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Award Number: DAMD17-98-C-8025

TITLE: Expression of Brucella Antigens in Vaccinia Virus to Prevent Brucellosis in Humans: Protection Studies in Mice

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REPORT DATE: August 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution unlimited;
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DTIC QUALITY INSPECTED 4

20001017 032

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
August 2000

3. REPORT TYPE AND DATES COVERED
Annual(1 Jun 99 - 31 Jul 00)

4. TITLE AND SUBTITLE
Expression of Brucella Antigens in Vaccinia Virus to Prevent Brucellosis in Humans: Protection Studies in Mice

5. FUNDING NUMBERS
DAMD17-98-C-8025

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Previous and present work indicates that WRvaccinia/*Brucella* antigen recombinants are not conferring protective immunity . Based on our present studies and the finding that *Brucella* Cu/ZN SOD and L7/L12 proteins are protective antigens and that the presence of IL-12 is necessary at the moment of immunization, we conclude that one systemic vaccination with rvaccinia expressing 1 or 2 protective *Brucella* antigens with or without simultaneous production of IL-12, will not confer protective immunity. Systemic revaccination with the rvaccinia will most probably not change this situation because of immunity developed the vaccinia vector. Mucosal revaccination may overcome this problem and induce protection; such experiments are underway. Systemic vaccination with rvaccinia does induce an immune response to the *Brucella* antigens insuring priming of animals. Revaccination of the rvaccinia-primed animals with other protective vaccines may significantly increase the protective level of such vaccines; such experiments are underway. An alternative approach, immunization of animals with an attenuated *B. melitensis* vaccine overexpressing *Brucella* protective antigens, is being actively pursued since this approach significantly enhanced the effectiveness of other vaccines. We demonstrate that such a vaccine can be made and are ready to test its effectiveness against B. melitensis infection in mice.

14. SUBJECT TERMS
Brucellosis, Brucella, Antigen

15. NUMBER OF PAGES
27

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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6/29/00

PI - Signature Date

Table of Contents

Cover..

SF 298... 2

Foreword.. 3

Introduction.. 5

Body... 5

Key Research Accomplishments.. 18

Reportable Outcomes... 19

Conclusions... 19

References... 20

Appendices.. 22

5. INTRODUCTION.

The current work has 2 overall objectives. One of the objectives is genetically engineering mono- and polyvalent, IL-12 producing vaccinia/*Brucella* recombinant vaccines using synthetic E/L promoters and to test such vaccinia recombinants for their ability to ultimately induce a protective immune response against challenge with virulent *Brucella* in a mouse model. If such vaccines are able to protect mice against challenge with *Brucella* there is a good possibility that such vaccines could be used to immunize humans against infection with *Brucella spp.* It may be necessary to combine vaccination with vaccinia/*Brucella* recombinants with the inoculation of DNA vaccines. The second objective (amended SOW-99) is achieving overexpression of homologous antigens in strain *B. melitensis*ΔpurE/ΔwboA (WRPP1) which is a rough strain.

2. BODY.

<u>Approved objectives of the project:</u>

<u>a. Recombinant vaccinia (rvaccinia) constructs.</u>

a.1. Construct vaccinia virus/*Brucella* recombinants expressing one or more demonstrated protective *Brucella* antigens (SOD & L7/L12 proteins) and potentially protective antigens (31 kDa protein, 18 kDa lipoprotein and PAL a 16.5kDa peptidoglycan associated lipoprotein and/or additional antigens). Use vaccinia strains Western Reserve (WR - with and without expression of IL-12) and potentially modified vaccinia virus Ankara (MVA) strain.

a.2. Construct similar vaccinia virus/*Brucella* recombinants expressing combinations of *Brucella* antigens simultaneously.

<u>b. Mice immunization and challenge.</u> Immunize BALB/c mice with the vaccinia/*Brucella* recombinants alone or in combination with other vaccines. Analyze the humoral and CMI responses to *Brucella* antigens. Challenge vaccinated mice with virulent *Brucella spp.* and test the ability of the vaccinations to induce protection.

<u>c. Antigen overexpression in *Brucella* vaccine strain candidates.</u>
(Addition see amendment SOW-99) Overexpression of homologous antigens in strain *B. melitensis*ΔpurE/ΔwboA. A strain of *B. melitensis* containing deletions in *purE* and *wboA* genes will be used for the overexpression homologous, protective antigens.

a. Recombinant vaccinia (rvaccinia) constructs.
a.1. Rvaccinia expressing a single *Brucella* antigen.

i) *Brucella* outer membrane18 kDa lipoprotein. Work on the construction and use of this rvaccinia has been reported in previous reports and has now been completed and published (1). Rvaccinia expressing this protein was unable to induce protection although the vaccine was able to induce *Brucella* antigen-specific lymphocytes which upon in vitro stimulation with the 18kDa protein produced interferon-gamma (INF-g). Vaccine strain RB51 with deleted 18kDa protein did not show any loss in protective ability and overexpression of this antigen did not result in enhanced protection. We concluded that failure of the rvaccinia to protect against challenge with *Brucella* is mainly due to the characteristics of the antigen which is non-protective (1).

ii) Heat shock *Brucella* protein GroEL. Previously reported work on the expression of GroEL in vaccinia has now been accepted for publication (2). In summary, the rvaccinia expressing *Brucella* GroEL was able to induce an anti-GroEL immune response but was not protective. It is now apparent (although this was not the case at the initiation of this particular recombinant work) that *Brucella* GroEL is not a protective antigen. Overexpression of GroEL in RB51 does not lead to better protection (data not published yet). Deletion mutants can not be produced because the deletion is lethal. We concluded that failure of the rvaccinia to protect against challenge with *Brucella* is mainly due to the characteristics of the antigen which is non-protective

iii) *Brucella* Cu/Zn SOD. R vaccinia strain WR/pMC02-SOD (WR vaccinia strain expressing *Brucella* Cu/Zn SOD under a synthetic Early/late (E/L)promoter) was constructed (following details described in the previous report) and was administered to mice in combination with a DNA-*Brucella* SOD vaccine since previous work carried out in our laboratory indicated that rvaccinia expressing *Brucella* SOD did not protect against *Brucella* challenge. Work with other infectious agents indicated that combination of rvaccinia with a DNA vaccine has results in protective immunity even if each independent vaccine was not protective. The experiment illustrated in table 1 was performed.

The results of this experiment indicate that the combination of a rvaccinia expressing a protective *Brucella* antigen (SOD) with a DNA based SOD vaccine did not lead to protection. We have previously demonstrated that Cu/Zn SOD is a protective antigen (3). It is possible that protection failure by rvaccinia/*Brucella* SOD is due to low SOD expression but, we have reached maximum SOD expression by using synthetic E/L promoters.

Table 1. Mouse protection/challenge experiment with Vaccinia/*Brucella*-SOD recombinant in combination with DNA-SOD vaccine

Group #	# of mice	Vaccine and schedule	CFU/spleen*
1	5	WR/pMC02-SOD day0 & DNA Vaccine week 7	5.71 0.22
2	5	DNA Vaccine day0 & WR/pMC02-SOD week 7	5.55 0.28
3	5	RB51 (pos. control) week 7	3.65 0.37
4	5	Saline (neg. control) week 7	5.38 0.32

Challenge week 14 and termination week 16 (Brucella CFU's/spleen determination).
8. Average CFU followed by +/- SD

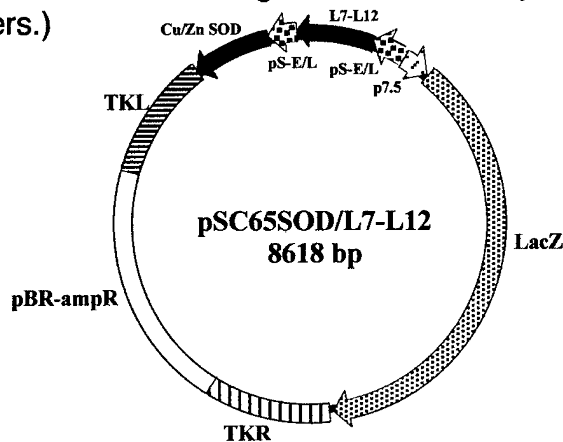
Purified SOD can protect mice from *Brucella* challenge particularly if administered concomitantly with IL-12 (4). Therefore, it is also possible that during rvaccinia replication, a certain levels of IL-12 is necessary to induce a protective immune response. Following this rationale, efforts have focused on the construction of vaccinia recombinants expressing IL-12 and costimulatory molecule B7.1 simultaneously with SOD and or L7-L12. *Brucella* L7-L12 has been reported to be a protective antigen by others.

v) Vaccinia expressing partial Listeriolysin containing protective epitopes (WRpLlo and WRIL-12/B7.1/pLlo). These constructs will be tested for their ability to protect against *Listeria* infection in mice to insure that vaccinia can be used as a vaccine vector against bacterial diseases caused by facultative intracellular parasites. They will also serve as controls for non-specific immunity. This work is in progress.

a.1 & a.2. Rvaccinia expressing one or two *Brucella* antigens.

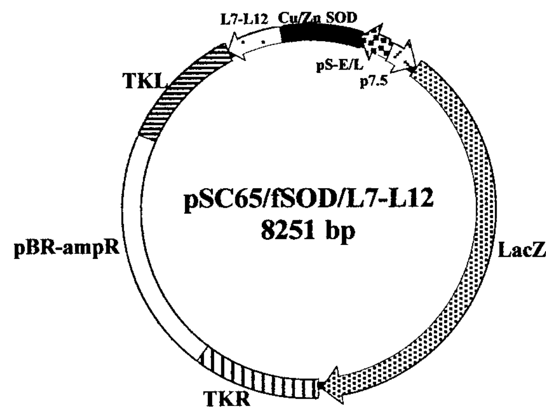
i) Description of the rvaccinia generation follows. The major problem encountered was generating pure recombinants in numbers sufficiently high to inoculate mice for *in vivo* protection and immune response experiments. This problem (unexplained at this moment) has introduced a moderate delay in initiating selected mice experiments. Sufficient amounts are now available for most constructs.

A. WRSOD/L7-L12 (Expression of SOD and L7-L12 genes are driven by two independent synthetic E/L promoters.)



B. WRIL-12/B7.1/SOD/L7- L12 (Expression of SOD and L7-L12 genes are driven by two independent synthetic E/L promoters)

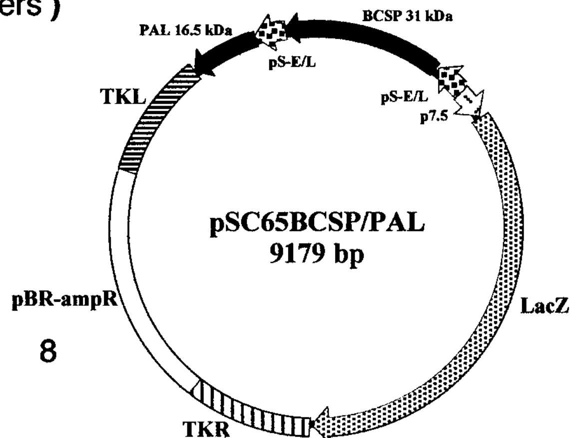
B. WRfusionSOD/L7-L12 (Expression of SOD L7-L12 fusion protein is driven by one synthetic E/L promoter).



C. WRIL-12/B7.1/fusionSOD/L7-L12. (Expression of SOD L7-L12 fusion protein is driven by one synthetic E/L promoter).

D. WRIL-12/B7.1/SOD (Expression of SOD is driven by a synthetic E/L promoter).

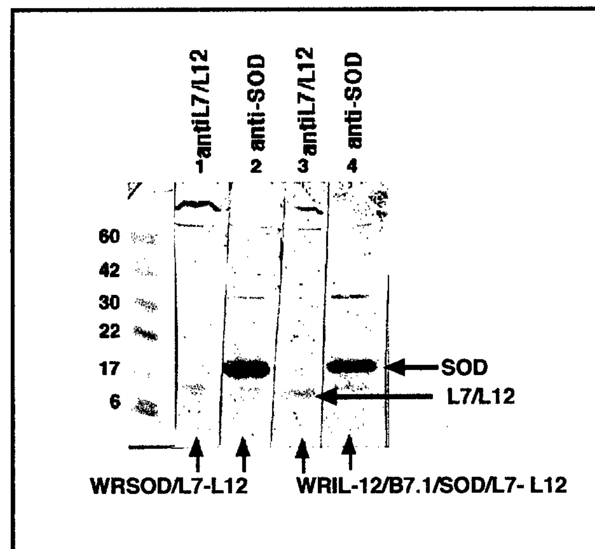
E. WR/31kDa/PAL (Expression of 31kDa and PAL genes are driven by two independent synthetic E/L promoters)



F. **WRIL-12/B7.1/31kDa/PAL**(Expression of 31kDa and PAL genes are driven by two independent synthetic E/L promoters)
G. **WRIL-12/B7.1/L7-L12** (Expression of L7-L12 is driven by one synthetic E/L promoter).
H. **WRL7-L12** (Expression of L7-L12 is driven by one synthetic E/L promoter)
I. **WRpLlo** (Expression of partial Listeriolysin is driven by one synthetic E/L promoter).
J. **WRIL-12/B7.1/pLlo** (Expression of partial Listeriolysin is driven by one synthetic E/L promoter).

Constructs I, J & K appear not to be stable and pure populations have not yet been obtained. Nevertheless, inoculation of mice with either of these stimulated the production of specific anti-L7-L12 and anti-pLlo antibodies respectively. Work is continuing to plaque - purify these strains; if this is not possible, constructs will be carried out using a natural E/L promoter instead of the synthetic promoter.

ii) Purified antigens and immunological assays. The above listed constructs were or are being used in mouse experiments. In order to study the immune response to the *Brucella* antigens, *Brucella* SOD and L7-L12 antigens had to be purified. Purified rSOD was produced (5) and purified L7-L12 was produced as a fusion protein (MBP-L7/L12). Residual E. coli LPS present in the recombinant, purified antigens, was removed by applying the antigen to an Affi-prep PolymixinB Matrix column (Bio-Rad). These antigens were used to assess INF-g production by splenocytes from immunized and control mice and to assess antibody levels by ELISA. Expression of SOD, L7/L12 or the SOD/L7-L12fusion protein in WR was assessed by western blot analysis (see figure 1). Anti SOD and L7-L12 antisera were prepared in mice using purified proteins as the immunizing antigens.
Figure 1. Expression of *Brucella* SOD and L7-L12 by rvaccinia.



iii) Recombinant MVA Strains.
The highly attenuated MVA strain will be used for the expression of *Brucella* antigens which demonstrate protective and/or CMI inducing abilities in mice. Work has been initiated to prepare MVA recombinants. Chicken fibroblast cell cultures necessary for the propagation of these recombinants have been started. Recombinant viruses are recognized using beta-glucoronidase expression which turns recombinant plaques greenish/blue. First we are trying to obtain MVA expressing the L7-L12 *Brucella* protein. We have succeeded in obtaining some primary recombinant plaques but have not been able to expand them. Work continuos along this line.

b. Mice immunization and challenge.

i) Immunization with WR/pMC02-SOD.
Immunization of mice with rvaccinia WR/pMC02-SOD is described under a.1. This experiment was initiated during the previous report period and a partial report was submitted last June; completion is reported under a.1.

ii) Challenge experiment of mice immunized with rvaccinia expressing *Brucella* fusion proteins with or without expression of IL-12 and B7.1.
Mice were vaccinated with these rvaccinia ip and challenged 6 weeks later with *B. abortus* strain 2308. This experiment did not involve immune response studies except, testing for antibodies to vaccinia to insure that vaccine was able to induce an immune response. All mice immunized with the rvaccinia responded to the vaccinia antigens.
Table 2. Protection experiment with rvaccinia WRfusionSOD/L7-L12 and WRIL-12/B7.1/fusionSOD/L7-L12.

Vaccine	Brucella CFU/spleen Average	SD
WRfusionSOD/L7-L12	5.98*	0.09
WRIL-12/B7.1/fusionSOD/L7-L12	5.94	0.23
WRIL-12/B7.1/SC65	5.80	0.21
Saline	5.76	0.05

*log10

The data indicated that a rvaccinia vaccine expressing two protective *Brucella* antigens as a single fusion protein with or without simultaneous expression of IL-12 is not protective if inoculated once into mice. Failure to induce protective immunity can not be explained at this time but, may be due to a poor CMI response. CMI was not measured in this experiment.

iii) Immunizations with rvaccinia combinations.
Magdalena et al (6) reported that IL-12 delivery coming from rvaccinia attenuates the vaccinia vector but also enhances the CMI response to the heterologous antigen (HIV-1 antigen expressed by the vaccinia vector)in a dose dependent manner. They also reported that 10^7 pfu of rvaccinia expressing a heterologous antigen but not IL-12 in combination with 10^4 rvaccinia expressing the heterologous antigen and IL-12 simultaneously, induced enhanced CMI responses to the heterologous antigen.

We carried out a preliminary experiment to test if such behavior would be observed with our recombinants. As summarized in table 3, mice were immunized with either 1x10^7pfu of WRfusionSOD/L7-L12, 1 x 10^7 of WRIL-12/B7.1/fusionSOD/L7-L12 or with a combination of 1x10^7pfu of WRfusionSOD/L7-L12 and 2 x 10^4 of WRIL-12/B7.1/fusionSOD/L7-L12 (doses as per recommendation of Magdalena et al for best enhancement of immune responses). Serum and splenocytes were obtained at 2 weeks post-immunization. Splenocytes were stimulated with purified SOD to measure INF-g production. Although INF-g production was obtained in all 3 groups upon stimulation with SOD, there where no significant differences among the 3 groups. Interestingly, SOD antibodies developed after immunization with WRfusionSOD/L7-L12 (expression of SOD as a fusion protein with L7-L12) but not with rvaccinia expressing native SOD (later experiments, see below).

The data suggest that the combination of rvaccinia expressing SOD as a fusion protein with L7-L12 co-administered with a low dose (10^4) of a similar rvaccinia also expressing IL-12, does not increase the CMI response to SOD. This prompted us to test if combinations with different doses of rvaccinia expressing IL-12 would lead to enhanced immune responses to SOD and/or L7-L12. This experiment was completed and is summarized in table 3. In this experiment, rvaccinia were expressing the SOD and L7-L12 as 2 distinct proteins and not as fusion proteins.

It can be concluded that WRSOD/L7-L12 induces lymphocytes which are able to specifically respond to the SOD antigen with the production of INF-g and that co-administration of WRIL-12/B7.1/SOD/L7- L12 in doses ranging of 10^2 ,10^4 and 10^5 did significantly enhanced INF-g production to SOD. No significant INF-g levels were observed when L7-L12 was used as the stimulatory antigen except when co-administration of WRIL-12/B7.1/SOD/L7- L12 increased to 10^5 pfu. On the other hand, rvaccinia immunizations did not induce antibodies to SOD while they did stimulate the production of antibodies to L7-L12. Experiments were carried out in duplicates.

Table 3. Immune response of mice to Brucella SOD and L7-L12 antigens after immunization with different combinations of rvaccinia strains.

Vaccination	INF-g (ng/ml)		Serology ELISA titers to(OD at 450nm)	
	SOD	L7-L12	SOD IgG	L7-L12 IgG
2x10^7 of A	2.74	0	0	0.28
2x10^7 of A + 10^2 of B	14.7	0	0	0.21
2x10^7 of A + 10^3 of B	0.0	0	0	1.24
2x10^7 of A + 10^4 of B	11.03	0	0	0.54
2x10^7 of A + 10^5 of B	18.4	10.52	0	0.03
WR	0	0	0	0

Based on the previous experiments which indicated that co-administration of 10^5 pfu of WRIL-12/B7.1/SOD/L7- L12 to a routine dose of 10^7 WRSOD/L7-L12 led to an increase of INF-g production upon lymphocyte stimulation with SOD and L7-L12, the experiment was repeated and a challenge step with Brucella was included to assess protection. Also, CpG (a genetic adjuvant leading to enhanced Th1 responses) was added to one of the groups. Results are summarized in table 4.

In this experiment, co-administration of the rvaccinia did not significantly increase the amount of INF-g produced upon in vitro stimulation with the Brucella antigens as observed in the previous experiment. Protection was not observed even after addition of CpG to the rvaccinia mixture. In general, the INF-g levels reached with lymphocytes obtained from the rvaccinia immunized mice and stimulated in vitro with SOD were 5 times lower than the levels obtained with RB51 vaccination. From these and previous experiments it is becoming clear that rvaccinia expressing one or two protective Brucella antigens as individual antigens or as fusion proteins are not able to protect mice against a challenge with virulent Brucella. This is also true if the antigens are expressed in vaccinia co-expressing IL-12 and B9.1 and if the rvaccinia are co-administered in different proportions with or without DNA vaccines or genetic adjuvants (CpG).

Table 4. Immune response of mice to Brucella SOD and L7-L12 antigens after immunization with different combinations of rvaccinia strains and their protection against challenge with B. abortus.

Vaccination	INF-g (ng/ml)		Serology ELISA IgG titers (OD at 450nm) to		CFU Brucella per spleen	
	SOD	L7-L12	SOD	L7-L12	Aver	SD
2x10^7 of A	3.99	0	0	0.54	5.61*	0.17
2x10^7 of A + 10^2 of B	6.9	0	0	0.33	5.37	0.28
2x10^7 of A + 10^5 of B	6.7	0	0	0.26	5.48	0.26
2x10^7 of B	5.4	0	0	0.55	5.33	0.24
2x10^7 of A + 10^2 of B + CpG	0	0	0	0.33	5.36	0.26
RB51SOD*	34.9	2.8	0.89	0.95	3.33	0.34
WR 2 x 10^7	0	0	0	0	5.31	0.12

* log10

The data indicate that rvaccinia expressing Brucella antigens are able to induce Brucella antigen-specific lymphocytes able to produce INF-g and antibodies. This indicates that the rvaccinia are able to prime mice to Brucella antigens but, this priming is not enough to induce protection. The fact that priming occurs, may be used to boost or significantly enhance the protective effect of other candidate Brucella vaccines. Based on this assumption we have initiated an experiment in which vaccination with rvaccinia is combined with an effective, attenuated Brucella vaccine (strain RB51). Mice were vaccinated once at the initiation of the experiment, revaccinated 3 weeks later and will be challenged with virulent Brucella 7 weeks later. Spleen CFU will be obtained 2 weeks post-challenge. Both, the antibody response and the CMI response (as reflected by in vitro, antigen specific INF-g production) will be followed. The experiment will be completed within the second week of September 2000. Table 5 depicts the experimental design.

Table 5. Ongoing protection experiment in mice using combinations of rvaccinia and RB51

Vaccination-wk 0	Vaccination-wk3	CMI studies-wk5	challenge-wk10	End-wk12
A 1x10^7 + B 1x10^2	RB51	yes	yes	CFUspleen
A 1x10^7 + B 1x10^5	RB51	yes	yes	CFUspleen
Saline	RB51	yes	yes	CFUspleen
RB51	A 1x10^7 + B 1x10^2	yes	yes	CFUspleen
RB51	A 1x10^7 + B 1x10^2	yes	yes	CFUspleen
RB51	none	yes	yes	CFUspleen
None	none	yes	yes	CFUspleen

iii) Mice "colonic" Immunization experiment.

Systemic vaccinia immunity induced by one immunization interferes with subsequent systemic immunizations eliminating the possibility of effective booster vaccinations (7). Such interference can be bypassed by re-immunization via the mucosal immune system (7). After sc. immunization with vaccinia virus the inductive sites of the mucosal immune system may still be naive to vaccinia antigens and thus, mucosal routes of immunization can be used for the induction of recombinant protein-specific response in animals with preexisting immunity (7) Based on (7), we have initiated a mouse immunization experiment using the colonic route as a booster route for a second administration of rvaccinia.. This study should answer the question if an effective booster vaccination with rvaccinia will be sufficient to induce protective immunity. Mice in the ongoing experiment have been boosted and will be challenged within the next 3 weeks to assess protection. Both, the humoral and CMI response is being studied. Booster immunizations were performed under full anesthesia.

iv) DNA vaccine immunizations and rationale.

Initially, based on the published literature, we selected 10 *Brucella* proteins able to induce immune responses and could therefore serve as protective antigens. If protective, they can be used in rvaccinia work. The DNA vaccines were tested in mice for their protective abilities. It was found that out of the 10 vaccines tested

three appeared to provide some marginal protection against challenge. They were *B. abortus* BaBCSP31, BaPAL16.5 and antigen BmCP24, a ribosome releasing factor from *B. melitensis.* The BCSP31 is a surface protein and PAL16.5 is a peptidoglycan-associated lipoprotein. During this period, we re-tested the promising BaBCSP31 and BaPAL16.5 vaccines again and also asked if they would enhance the protective abilities of RB51. This approach gives more sensitivity to assess the role of these antigens in protective immunity. If they enhance protection, they are able to sensitize the immune system of the animal in the right direction and must be playing some role in protection.
The DNA vaccines were generated by amplifying the required genes using specific primers designed, so that they could be easily cloned into expression system of the pcDNA 3 vector as described in the previous report.

Six week-old BALB/c female mice were vaccinated with the DNA vaccines intra-muscularly at two sites (50 ug/site) using 100 ug of highly purified (using Quagen plasmid purification kit) pCDNA-Brucella recombinants. Each of the DNA vaccine was given to two groups of seven mice each. All the mice were anaesthetized. A L7/L12 DNA vaccine was included as a positive control for the protection studies. DNA vaccine was given either alone three times I/M or two doses followed by a booster of RB51. All of the vaccinated mice were challenged with 1 .8 x 10^4 cfu of *B. abortus* 2308 ip at week 20 (10 weeks post last immunization). Two weeks after challenge, the experiment was terminated and spleen CFU obtained. Average cfu for each group was calculated and a student "t" test was used to determine significance. In addition, two mice were killed two weeks before the challenge to perform Lymphocyte proliferation assay. The results from this effort are being finalized and will be reported in our next progress report. Experimental design and results are depicted in table 6.

Western blot analysis demonstrated no antibody production as expected from i.m. DNA-vaccines. Samples from mice vaccinated with two-DNA-vaccine and boosted with a dose of RB51 developed antibodies against several Brucella antigens. Studies are under way to determine if these antigens (DNA vaccine candidates) had any effect on the cell mediated immunity in LPA.

From table 6 it can be concluded that DNA vaccines of BCSP31, PAL16.5 and L7/L12 did not provide significant protection when given either alone or in combination with other DNA vaccines. Brucella antigen PAL16.5 did not provide significant protection when given in combination with RB51 as a booster but, BCSP31 and L7/L12 significantly enhanced the protection provided by RB51 alone. Enhancement was not observed when the RB51 was the primary and the DNA-vaccines were used as the booster injections.

Table 6: *B. abortus* DNA vaccine trial in BALB/c mice.

Group Number	Vaccination at 0 Week	Vaccination at 3 week	Vaccination at 10 week	*B. abortus* Challenge at 20 weeks	Protection (Difference in Log CFU*) at 22 weeks
1a	saline	saline	saline	1×10^4	NA
2a	cDNA	cDNA	cDNA	1×10^4	0.5
3a	PAL	PAL	PAL	1×10^4	0.1
4a	BCSP	BCSP	BCSP	1×10^4	0.11
5a	PAL+BCSP	PAL+BCSP	PAL+BCSP	1×10^4	0.06
1b	saline	saline	RB51	1×10^4	1.19
2b	cDNA	cDNA	RB51	1×10^4	1.47
3b	PAL	PAL	RB51	1×10^4	1.47
4b	BCSP	BCSP	RB51	1×10^4	<u>2.44</u>
5	RB51	PAL+BCSP	PAL+BCSP	1×10^4	0.97
6	PAL+BCSP	PAL+BCSP	RB51	1×10^4	<u>1.92</u>
7	none	none	RB51	1×10^4	1.26
8	L7/L12	L7/L12	L7/L12	1×10^4	0.07
9	L7/L12	L7/L12	RB51	1×10^4	<u>1.84</u>

All of the DNA vaccines were administered I/M 100ug/mouse at two sites

* CFU $\log_{10}$ of non-immunized – CFU$\log_{10}$ of immunized group

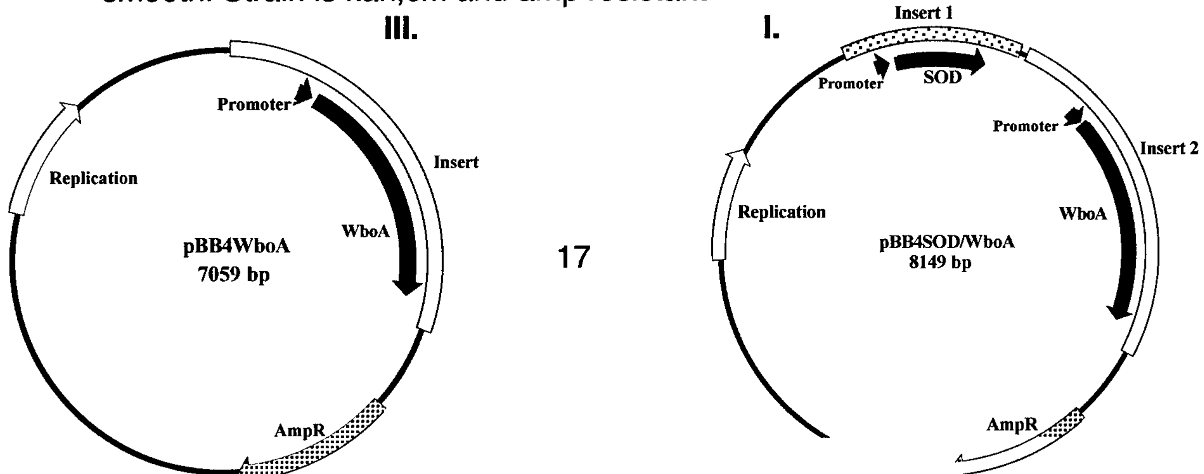
<u>c. Antigen overexpression in *Brucella* vaccine strain candidates.</u>

The use of attenuated *Brucella* strains as a possible human vaccine is being investigated by various groups. A *B. melitensis* mutant with a deletion in the purE gene (*B. melitensis*ΔpurE) has proven to be a smooth, attenuated strain able to afford protection against systemic and respiratory challenge with *B. melitensis* in the mouse and monkey models. Strain *B. melitensis*ΔpurE has been made rough by introducing a deletion in the wboA gene generating strain WRRP1 (strain *B. melitensis*ΔpurE/ΔwboA). This strain is extremely attenuated to the point that it is also unable to induce protective immunity. On the other hand, strain *B. melitensis*

ΔwboA is attenuated and rough and is able to induce immunity against a systemic challenge but not a respiratory challenge. Our group has demonstrated that, overexpression of a protective *Brucella* protein (i.e. Cu/Zn SOD) in a *Brucella* vaccine strain (RB51 – a rough organisms with an insertion sequence-IS711- interrupting the wboA gene) significantly enhances the protective ability of the vaccine (5). Also, it was demonstrated that complementation of vaccine strain RB51 with wboA increases its protective ability (8). Based on this information we are creating a WRPP1 strain overexpressing SOD and complemented for wboA to acquire O-chain expression(WRRP1pBBSOD/wboA). This strain should have the attenuation characteristics of *B. melitensis*ΔpurE but should have enhanced protective abilities. Induction of specific immune responses and protection against challenge with *B. abortus* and *B. melitensis* will be tested in the mouse model.

Since WRRP1 contains kanamycin (kan) and chloramphenico (cm) resistance genes (used for deleting the purE and wboA genes respectively), we have selected plasmid pBBR4MCS for use in the overexpression of SOD and complementation of wboA gene (pBBR4MCS is a broad host range plasmid containing ampicillin (amp) resistance marker gene). The wboA and sodC genes along with their own promoters were cloned either separately or together into pBBR4MCS to generate pBB4WboA, pBB4SOD and pBB4SOD/WboA plasmids (the schematic diagrams are shown). The recombinant plasmids were electroporated into WRRP1 strain and the plasmid-containing colonies were selected on a TSA plate containing ampicillin. The following recombinant strains were generated and are being characterized to start mouse protection and clearance experiments.

I. WRRP1pBB4SOD/wboA (*B. melitensis*ΔpurE overexpressing SOD and expressing O-chain). This strain is smooth as determinated by acriflavin agglutination tests, crystal violet staining of colonies and appearance of O-chain in western blot analysis. Strain is kan, cm and amp resistant.
II. WRRP1pBB4SOD (*B. melitensis*ΔpurE overexpressing SOD). This strain is Rough. Strain is kan,cm and amp resistant.
III. WRRP1pBB4wboA (*B. melitensis*ΔpurE expressing O-chain). This strain is smooth. Strain is kan,cm and amp resistant



II.

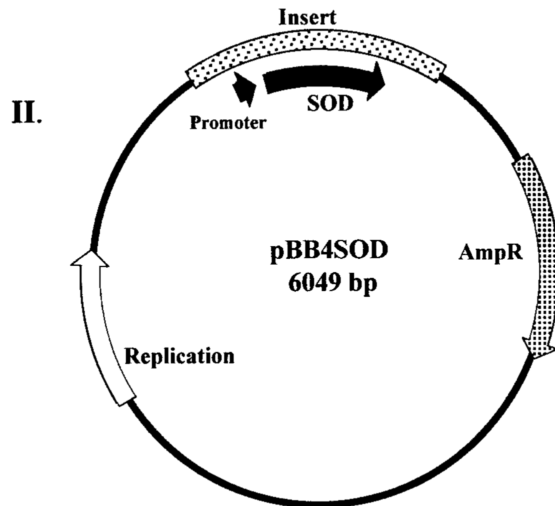
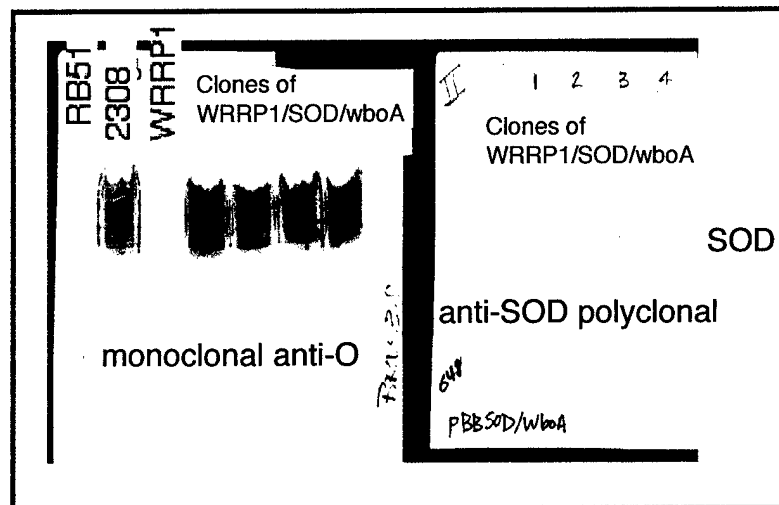


Figure 3 (below) illustrates the expression of O-chain and SOD in 4 WRRP1pBB4SOD/wboA. The western blot on the left clearly shows no O-chain in RB51 and WRRP1 as expected but expression in the 4 complemented clones. The western blot on the right suggests overexpression of SOD particularly in clones 3 and 4 of WRRP1pBB4SOD/wboA



7. KEY RESEARCH ACCOMPLISHMENTS DURING THIS PROJECT.

- Tested various vaccinia/*Brucella* antigen recombinants for their ability to induce immune responses and protect mice against *Brucella* challenge; demonstrated that rvaccinia expressing *Brucella* 18kDa protein, GroEL and SOD could not protect if given once to mice with or without DNA vaccines.
- Finished construction of all WR-rvaccinia planed for this study including vaccinia expressing two protective *Brucella* antigens simultaneously with or without simultaneous expression of IL-12 and B7.1. Several have been tested

in mice for immune response induction and protection, others are in progress. Although not all studies are completed, immunization with these constructs has not lead to protective immunity.

- Initiated production of MVA vaccinia/ *Brucella* antigen recombinants.
- Retested DNA vaccines to discover additional protective *Brucella* antigens. At least one additional antigen was found to have protective abilities and has been cloned into vaccinia for protection testing.
- Initiated experiments to test if mucosal exposure to rvaccinia can be used as a booster route. It is very likely that in order to induce protective immunity with rvaccinia against *Brucella* infection an effective booster is necessary.
- Initiated experiments to test if the developed rvaccinia are able to significantly increase protection of existing attenuated *Brucella* vaccines.
- Construction of a potential *Brucella* vaccine for humans (overexpressing a *Brucella* protective antigen) based on a *B. melitensis* vaccine candidates developed by has been completed and will be tested in mice.

8. REPORTABLE OUTCOMES.

Publications and presentations.

Vemulapalli R, Cravero S, Calvert CL, Toth TE, Sriranganathan N, Boyle SM, Rossetti OL, Schurig GG. Characterization of specific immune responses of mice inoculated with recombinant vaccinia virus expressing an 18-kilodalton outer membrane protein of Brucella abortus. Clin Diagn Lab Immunol 7:114-118. 2000.

S. Baloglu, T. E. Toth, G. G. Schurig, N. Sriranganathan, S. M. Boyle. Humoral immune response of Balb/C mice to a vaccinia virus recombinant expressing *Brucella abortus* GroEl does not correlate with protection against a *B. abortus* challenge. S. Baloglu, T. E. Toth, G. G. Schurig, N. Sriranganathan, S. M. Boyle. Veterinary Microbiology In Press 2000

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9. CONCLUSIONS.

Previous and present work indicates that WRvaccinia/*Brucella* antigen recombinants are not conferring protective immunity . This is not necessarily due to promoter selection as demonstrated previously. Based on the finding that *Brucella* Cu/ZN SOD and L7/L12 protein are protective antigens and that

presence of IL-12 is necessary at the moment of antigen presentation, we are fairly confident that one systemic vaccination with rvaccinia expressing 1 or 2 protective *Brucella* antigens with or without simultaneous production of IL-12 will not confer protective immunity. Systemic revaccination with the rvaccinia will most probably not change the situation since animals will already be immune to the vaccinia vector. Mucosal revaccination may overcome this problem and may result in protection; such experiments are underway. Systemic vaccination with rvaccinia does induce an immune response to the *Brucella* antigens in mice insuring that the animals are primed. Revaccination of the primed animals with non-rvaccinia protective vaccines may significantly increase the protective level of such vaccines; such experiments are underway. An alternative approach, immunization of animals with an attenuated *B. melitensis* vaccine overexpressing *Brucella* protective antigens and complemented with wboA, is being pursued due to promising results reported for a similar B.abortus (RB51)vaccine. It is feasible to construct such a vaccine as demonstrated in this project.

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10. APPENDIX

CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 2000, p. 114–118 Vol. 7, No. 1
1071-412X/00/$04.00+0
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Characterization of Specific Immune Responses of Mice Inoculated with Recombinant Vaccinia Virus Expressing an 18-Kilodalton Outer Membrane Protein of *Brucella abortus*

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Received 16 August 1999/Returned for modification 27 September 1999/Accepted 4 November 1999

Using the shuttle vector pMCO2 and the vaccinia virus wild-type WR strain, we constructed a recombinant virus expressing an 18-kDa outer membrane protein of *Brucella abortus*. BALB/c mice inoculated with this virus produced 18-kDa protein-specific antibodies, mostly of immunoglobulin G2a isotype, and in vitro stimulation of splenocytes from these mice with purified maltose binding protein–18-kDa protein fusion resulted in lymphocyte proliferation and gamma interferon production. However, these mice were not protected against a challenge with the virulent strain *B. abortus* 2308. Disruption of the 18-kDa protein's gene in vaccine strain *B. abortus* RB51 did not affect either the strain's protective capabilities or its in vivo attenuation characteristics. These observations suggest that the 18-kDa protein plays no role in protective immunity.

Brucellosis is a zoonotic disease caused by the members of genus *Brucella*, which are gram-negative, facultatively intracellular bacteria. In domestic and wild mammals, brucellosis often results in abortions and infertility. Humans usually acquire the infection by consuming contaminated dairy products or by coming in contact with an infected animal's tissues and secretions (1). In humans the disease manifests itself as a chronic infection with undulant fever and general malaise. It is generally accepted that cell-mediated immunity (CMI) is necessary for effective protection against brucellosis, although antibodies, especially against the O side chain of the lipopolysaccharide, appear to enhance resistance against infection, at least in certain host species (2, 3). Attenuated, live *Brucella* strains such as *Brucella abortus* RB51 and 19 and *Brucella melitensis* Rev 1 are being used as vaccines to control brucellosis in domestic animals. However, these vaccines are not suitable for humans; strains 19 and Rev 1 can cause disease in humans and there is no study ascertaining the safety of strain RB51, although human infections with this vaccine strain have not been reported. An efficient and safe vaccine is needed for the prophylaxis of human brucellosis, which is a major zoonotic health risk in many countries (8, 27) and a potential tool for biological warfare (5, 12).

Immune mechanisms operative in protection against brucellosis can be discerned by using a mouse model of vaccination and challenge infection. Development of resistance to brucellosis has been associated with the induction of a Th1-type immune response (21). However, with the exception of L7/L12 ribosomal protein and Cu/Zn superoxide dismutase, which have been shown to induce certain levels of protection (18, 19, 23), the specific proteins involved in the stimulation of a protective response have not been identified. A potential candidate for protective antigens is an 18-kDa (referred to as 19 kDa by some researchers) lipoprotein present on the surface of *Brucella* (14, 25). Infected mice, sheep, goats, and dogs develop antibodies to this antigen, indicating the immunological recognition of the 18-kDa protein in brucellosis of several animal species (14, 25). Humans infected with either *B. abortus* or *B. melitensis* also develop antibodies to this antigen (14). Preliminary studies carried out in our laboratory detected CMI responses to this protein in strain RB51-vaccinated mice. In order to develop an efficient recombinant vaccine for humans, we are studying the possibility of using vaccinia virus as a delivery vector for *Brucella* proteins of protective potential. In the present work, we constructed a recombinant vaccinia virus that can express the 18-kDa protein and characterized the specific humoral and selected CMI responses of mice vaccinated with this virus. Our studies indicated that mice vaccinated with the recombinant virus developed Th1-type immune responses to the 18-kDa protein. However, these immune responses did not lead to any level of protection against challenge infection with virulent *B. abortus* 2308. Further, using vaccine strain *B. abortus* RB51 with an interrupted gene for the 18-kDa protein, we showed that the 18-kDa protein does not appear to have any protective role in brucellosis.

Expression of *B. abortus* 18-kDa protein by recombinant vaccinia virus. The gene for the 18-kDa protein was initially obtained from a genomic library of *B. abortus* 19 (unpublished data) and was subsequently sequenced (GenBank accession no. L42959). Further, the complete open reading frame of this gene (from nucleotides 282 to 833 of sequence L42959) was previously PCR amplified and cloned into plasmid pBK-CMV (Stratagene, La Jolla, Calif.). From this plasmid, the 18-kDa antigen gene was excised with a *Kpn*I digestion and subcloned into a vaccinia shuttle vector, pMCO2 (4). The pMCO2 plasmids containing the gene for the 18-kDa protein in the proper as well as in the reverse orientation with regard to the pro-

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moter were used in making the recombinant vaccinia viruses. The vaccinia virus recombinants were produced according to previously described methods (4, 6, 26). The recombinant vaccinia viruses containing the gene for the 18-kDa protein in proper and reverse orientation were designated v18-1 virus and v18-2 virus, respectively. The 18-kDa protein expression in the v18-1 virus- but not the v18-2 virus-infected cell culture lysates was confirmed by Western blot analysis with sera from *B. abortus* RB51-vaccinated mice and rabbit antiserum to the 18-kDa protein. The rabbit antiserum was prepared according to procedures described elsewhere (13). However, this protein was slightly higher in molecular weight than the native 18-kDa protein from *B. abortus* (Fig. 1A). This is probably because of the intact prokaryotic signal sequence of the 18-kDa protein and/or glycosylation of the expressed protein; a region containing weak homology with the consensus eukaryotic N-glycosylation site was identified in the deduced amino acid sequence of the 18-kDa protein by computer analysis.

Immune responses of mice inoculated with the recombinant vaccinia virus. Two separate mouse experiments were performed. In each experiment, four groups of eight BALB/c female mice (Charles River Laboratories, Wilmington, Mass.) of 6 to 8 weeks of age were used. The mice of two groups were each injected with a 10^7-CFU median tissue culture infective dose of either v18-1 or v18-2 virus. In the first experiment an intraperitoneal route was used for inoculating animals with the vaccinia viruses, whereas in the second experiment an intradermal route was used. As a positive control, one group was injected with 10^8 CFU of *B. abortus* RB51, and as a negative control, another group was injected with saline alone. All mice were bled at 6 weeks postinoculation (p.i.) to obtain sera for enzyme-linked immunosorbent assay (ELISA) and Western blot analyses. Three mice from each group of experiment 1 were sacrificed at 12 to 14 weeks p.i., and their splenocytes were used for in vitro CMI assays. At 7 weeks p.i., five mice from each group were challenge-infected with 2×10^4 CFU of *B. abortus* 2308 intraperitoneally. Two weeks after the challenge infection, the mice were killed, bacteria from their spleens were recovered, and CFU counts were determined. In conducting research using animals, the investigators adhered to guidelines set forth by a committee of the Institute of Laboratory Animal Resources, National Research Council (7a).

For use in ELISA and CMI assay, the 18-kDa protein of *Brucella* was expressed in *Escherichia coli* DH5α (Gibco BRL, Bethesda, Md.) as a fusion with maltose binding protein (MBP) by using expression vector pMalC$_2$ (New England Biolabs Inc., Beverly, Mass.). The fusion protein (MBP–18-kDa protein) was purified by affinity chromatography on amylose resin. To achieve this, the gene for the 18-kDa protein was amplified by PCR from the genomic DNA of *B. abortus* 2308. A primer pair consisting of one forward primer (5′ GGA TCC CAG AGC TCC CGG CTT GGT 3′) and one reverse primer (5′ AAG CTT CCC TCT TCA TCG TTT CCG 3′) was designed based on the nucleotide sequence of the 18-kDa protein gene sequence. The forward primer of the gene was selected such that the coding sequences for the signal peptide part of the antigen were not included in the amplification. PCR and cloning of the amplified product in pMalC$_2$ were performed as previously described (28). The protein expression and purification were performed according to the manufacturer's suggested procedure. Expression of the MBP–18-kDa protein fusion was confirmed by a Western blot analysis with the 18-kDa protein-specific rabbit antiserum. Similarly purified recombinant MBP was used as a control in the lymphocyte proliferation assays.

The presence of serum immunoglobulin G (IgG), IgG1, and IgG2a isotypes with specificity to the 18-kDa protein was determined by indirect ELISA following the standard procedures (7). Purified MBP–18-kDa protein in carbonate buffer, pH 9.6, was used to coat polystyrene plates (0.5 μg/well, Nunc-Immuno plates with MaxiSorp surfaces). Isotype-specific goat anti-mouse horseradish peroxidase conjugates (Caltag Laboratories, San Francisco, Calif.) and TMB Microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) were used in the assay. The enzyme reaction was stopped by adding 100 μl of stop solution (0.185 M sulfuric acid), and the absorbance at 450 nm was recorded with a microplate reader (Molecular Devices, Sunnyvale, Calif.).

Lymphocyte proliferation and gamma interferon (IFN-γ) quantification assays were performed as previously described (28). Splenocytes were cultured in the presence of 5 μg of recombinant MBP–18-kDa protein, 5 μg of MBP, 10 μg of *B. abortus* RB51 crude extract (28), 0.5 μg of concanavalin A, or no additives (unstimulated control). After culturing for 5 days, supernatants of the cultures were collected for quantitating IFN-γ by a sandwich ELISA, the cells were pulsed with 1 μCi of [^{3}H]thymidine/well for 18 h and harvested onto glass fiber filters, and the radioactivity was measured in a liquid scintillation counter. The assays were performed in triplicate. Results of the proliferation assay were expressed as a stimulation index (mean counts per minute from wells of test antigen/mean counts per minute of unstimulated control wells).

Mice that were inoculated with either the v18-1 virus or *B. abortus* RB51 developed antibody and CMI responses to the 18-kDa protein. Western blots shown in Fig. 1 demonstrate that mice inoculated with v18-1 or v18-2 virus developed IgG antibodies to the vaccinia viral proteins but only the v18-1 virus- or *B. abortus* RB51-inoculated mice developed antibodies to the 18-kDa protein of *B. abortus*. As shown in Table 1, IgG2a was the predominant isotype of antibody to the 18-kDa protein in mice inoculated with either the v18-1 virus or *B. abortus* RB51. However, the 18-kDa protein-specific IgG and IgG2a levels were highest in mice vaccinated with *B. abortus* RB51. In contrast to the mice inoculated with v18-1 virus, the mice inoculated with strain RB51 had essentially no specific IgG1 isotype antibodies to the 18-kDa protein.

Splenocytes from mice inoculated with strain RB51 or v18-1 virus proliferated (Fig. 2) and produced IFN-γ (Table 2) upon stimulation with either MBP–18-kDa protein or strain RB51 antigen extract. Splenocytes from the control mice (v18-2 virus or saline injected) did not respond to stimulation with either strain RB51 antigen extract or MBP–18-kDa protein. Splenocytes from mice inoculated with v18-1 virus or strain RB51 responded with similar levels of IFN-γ production upon stimulation with MBP–18-kDa protein. However, when stimulated with strain RB51 antigen extract, splenocytes from strain RB51-inoculated mice produced much higher levels of IFN-γ than those from the v18-1 virus-inoculated mice. A similar tendency was also observed in the lymphocyte proliferation assay.

No difference in challenge strain *B. abortus* 2308 counts between spleens from mice inoculated with saline, v18-1 virus, or v18-2 virus was observed (data not shown). This indicates that the detected humoral and cell-mediated immune responses against the 18-kDa protein were unable to protect mice against the challenge infection. As expected, strain RB51-vaccinated mice were protected significantly against challenge infection (20).

Effect of disruption of the gene for the 18-kDa protein on protective capabilities of strain RB51. Since the mice inoculated with v18-1 virus were not protected against the challenge infection, we verified the lack of a potential protective role for

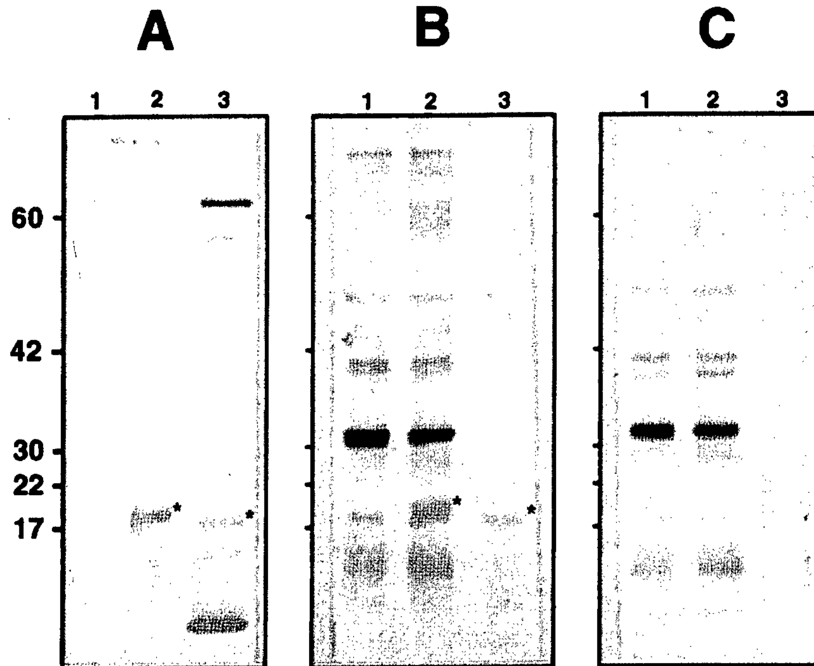

FIG. 1. Western blot analysis of sera (6 weeks p.i.) from mice vaccinated with *B. abortus* RB51 (A), v18-1 virus (B), or v18-2 virus (C). Lanes 1 and 2, antigens from lysates of HuTK$^-$ cells infected with v18-2 and v18-1 viruses, respectively. Lanes 3, antigens of *B. abortus* RB51. Numbers at left of panel A are approximate protein molecular masses, in kilodaltons. Asterisks in panels A and B indicate the 18-kDa antigen band.

the 18-kDa protein in strain RB51. We disrupted the gene encoding the 18-kDa protein by inserting a kanamycin cassette. This was achieved by cloning a 994-bp DNA fragment containing the gene for the 18-kDa protein (GenBank accession no. L42959) in *Eco*RI and *Nco*I sites of pRSETC (Invitrogen) to generate pRS18. A kanamycin cassette obtained from pUC4K (Pharmacia Biotech, Piscataway, N.J.) was inserted into the *Sac*I site present within the open reading frame (72 bp from the start codon) of the 18-kDa protein gene to create pRS18Δkan. The pRS18Δkan plasmid was electroporated into *B. abortus* RB51 (16) to replace the native gene by homologous recombination. The kanamycin-resistant colonies were screened for the presence of the kanamycin cassette and the disruption of the gene for the 18-kDa protein by Southern blot hybridization according to methods previously described (reference 15 and data not shown). The disruption mutant was designated RB51Δ18kDa. To determine the infectivity and protectivity characteristics of strain RB51Δ18kDa and to compare them with those of strain RB51, experiments with mice were performed as previously described (17, 20). Strain RB51Δ18kDa did not express the 18-kDa protein, as determined by the Western blot analysis (data not shown). Our studies also revealed that the ability of strain RB51Δ18kDa to survive within mice was similar to that of strain RB51 (data not shown), indicating that the disruption had no influence on the in vivo survival of this vaccine strain. Mice inoculated with strain RB51Δ18kDa produced neither antibody nor CMI responses to the 18-kDa protein, yet they were protected to the same extent as the strain RB51-inoculated mice (data not shown).

TABLE 1. ELISA detection of serum IgG, IgG2a, and IgG1 antibodies specific to the 18-kDa protein in mice at 6 weeks after inoculation with either of two recombinant vaccinia viruses or with *B. abortus* RB51

Vaccination	18-kDa antigen-specific immunoglobulin level[a]		
	Total IgG	IgG2a	IgG1
v18-1 virus	1.158 ± 0.444	0.743 ± 0.245	0.167 ± 0.015
v18-2 virus	0.076 ± 0.063	0.008 ± 0.013	0.014 ± 0.004
Strain RB51	1.909 ± 0.256	1.354 ± 0.211	0.003 ± 0.002
Saline	0.002 ± 0.001	0	0

[a] Values are mean absorbance at 450 nm ± standard deviations for three mouse serum samples.

Inoculation of mice with live v18-1 virus resulted in 18-kDa protein-specific antibodies predominantly of subisotype IgG2a, suggesting the induction of a Th1 type of immune response (22). Furthermore, the proliferation and production of IFN-γ by splenocytes of these mice upon in vitro stimulation with the specific antigen demonstrated a Th1 response to the 18-kDa protein. However, these immune responses did not result in protection against a challenge infection with virulent *B. abortus* 2308. We do not expect the 18-kDa protein-specific antibodies to have a protective role, since passive transfer of strain RB51-induced antibodies, which include antibodies to the 18-kDa protein, did not confer protection to challenge with *B. abortus* 2308 (11). The lack of protection could be due to inappropriate posttranslational processing of the vaccinia virus-expressed 18-kDa protein resulting in an altered antigenicity or loss of crucial CMI-inducing protective epitopes. As previously mentioned, the 18-kDa protein in *Brucella* appears to be a lipoprotein (25); this lipid modification would not have occurred in the vaccinia virus-expressed 18-kDa protein. Also, any glycosylation of the 18-kDa protein might have affected the antigen processing by the host antigen-presenting cells. Nevertheless, disruption of the 18-kDa protein gene in vaccine strain *B. abortus* RB51 had no influence on the strain's ability to induce protection in vaccinated mice. Since strain RB51Δ18kDa was constructed by inserting a kanamycin cas-

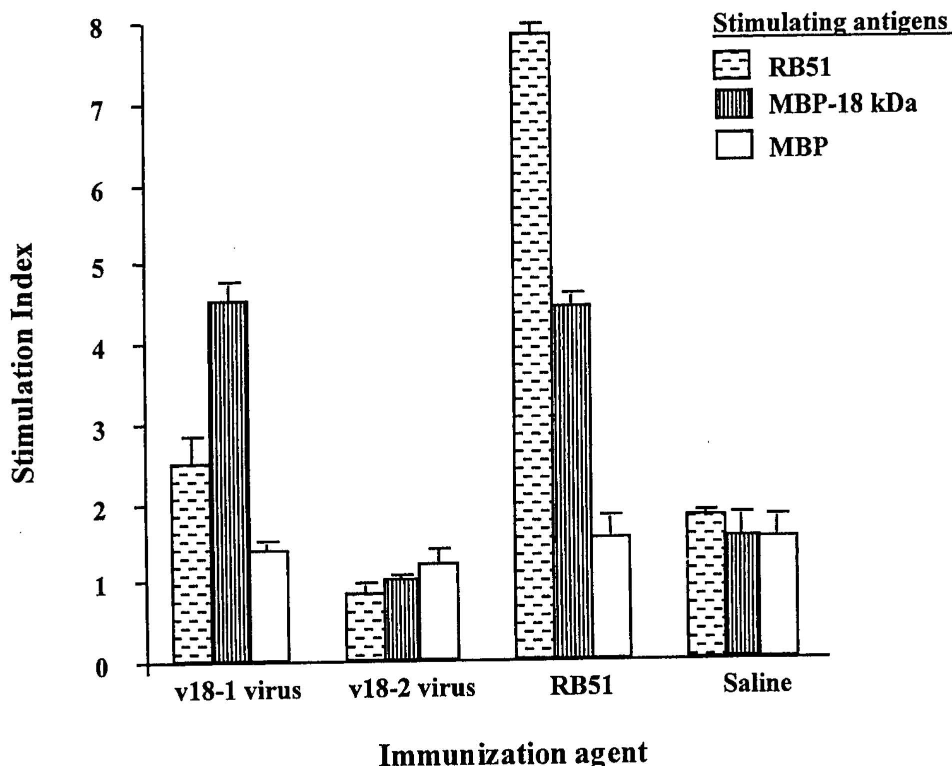

FIG. 2. In vitro proliferative responses of splenocytes from vaccinated and control mice. The assays were set up in triplicate, and the cells were either left unstimulated (media alone) or stimulated with antigens as described in Materials and Methods. Results are mean stimulation indices ± standard deviations (error bars) ($n = 3$).

sette at 72 bp (24 codons) downstream of the start site of the gene for the 18-kDa protein, this mutant is capable of synthesizing a peptide consisting of the first 24 amino acids of the 18-kDa protein. The first 20 of these 24 amino acids form the signal peptide sequence of the 18-kDa protein (14, 25). In *Brucella*, this signal peptide is cleaved off and the rest of the polypeptide is subjected to lipid modification (25). Therefore, there is a possibility that this signal peptide contains crucial protective epitopes and we failed to detect this immune response in mice inoculated with our disruption mutant since our recombinant 18-kDa fusion protein also lacked these 24 amino acids. However, ongoing studies in our laboratory indicate that overexpression of the 18-kDa protein with its signal sequence in strain RB51 did not enhance its protective ability, while overexpression of known protective antigens did (Vemulapalli et al., unpublished results). Taken together, it appears that the 18-kDa outer membrane protein of *B. abortus* is not involved in mediating protective immunity, although it is able to induce low levels of IFN-γ production from specific immune lymphocytes. Production of IFN-γ by specific antigen stimulation of lymphocytes is considered to be important in protection, since it is able to activate macrophages which are able to contain *Brucella* replication (29, 30).

Based on the results of this study and in accordance with other published findings (9, 10, 24), it appears that many *Brucella* proteins to which infected or vaccinated animals develop appropriate immune responses may not play a crucial role in host-acquired protective immune mechanisms to brucellosis. Until a reliable in vitro assay that correlates positively with the protective potential of a *Brucella* protein is developed, the present trial-and-error strategy is the only means available to verify the protective role of a *Brucella* protein antigen.

TABLE 2. IFN-γ production by splenocytes of inoculated mice after in vitro stimulation with specific antigens or mitogen

Antigen	IFN-γ concn (ng/ml)[a] in culture supernatant of splenocytes from mice vaccinated with:			
	v18-1 virus	v18-2 virus	RB51	Saline
Media	—[b]	—	—	—
Concanavalin A	26 ± 6.2	28 ± 4.3	24 ± 7.4	24 ± 4.0
RB51	1.78 ± 0.81	—	18 ± 6.0	—
MBP–18-kDa	0.3 ± 0.13	—	0.16 ± 0.1	—
MBP	—	—	—	—

[a] Values are means ± standard deviations for three experiments.
[b] —, below lower detection limit (<100 pg/ml).

This project was supported by the U.S. Army Medical Research, Development, Acquisition and Logistics Command (Prov.) under contract no. DAMD 17-94-C-4042.

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