51 infection was determined to be cleared in mice within 3 to 4 weeks (Chapter I). One group of six mice were infected with the cow isolate and the clearance



TABLE XVI

## Bovine Experiment I &amp; II

## LYMPH NODE BIOPSY RESULTS FROM S.C. INFECTED CATTLE

COW	Tissue Obtained			Organisms/gm tissue		
	7 dpi	15 dpi	22 dpi	7 dpi	15 dpi	22 dpi
N0124	yes	yes	no	$4 \times 10^2$	$2 \times 10^1$	ND
N0132	yes	yes	no	$4 \times 10^3$	$7 \times 10^3$	ND
N0133	yes	yes	no	$6 \times 10^1$	$7 \times 10^3$	ND
Y141	yes	yes	no	$5 \times 10^2$	NEG	ND
Y148	no	no	yes	ND	ND	$3 \times 10^2$
Y150	no	no	yes	ND	ND	$2 \times 10^4$
Y151	no	no	yes	ND	ND	$7 \times 10^3$
Y154	no	no	yes	ND	ND	$2 \times 10^3$

TABLE XVII

## Bovine Experiment I

ACRIFLAVINE AGGLUTINATION (AGG), CRYSTAL VIOLET UPTAKE (C.V.), AND  
RIFAMPIN RESISTANCE (R.R.) OF LYMPH NODE ISOLATES.

COW	7 dpi AGG	7dpi C.V.	15 dpi AGG	15 dpi C.V.	7 dpi R.R. 400 ug/ml	15 dpi R.R. 400 ug/ml
N0133	+	+	+	+	+	+
N0132	+	+	+	+	+	+
N0124	+	+	NA <sup>b</sup>	NA <sup>b</sup>	+	NA <sup>b</sup>
Y141	+	+	NA <sup>a</sup>	NA	+	NA

a. Na isolates not recovered.

b. Isolates not received from L.S.U., but were recovered from the lymph node.

TABLE XVIII

## Bovine Experiment I

REACTION OF LYMPH NODE ISOLATES IN THE RAPID IDENTIFICATION ASSAY  
WITH MONOCLONAL ANTIBODIES BRU 38 AND BRU 48.

COW	7 dpi		15 dpi		7 dpi		15 dpi	
	BRU38	BRU48	BRU38	BRU48	BRU38	BRU48	BRU38	BRU48
N0133	-	+	-	+	-	+	-	+
N0132	-	+	-	+	-	+	-	+
N0124	-	+	NA <sup>a</sup>	NA <sup>a</sup>	-	+	NA <sup>a</sup>	NA <sup>a</sup>
Y141	-	+	NA <sup>b</sup>	NA <sup>b</sup>	-	+	NA <sup>b</sup>	NA <sup>b</sup>

- a. Isolates not received from L.S.U., but were recovered from the lymph node.
- b. Isolates not recovered from lymph node.

Figure VII

Bovine Experiment I

RAPID IDENTIFICATION ASSAY OF BOVINE LYMPH NODE ISOLATES UTILIZING

MONOCLONAL ANTIBODIES BRU 38 (ANTI-O-SIDE CHAIN)

AND

BRU 48 (ANTI-CORE DETERMINANTS)

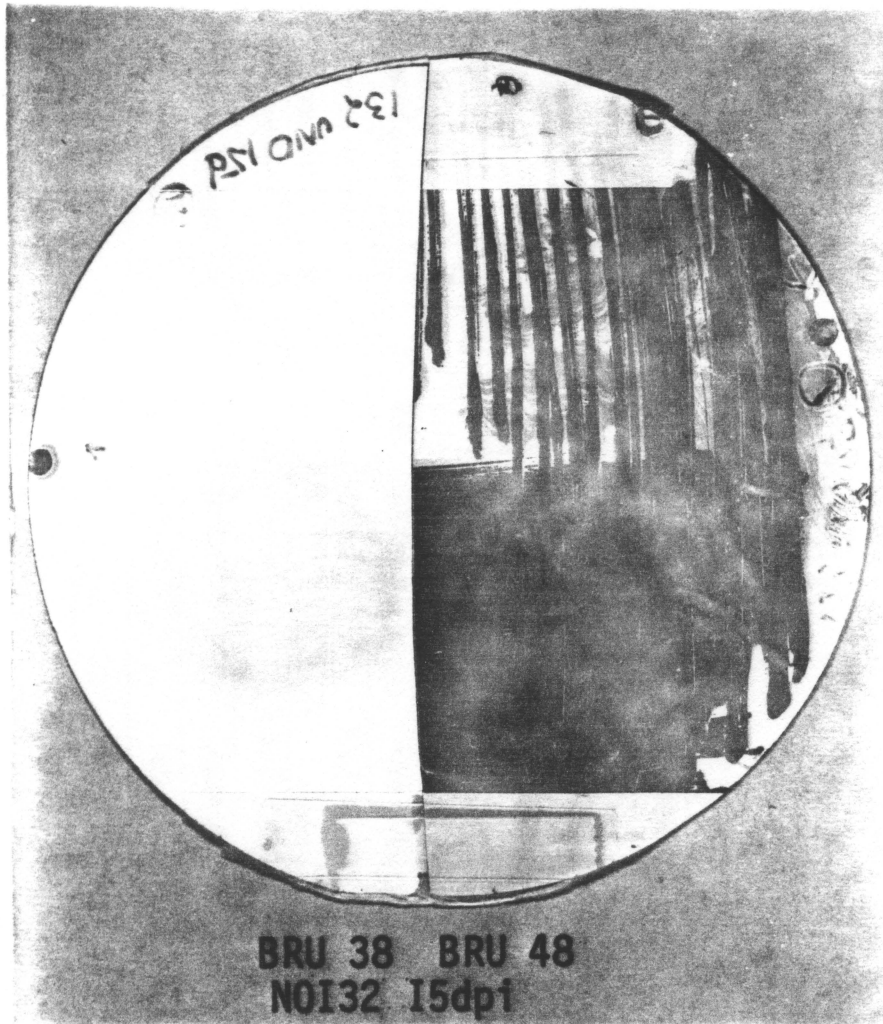


TABLE XIX

## Bovine Experiment I

REACTIONS OF CALF TISSUE CULTURE ISOLATES IN THE ACRIFLAVINE,  
CRYSTAL VIOLET, RIFAMPIN RESISTANCE AND RAPID IDENTIFICATION ASSAYS.

Y141 Calf	Sample	AGG	C.V.	R.R.	BRU38	BRU48
	Prescapular Lymph Node	+	+	+	-	+
	Lung	+	+	+	-	+
	Abomasal Fluid	+	+	+	-	+
	Abomasal Fluid	+	+	+	-	+
	Cotyledon	+	+	+	-	+
	Lung	+	+	+	-	+

of the isolate was compared with a group of 6 mice inoculated with stock RB51. Five mice from each group had cleared the infection. One mouse from each group was infected with approximately 80 organisms. The cfu were tested in the RIT (64) and the organisms from both mice reacted with Bru 48 but not Bru 38. The results are summarized in Table XX. No difference in the rate of clearance was observed.

### Serology

Serum samples were tested in the SAT test for the presence of O-side chain antibodies. Weekly samples were obtained from cows for over thirteen months and all were tested through the thirteenth month. The results from the SAT of only 1 representative sample from each month are presented in Table XXI. Serum from the dead calf was also obtained and tested in the SAT. These results are included in Table XXI. The highest complete agglutination reaction of any of the four cows achieved was 1:25, although incomplete agglutinations at a serum dilution of 1:50 did occur. The RB51 W.C. antibody titers for Experiment I are included in Table XX.

Selected serum samples from Experiment I were sent to the State Laboratory in Wytheville, VA for confirmation of SAT test results and evaluation in various other assays, including the Buffered antigen plate agglutination (BAPA) test. These results are presented in Table XXII. Of 16 samples sent from Experiment I, 1 sample from 2 different cows showed an incomplete reaction at a dilution of 1:50 in the SAT. These were samples from cow N0133 on 3/23/88 and Y141 on 7/18/88. All of the

## TABLE XX

## Bovine Experiment I

## CLEARANCE OF ISOLATED RB51 IN MICE

	inoculum	25 dpi clean/infected spleens	number organisms recovered	react with BRU48
GROUP 1	isolate	5/1	80	+
GROUP 2	stock	5/1	80	+

TABLE XXI

## Bovine Experiment I

## RB51 W.C. ANTIBODY TITERS AND SAT RESULTS.

DATE	dpi	N0132		N0124		N0133		Y141		CALF	
		a	b	a	b	a	b	a	b	a	b
1/20/88	(pre)	<20 <sup>c</sup>	<25	<20	<25	<20	<25	<20	<25		
2/04/88	(15)	640	<25	640	<25	320	<25	320	<25		
3/23/88	(62)	640	<25	640	<25	320	<25	320	<25		
4/04/88	(74)	2560	<25	2560	<25	320	<25	1280	<25		
4/20/88	(90)	5120	<25	2560	<25	1280	<25	1280	<25		
5/1/88	(118)	2560	<25	2560	<25	2560	<25	2560	<25		
5/25/88	(125)	2560	<25	2560	<25	2560	<25	2560	<25		
6/7/88	(138)	2560	<25	2560	<25	2560	<25	2560	<25		
6/20/88	(151)	640	<25	2560	<25	2560	<25	2560	<25		
7/18/88	(179)	640	251 <sup>d</sup>	320	<25	160	<25	2560	<25		
8/15/88	(207)	160	25	160	<25	160	<25	1280	<25	0	<25
9/14/88	(236)	160	25	80	25	80	25	1280	<25		
10/25/88	(277)	160	25	20	25	80	25	640	<25		
11/09/88	(292)	160	25	40	25	80	25	320	<25		
12/16/88	(329)	died		40	25	80	<25	320	<25		
1/14/89	(358)			40	25	80	25	320	25		
2/17/89	(392)			40	25	80	25	320	501 <sup>e</sup>		
2/23/89	(398)			40	25	80	25	320	501 <sup>e</sup>		

a. RB51 W.C. antibody titer.

b. SAT results.

c. All numerical values expressed as reciprocals.

d. Incomplete agglutination at a dilution of 1:25.

e. Incomplete agglutination at a dilution of 1:50.



TABLE XXII  
Bovine Experiment I

## STATE LABORATORY SAT RESULTS AND BAPA RESULTS

Code#	Sample	STT <sup>b</sup>	BAPA
9	N0132 pre	N	N
3	N0132 3/23	N	N
13	N0132 7/18	N	N
7	N0132 10/5	N	N
4	N0124 pre	N	N
10	N0124 3/23	N	N
1	N0124 7/18	N	N
8	N0124 9/14	N	N
12	N0133 pre	N	N
2	N0133 3/23	501 <sup>a</sup>	N
11	N0133 7/18	N	N
15	N0133 2/17	N	N
6	Y141 pre	N	N
16	Y141 3/23	N	N
14	Y141 7/18	501 <sup>a</sup>	N
5	Y141 2/17	N	N
17	Rabbit 3/89	N	N
18	Steer 66	N	N
19	Steer 121	200	+
20	Goat 48	N	N

a. Incomplete agglutination at a dilution of 1:50

b. Numerical values expressed as reciprocals.

other samples and the st. RB51 positive controls were negative in the SAT. One animal Steer 121, which was a st. 19 positive control, demonstrated a titer of 1:200. In the BAPA, all experimental serums and st. RB51 positive control animals were negative. Steer 121 was positive in this test as well as all other tests run by the State Laboratory.

#### Western Blot Analysis

Western Blot analysis against RB51 W.C. and Y. enterocolitica 0:9 LPS was performed on many serum samples. Representative blots are presented in Figures VIII, IX and X. The cows demonstrated a strong core response early in the experiment, and reaction with a variety of high molecular weight antigens. Over time, these responses decreased in intensity. No reaction with the smooth LPS was observed at any point in the experiment. In Figure XI, positive control animals are shown. Goat 35 and Steer 66, RB51 positive controls did not react with 0:9 LPS. Steer 121, st. 19 positive control did react with Y. enterocolitica 0:9 LPS.

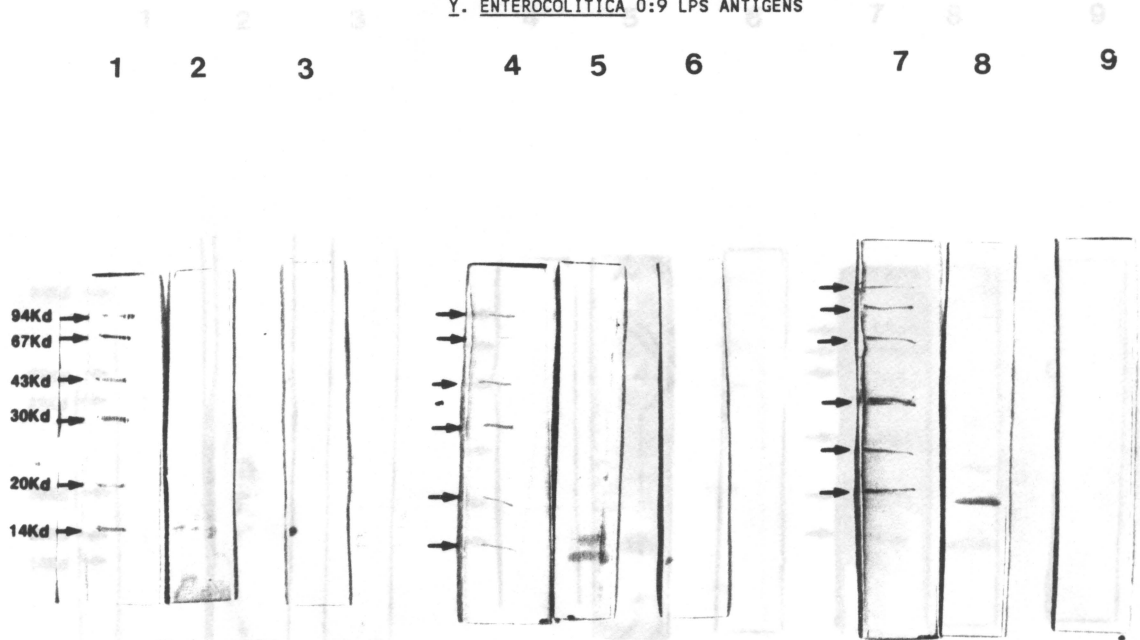
Selected serum samples from Experiment I were chosen for absorption studies. One or 2 samples from each cow collected on 4/6/88 and/or 5/12/88 were chosen for these experiments. The serum was absorbed with live st. RB51 or with live Y. enterocolitica 0:9. Upon Western Blot analysis against RB51 W.C. antigen my observation was that absorption with live RB51 removed all reactivity from the serum samples

Fig. 8  
Bov. Figure VIII

WESTERN BLOT ANALYSIS OF Bovine Experiment I C. AND E. ENTEROCOLITICA

WESTERN BLOT ANALYSIS OF N0133 AGAINST RB51 AND W.C. AND

Y. ENTEROCOLITICA 0:9 LPS ANTIGENS



<u>ANTIGEN</u>	<u>SAMPLE DATE</u>
Lane 1 Molecular Weight	
Lane 2 RB51 W.C.	03/23/88
Lane 3 0:9 LPS	03/23/88

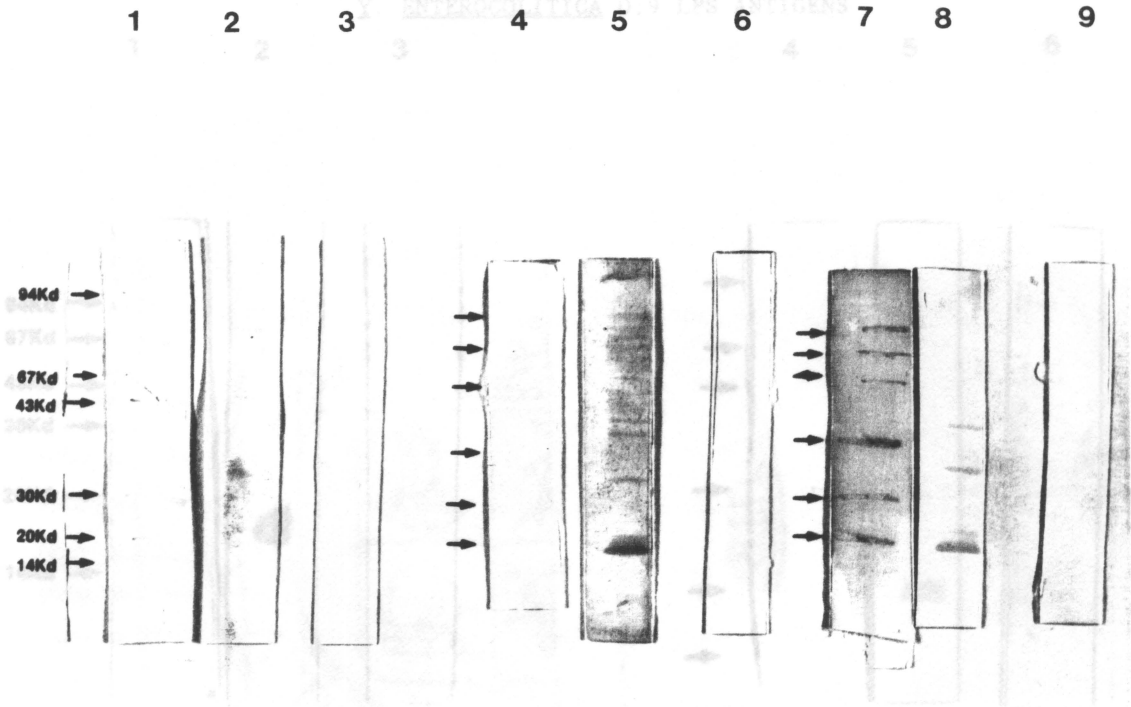
<u>ANTIGEN</u>	<u>SAMPLE DATE</u>
Lane 4 Molecular Weight	
Lane 5 RB51 W.C.	11/09/88
Lane 6 0:9 LPS	11/09/88
Lane 7 Molecular Weight	
Lane 8 RB51 W.C.	04/26/89
Lane 9 0:9 LPS	04/26/89

Figure IX

Bovine Experiment I

WESTERN BLOT ANALYSIS OF Y141 AGAINST RB51 W.C. AND Y. ENTEROCOLITICA

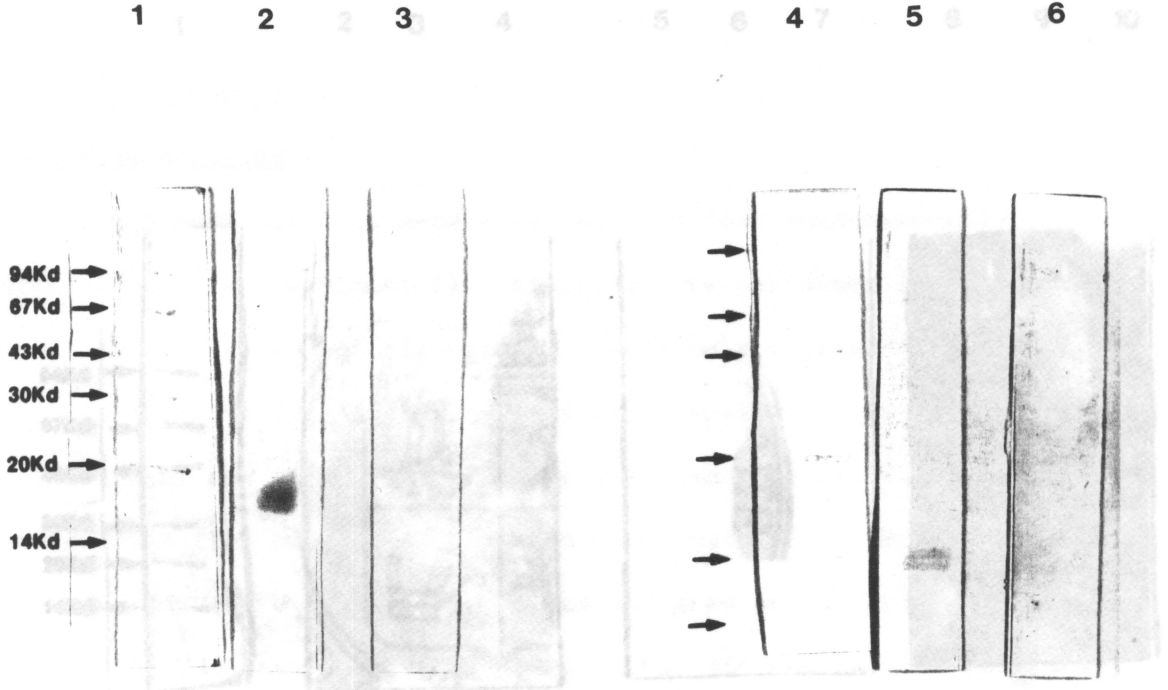
0:9 LPS ANTIGENS



ANTIGEN	SAMPLE DATE	ANTIGEN	SAMPLE DATE
Lane 1 Molecular Weight		Lane 4 Molecular Weight	
Lane 2 RB51 W.C.	03/23/88	Lane 5 RB51 W.C.	11/09/88
Lane 3 0:9 LPS	03/23/88	Lane 6 0:9 LPS	11/09/88
Lane 4 Molecular Weight		Lane 7 Molecular Weight	
Lane 5 RB51 W.C.		Lane 8 RB51 W.C.	04/26/89
Lane 6 0:9 LPS		Lane 9 0:9 LPS	04/26/89

Figure X

WESTERN BLOT ANALYSIS OF POSITIVE CONTROL ANIMALS AGAINST RB51 LPS,  
 Bovine Experiment I  
 Y. ENTEROCOLITICA 0:9 LPS, AND RB51 W.C. ANTIGENS  
 WESTERN BLOT ANALYSIS OF N0132 AGAINST RB51 W.C. AND  
 Y. ENTEROCOLITICA 0:9 LPS ANTIGENS



ANTIGEN

SAMPLE DATE

Lane 1 Molecular Weight

Lane 2 RB51 W.C.

Lane 3 0:9 LPS

Lane 4 Molecular Weight

Lane 5 RB51 W.C.

Lane 6 0:9 LPS

03/23/88

03/23/88

11/09/88

11/09/88

ANTIGEN	SERUM
Lane 6 0:9 LPS	Stear 121
Lane 7 RB51 W.C.	Stear 121
Lane 8 RB51 LPS	Stear 06
Lane 9 0:9 LPS	Stear 06
Lane 10 RB51 W.C.	Stear 06

Figure XI

WESTERN BLOT ANALYSIS OF POSITIVE CONTROL ANIMALS AGAINST RB51 LPS, Y. ENTEROCOLITICA 0:9 LPS, AND RB51 W.C. ANTIGENS

1 2 3 4 5 6 7 8 9 10

Bovine Experiment II

Lymph Node Biopsies

Lymph node isolates were obtained from four subcutaneously infected cows in Experiment II. Biopsies were performed... The results from... flavins agglutination... summarized in Table... were positive in the... and grew on rifampin... but did not react...

Serology

SAT results and RB51 W.C. antibody titers are presented in Table XXIV. The highest complete SAT reaction of any of the 4 cows achieved was 1:50. RB51 W.C. antibody titers rose for approximately two months, then began to decline.

<u>ANTIGEN</u>	<u>SERUM</u>	<u>ANTIGEN</u>	<u>SERUM</u>
Lane 1 Molecular Weight		Lane 6 0:9 LPS	Steer 121
Lane 2 RB51 LPS	Goat 35	Lane 7 RB51 W.C.	Steer 121
Lane 3 0:9 LPS	Goat 35	Lane 8 RB51 LPS	Steer 66
Lane 4 RB51 W.C.	Goat 35	Lane 9 0:9 LPS	Steer 66
Lane 5 RB51 LPS	Steer 121	Lane 10 RB51 W.C.	Steer 66

of every cow. When the serum absorbed with live Y. enterocolitica 0:9 was tested against this antigen no reactivity of the serum samples was lost.

## Bovine Experiment II

### Lymph Node Biopsies

Lymph node isolates were obtained from four subcutaneously infected cows in Experiment II. Biopsies were performed 22 days after inoculation. The number of organisms isolated are presented in Table XVI. The results from the RID, acriflavine agglutination, crystal violet uptake and rifampin resistance are summarized in Table XXIII. The isolates from all 4 cows biopsied were positive in the acriflavine agglutination and crystal violet tests and grew on rifampin at 400 ug/ml. The isolates reacted with Bru 48 but did not react with Bru 38.

### Serology

SAT results and RB51 W.C. antibody titers are presented in Table XXIV. The highest complete SAT reaction of any of the 4 cows achieved was 1:50. RB51 W.C. antibody titers rose for approximately two months, then began dropping.

## TABLE XXIII

## Bovine Experiment II

ACRIFLAVIN AGGLUTINATION (AGG), CRYSTAL VIOLET UPTAKE (C.V.),  
RIFAMPIN RESISTANCE (R.R.) AND RAPID IDENTIFICATION TESTS OF 22  
dpi LYMPH NODE BIOPSY.

COW	AGG	C.V.	R.R. 400ug/ml	BRU38	BRU48
Y148	+	+	+	-	+
Y150	+	+	+	-	+
Y151	+	+	+	-	+
Y154	+	+	+	-	+



TABLE XXIV

## Bovine Experiment II

## RB51 W.C. ANTIBODY TITERS AND SAT RESULTS

DATE	dpi	Y148		Y150		Y151		Y154	
		a	b	a	b	a	b	a	b
10/10/88	(pre)	<20 <sup>c</sup>	<25	<20	<25	<20	<25	<20	<25
11/9/88	(30)	320	251 <sup>d</sup>	80	<25	160	<25	40	<25
12/7/88	(58)	320	251 <sup>d</sup>	80	<25	160	<25	40	<25
1/6/89	(88)	160	25	40	<25	320	<25	40	251 <sup>d</sup>
2/23/89	(136)	80	50	40	50	40	<25	40	251 <sup>d</sup>

- a. RB51 W.C. antibody titer.
- b. SAT titer.
- c. All values expressed as reciprocals.
- d. Incomplete agglutination at a dilution of 1:25.

### Bovine Experiment III

#### Serology

The results of the SAT test for Experiment III are summarized in Table XXV. The highest complete SAT reaction of any of the four cows achieved was 1:50. The RB51 W.C. antibody titers are also in Table XXV. In 2 animals, Y149 and Y145, antibody titers began dropping after 3 months post exposure. Y147 began dropping after 2 months and Y146 maintained the same titer throughout the months tested. This group of cows never attained the level of antibody titers seen in Experiments I and II.

### DISCUSSION

#### Bovine Experiment I

In Experiment I, all four cows yielded st. RB51 isolates 7 days post subcutaneous inoculation. The number of organisms recovered from the biopsy samples ranged from  $6 \times 10^1$  to  $3.8 \times 10^3$  organisms/g of tissue. At 15 days post inoculation organisms were recovered from 3 of the 4 cows. The number of organisms ranged from  $2.1 \times 10^1$  to  $7 \times 10^3$ /g of tissue. The entire prescapular lymph node was not cultured, therefore, these figures are not necessarily representative of the total number of organisms in the lymph node present at those times because it was not known if st. RB51 will distribute itself homogeneously throughout an infected lymph node. The purpose of

TABLE XXV

## Bovine Experiment III

## RB51 W.C. ANTIBODY TITERS AND SAT RESULTS

DATE	dpi	Y145		Y146		Y147		Y149	
		a	b	a	b	a	b	a	b
10/10/88	(pre)	<20 <sup>c</sup>	25	<20	25	<20	<25	<20	<25
11/9/88	(30)	80	25	40	25	80	<25	160	<25
12/7/88	(58)	40	25	40	<25	80	<25	160	<25
1/6/89	(88)	80	25	40	50	40	<25	160	<25
2/23/89	(136)	20	25	40	<25	40	<25	80	<25

- a. RB51 W.C. Antibody Titer.
- b. SAT titer.
- c. All values expressed as reciprocals.

the biopsy was to determine the presence (survivability) and condition (rough or smooth) of st. RB51 in cattle, not replication capabilities or total surviving numbers.

Subcutaneously infected cattle were biopsied 22 days post inoculation (Bovine Experiment II). Four of four yielded st. RB51 organisms at that time. From the samples, the numbers of organisms ranged from  $10^2$  to  $10^4$  bacteria with the average being  $6.9 \times 10^3$  organisms/sample. These data indicated that the population of st. RB51 in the lymph node was not being eliminated by 22 days post inoculation. Thus, st. RB51 can survive in cattle for a minimum of three weeks after inoculation.

The lymph node isolates were grown on TSBA planes trypticase streaked from the original cultures and tested in a variety of assays. The goal was to determine if the organism had maintained its rough colony morphology (not reverted to smooth in vivo), and if st. RB51 maintained its rifampin resistance marker after passage in cattle.

White et al. (38) showed that smooth and rough colonies can be differentiated by their crystal violet dye uptake. Smooth individual colonies will not stain when flooded with crystal violet where rough colonies do take up the dye. Braun et al. (37) demonstrated the effect of suspending smooth or rough colonies of B. abortus in neutral acriflavine. Rough colonies agglutinate macroscopically where smooth colonies do not. These tests were performed on the lymph node isolate cultures from 7, 15 and 22 days post inoculation. Several individual

colonies from each sample were utilized. All of the isolates tested were positive in both tests. These standards indicated that st. RB51 had maintained its rough colony morphology up to 22 days post inoculation. An important biochemical marker for differentiating st. RB51 from other B. abortus strains is its resistance to the antibiotic rifampin. Strain 2308, the parent strain of st. RB51, is sensitive, as is st. 19 to as little as 50 ug/ml rifampin. The isolates were tested for this marker to determine if the organisms had undergone a mutation thereby losing the capability of surviving in the presence of the antibiotic. All of the isolates were resistant to rifampin at 400 ug/ml. These cultures as well as stock RB51 were not tested for resistance at any higher concentration of the antibiotic. Strain 2308 and st. 19 did not grow in 50 ug/ml, 100 ug/ml, 200 ug/ml or 400 ug/ml of rifampin. All cultures grew on TSBA plates without rifampin. The isolated organisms had not lost their original capability to grow in the presence of rifampin.

All lymph node isolates, as well as the growth from the rifampin plates were tested in the RIT. The primary antibodies used for the test were monoclonal antibodies Bru 38 and Bru 48. Bru 38 is a rat monoclonal antibody whose specificity is directed to antigenic determinants present on the O-side chain of smooth B. abortus LPS complex (39). Bru 48 is a mouse monoclonal antibody whose

specificity is believed to be the core portion of the LPS complex of st. RB51 (74). The monoclonal antibody was derived from mice infected with st. RB51.

Bru 38 did not react with any antigenic determinants present on st. RB51 in the RIT because this organism does not possess O-side chain. Bru 38 does react with smooth strains of B. abortus and with Y. enterocolitica serotype 0:9. Bru 48 usually does not react with smooth strains of B. abortus in the RIT because of the stearic hinderance created by the O-side chain on the core. The core determinant is not usually exposed on smooth organisms for the monoclonal to react with it in the RIT.

All of the tissue isolates did not react with Bru 38 monoclonal antibodies but did react with Bru 48 monoclonal antibodies (Figure VII). These tests were an additional indication that the isolated organisms had not reverted to a smooth form. Thus, rough st. RB51 was stable in cattle for at least 3 weeks post inoculation.

An isolate, obtained 15 days post inoculation, was chosen to reinfect mice. The goal of this experiment was to determine if the isolates had any increased capability to survive in mice which may indicate an increase in virulence. A primary inoculation of st. RB51 is cleared in mice in 3 to 4 weeks (Chapter I and reference 73). One group of mice were infected with the isolate and clearance was compared with that of a group who received stock RB51. The mice were killed 25 days post infection. Because no differences were observed among the 2 groups

at a time in which clearance of the stock RB51 was being completed (day 25), I concluded that the isolated st. RB51 had not become more virulent during its colonization in cattle.

Approximately 7 months after the start of Experiment I, a full term calf was born to cow Y141. From the onset of the experiment this cow was not known to be pregnant while being immunized with st. RB51. The opinion of a co-investigator who preformed the necropsy was that the calf was more than likely born alive and died shortly after birth. This opinion is based on the findings of the necropsy. The cause of death was not determined. Several tissue and fluid samples were obtained from the calf as well as a blood sample for serum. The calf was born to the cow, Y141, that tested negative in the lymph node biopsy at 15 dpi.

Strain RB51 was cultured from all 6 calf tissue samples. These samples were 1 from the prescapular lymph node, 2 from abomasal fluid, 2 from the lung, and 1 from cotyledon. All 6 isolates were tested with acriflavine and crystal violet, susceptibility to rifampin, and in the RIT Assay with monoclonal antibodies Bru 38 and Bru 48. Test results suggested that the isolated st. RB51 was still rough and presence of O-side chain was not detected, as demonstrated by the failure of Bru 38 to react with any of the isolates. All isolates had maintained their ability to grow in the presence 400 ug/ml rifampin. In this calf, st. RB51 survived and most probably replicated for a period of 7 months without changing.

SAT's were performed on the sera that was obtained through out the experiment. For Experiment I all samples were tested weekly for the first nine months of the experiment, monthly thereafter. For Bovine Experiment II and III, monthly samples were tested. A majority of the samples were negative in the SAT. The negative results were indicative of the absence of O-side chain antibodies. There are some samples that showed a very low titer, 1:25 or 1:50, to the smooth Brucella antigen. These low positive reactions were the result of IgM antibodies because reaction on Western Blot which detects only IgG, were negative. In Experiment I, of the samples tested none showed a higher titer than 1:25. Serum obtained from the calf was negative in the SAT.

Serum samples from Experiment I and four control serums were selected and randomly number coded for the State Lab. Assays performed on the samples were the BAPA, SAT, SPT, CFT and the card test. All samples were tested in the BAPA and SAT, but only some samples were tested in the remaining three tests due to an insufficient quantity of serum (SPT, CFT and Card test results not included in Results). All experimental cow samples were negative in the BAPA. This test is considered the most sensitive test for the detection of antibodies to the O-side chain, particularly those of the IgG type. Rabbit 3189, Steer 66 and Goat 48, hyperimmunized st. RB51 positive control animals, were negative in the BAPA. Steer 121 a hyperimmunized st. 19 positive control was positive.



Two experimental samples showed an incomplete reaction at 1:50 in the SAT. These were N0133 3/23/88 and Y141 7/18/88. These results are not in agreement with results obtained in our laboratory. It is my opinion that these incomplete agglutination results from the State Lab are, in fact, correct. But, as stated previously, may be the result of IgM titers. Also, one to two double dilution difference in reading is considered within the normal error of the SAT. The st. RB51 positive control animals were negative in the SAT. Steer 121, st. 19 positive control, was positive in the SAT with a titer of 1:200.

The results further substantiate the fact that st. RB51 appears not induce anti-O-side chain antibody titers. In the case of the two low titers observed, evidence showed that these titers were transient or IgM titers because samples at earlier and later bleedings in both cases were negative. Sample Y141 7/18/88 was also negative in the SPT, CFT and the Card test (results not shown). N0133, 3/32/88 was not tested in the three latter assays due to an insufficient quantity of serum.

Absorption studies of the bovine serum were performed with live st. RB51 and live Y. enterocolitica 0:9. Reactivity in Western Blot with RB51 W.C. antigen was lost after absorption with live st. RB51. No reactivity was lost after absorption with live Y. enterocolitica 0:9. The absorption studies further strengthened the observation that st. RB51 did not induce O-side chain antibodies and that B. abortus and Y. enterocolitica 0:9 did not share core determinants.

Western Blot analysis was performed on many serum samples using RB51 W.C. and Y. enterocolitica 0:9 as antigens (Again, for the sake of brevity, only a few of these Western's from Experiment I are presented in these figures). All cattle from Experiment I reacted to a variety of antigens after receiving st. RB51. Figures VIII, IX and X demonstrate these reactions from three of the cows over time. The 3/23/88 serum sample was obtained just before the second inoculation. My observations were that the cow's reactions do not differ much over the duration of the experiment. The low molecular weight antigen, approximately 14-18 Kd, was the "core" antigen described in the previous experiments (Chapter 2, Experiments II, III, IV). These serum samples reacted with st. RB51 purified LPS in the same region. All the cattle reacted strongly and somewhat more diffusely with this antigen early in the experiment. As samples were tested from later dates, the core reaction became more focused and distinct. The number of specific higher molecular weight antigens, (in the 43-85 Kd range) recognized by the cattle varied among the animals. The cows lose and gain reactivity with different high molecular weight antigens over time with no apparent tendency.

The Figures also include Western Blot analysis of the same samples against Y. enterocolitica 0:9 LPS. These samples as well as many other samples tested against this LPS, clearly showed no reactivity with this antigen. This further demonstrated the lack of O-side chain antibodies

in the serum samples. These results also indicated that st. RB51 survived in the cattle (a minimum of 22 days) remains stable (rough) because st. RB51 was unable to induce anti-O antibodies.

Positive control animals hyperimmunized with st. RB51 are depicted in Figure XI and showed reactivity with a variety of antigens present in the RB51 W.C. preparation as well as with st. RB51 LPS in the core region. They did not react with smooth 0:9 LPS. Steer 121, a st. 19 positive control which reacted with smooth 0:9 LPS, and very faintly with st. RB51 LPS (Figure XI).

All four cows demonstrated a similar rise in their antibody titers to RB51 W.C. as depicted in Table XIX following the first st. RB51 inoculation. Approximately one week following the second inoculation three cows showed a two-fold increase in titer. The fourth cow, N0133, demonstrated a similar increase slightly later. These high titers persisted for more than three months. Four months following the booster immunization, titers began to drop rapidly in all of the cows, and they maintain very low titers to RB51 W.C. thirteen months after the initial injection. Y141 which gave birth to the infected calf maintained a somewhat higher titer than the other cows and also maintained the highest titer when all the other cows showed a consistent decline in their antibody titer. After the birth of the calf, her titer began to decline.

Thus, kinetics of the antibody response of the cows to RB51 W.C. following the first and second inoculations of the organism was indicative of a classic primary and secondary antibody response. The long persistence of titers and the existence of an infected calf also suggested that st. RB51 was probably able to survive in cattle normally for more than 22 dpi.

#### Bovine Experiments II and III

The lymph node biopsies from Experiment II were discussed in the Experiment I discussion. In bovine Experiment II and III, selected monthly serum samples were tested in the SAT.. As in bovine Experiment I, a majority of samples were negative. The positive results may be due to nonspecific IgM titers. The antibody titers against RB51 W.C. for Experiment II and III are reported in Table XXIV and XXV, respectively. In Experiment II cows Y151 and Y148 demonstrated three- and four-fold increases in titer one month following inoculation. These reactions were similar to those in Experiment I. Both cows began declining four months post inoculation, as did the cows in Experiment I following the second inoculation. The other two cows, Y150 and Y154, never showed as high of a titer as the first two cows. By four months post inoculation, these cows' titers' were decreasing as well.

The conjunctivally infected cows (Experiment III) never demonstrated titers as high as the once or twice subcutaneously infected animals. Thus, boosting with the organism did increase the immune

response. They do show a similar rise and fall four months after inoculation, but the highest titer attained was 1:160 by Y149. The lesser response of these animals may have been the result of the route of infection even if the animals received a dose similar to the one in the previous two experiments. Also, the whole dose of organisms possibly did not enter the body because of the method of application, whereas with subcutaneous injection the animal received the whole dose. Also by inoculating conjunctivally, the animal may possibly be capable of clearing the organism more rapidly or this route permitted less dissemination of the bacteria, thus the animal developed a lower humoral response.

#### CONCLUSIONS

The results from bovine Experiments I, II and III led to the following conclusions.

1. Strain RB51 was able to survive in cattle for a minimum of 22 days.
2. During that time, st. RB51 remained stable (rough) and rifampin resistant.
3. Two subcutaneous injections of st. RB51 led to higher antibody titers than one subcutaneous or one conjunctival inoculation.

4. The organism was able to induce a humoral immune response to a variety of whole cell antigens distinct from the O-side chain.
5. No appreciable antibody response was induced that was directed toward the O-side chain. If a response was induced, it remained slight and transitory.

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