National Journal of Physiology, Pharmacy and Pharmacology

RESEARCH ARTICLE

Effect of levamisole administration on immunogenic and protective capacity of *Brucella abortus* RB_{51}

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Received: November 05, 2017; **Accepted:** December 04, 2017

ABSTRACT

Background: *Brucella abortus* strain RB_{51} is a rough strain that was derived after multiple passages of virulent strain 2308. Levamisole, a potent anthelmintic that is widely used in veterinary medicine, is also known as an immune stimulant. **Aim and Objectives:** Determine the capability of levamisole to enhance the humoral antibody response and cell-mediated immunity as well as protective capacity of RB_{51} vaccine in mice against challenge with virulent *Brucella melitensis* strain. **Materials and Methods:** Six groups of 7–8-week-old female Bulb/C mice were used. Group I was vaccinated I/P with 5 × 108 colony-forming units (CFU) of RB_{51} strain. Group II was vaccinated I/P with 5 × 108 CFU of RB_{51} strain simultaneously injected (at 0 day) s/cu with 12.5 mg/Kg levamisole. Group III was vaccinated I/P with 5 × 108 CFU of RB_{51} strain and injected at 7-day postvaccination with 12.5 mg/Kg levamisole. Group IV was vaccinated I/P with 5 × 108 CFU of RB_{51} strain and injected at the day of vaccination (0 day) and 7-day postvaccination with 12.5 mg/Kg levamisole. Group V was injected with levamisole alone. Group VI was kept as control group. 8-week postvaccination; all vaccinated as well as control animals groups received I/P challenge of 2 ml dose of 2 × 104 CFU/ml of virulent strain of *B. melitensis* biovar 3. **Results:** (Groups III and IV) At the day of vaccination (0 day) and 7-day postvaccination showed the highest significant serum antibody titer measured by ELISA all over the experimental period as well as increase in delayed type hypersensitivity (DTH) response 48 h after elicitation by intradermal inoculation of *B. abortus* soluble antigen compared to vaccinated control group (Group I). While RB_{51} vaccinated and simultaneously treated with levamisole (Group II) showed mild elevation in antibody titer as well as the lowest significant increase in DTH response compared to vaccinated control group. Bioassay test of challenged mice groups showed significant improvement in the resistance of vaccinated groups treated with levamisole either 7-day postvaccination (Group III) or 0 and 7 days postvaccination (Group IV) which showed 10% bacteriologically positive mice, whereas only 30% of the vaccinated control mice (Group I) and vaccinated simultaneously treated with levamisole (Group II), were bacteriologically positive.

KEY WORDS: Levamisole; *Brucella*; Immunogenic Capacity; Protective Capacity; RB_{51}

INTRODUCTION

Brucellosis affects many animals' species as well as human in most countries. *Brucella abortus* is the most important cause of bovine brucellosis, while both *Brucella melitensis* and *Brucella ovis* cause brucellosis in sheep.[1]

Vaccination represents an essential element in the control of bovine and ovine brucellosis. Live attenuated *B. abortus* strain 19 and *B. melitensis* Rev 1 have served as efficacious vaccine strain for cattle and sheep, respectively.[2,3] However, both vaccines have the disadvantages of inducing O-polysaccharide (OPS)-specific antibody responses that interfere with the serological diagnosis of disease.[4] They may cause abortion in vaccinated animals under some circumstances[5] and of being pathogenic for humans.[6]

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Website: www.njppp.com

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Doi: 10.5455/njppp.2018.8.1143004122017

In recent years, Schurig and his coworkers produced a stable rough variant of virulent *B. abortus* 2308 that was designated RB_{51}.[7] Strain RB_{51} diminished virulence in comparison with strains 2308 and[8] and did not induce the formation of OPS-specific antibodies. Recent experiments have indicated that strain RB_{51} may serve as an alternative vaccine for cattle;[9] however, there is interest in attempting to enhance its efficacy using immune-stimulating agents.

Levamisole is an immunopotenciator drug which is used as an anthelmintic drug as well as very effective remedy on cellular immunity compared with humoral immunity.[10] Levamisole, a potent anthelmintic that is widely used in veterinary medicine, is also known as an immune stimulant. Because levamisole act at the cellular level, cells of immune-regulatory mechanisms are affected.[11] Under selective condition, levamisole can enhance immune responses to viral antigens.[12] Levamisole is thought to have its greatest immunostimulating effect in immunosuppressed animals and reduced the frequency of some diseases.[13]

The aim of this study is to determine the capability of levamisole to enhance the humoral antibody response and cell-mediated immunity as well as protective capacity of RB_{51} vaccine in mice against challenge with virulent *B. melitensis strain*.

MATERIALS AND METHODS

Vaccine

B. abortus RB_{51} vaccine (Professional Biological Company, Denver, Co, USA).

Vaccination, Treatment, and Challenge Infection of Experimental Animal

Six groups of 7–8-week-old female Bulb/C mice obtained from VACSERA (Helwan) weighing 21 ± 5 g were used.

1. Group I was vaccinated I/P with 5×10^8 colony-forming units (CFU) of RB_{51} strain (vaccinated control group).
2. Group II was vaccinated I/P with 5×10^8 CFU of RB_{51} strain simultaneously injected (at 0 day) s/cu with 12.5 mg/Kg levamisole (levamisole hydrochloride ICI Company, England).[11]
3. Group III was vaccinated I/P with 5×10^8 CFU of RB_{51} strain and injected at 7-day postvaccination with 12.5 mg/Kg levamisole.
4. Group IV was vaccinated I/P with 5×10^8 CFU of RB_{51} strain and injected at the day of vaccination (0 day) and 7-day postvaccination with 12.5 mg/Kg levamisole.
5. Group V was injected with levamisole alone.
6. Group VI was kept as control group (negative control).

8-week postvaccination; all vaccinated as well as control animals groups received I/P challenge of 2 ml dose of 2×10^4 CFU/ml of virulent strain of *B. melitensis* biovar 3.

Collection of Blood and Tissue Samples

1. Blood was collected for the separation of sera from mice in all groups. The first samples were taken prevaccination, then, at intervals of 2, 4, 6, and 8 weeks postvaccination for serological examination.
2. Spleens, livers, kidneys, and lungs were collected by sacrificed 10 animals 2 weeks after challenge from each group and exposed to bacteriological examination.

Evaluation of Humoral Immune Response using ELISA Test

Brucella-specific antibodies were detected by the use of whole cell RB_{51} ELISA. Microtitration plates for the whole cell RB_{51} ELISA were coated with killed *B. abortus* RB_{51} organisms which were prepared from plate (Trypticase soy agar) grown cells that were killed with methanol,[14] and mice sera were serially diluted in phosphate-buffered saline solution (PBS) – 0.05% tween 80 were tested using goat anti-mouse IgG labeled with horseradish peroxidase for reactivity against rough antigens. A test result was considered positive if the sample gave a reading equal twice that obtained of known negative control serum samples.

Evaluation of Cell-mediated Immunity by Delayed Type Hypersensitivity (DTH) Test

DTH reaction was determined by the method described by Arya *et al.*,[15] using *B. abortus* soluble antigen (BASA) which prepared from live cells of strain RB_{51} that were suspended in PBS and autoclaved to extract BASA.[16] 14-day postvaccination, 10 mice from each group were injected with BASA (20 mg in 20 µL of PBS) in the right footpad. Thicknesses were measured 24, 48, and 72 h later with Hauptner dial caliper. A difference in footpad thickness of ≥2 U (Iu = 0.1 mm) was regarded as positive reaction.

Bacteriological Examination for Isolation of *B. melitensis* Biovar 3 from Mice

The isolation of *B. melitensis* biovar 3 from spleen, liver, kidney, and lung was carried out according to the method explained by.[17]

RESULTS

For evaluation of the humoral immune response in mice groups vaccinated with RB_{51} vaccine and groups vaccinated with RB_{51} vaccine and treated with levamisole as well as control group, the ELISA test was performed.

Mice vaccinated I/P with RB_{51} strain (5×10^{8c} CFU) showed high serum antibody titers to the surface antigen of intact RB_{51} bacteria from week 1 to week 8 postvaccination [Table 1].

Table 1: Antibody profile of examined mice groups using ELISA

Animal groups	Weeks postvaccination							
	1	2	3	4	5	6	7	8
I	68±6.11	96±6.5	240±5.9	544±7.2	704±8.51	512±8.53	256±6.8	208±5.1
II	83±6.63	132±6.4	310±7.4	715*±6.8	952*±7.32	576*±6.1	320*±7.1	230*±5.8
III	95**±6.90	192**±6.72	430**±6.3	960**±5.89	983**±8.1	768**±6.01	488**±7.4	320**±5.31
IV	160***±7.1	272***±6.31	640***±6.21	1216***±9.3	1088**±8.56	832**±5.3	510***±6.31	352***±6.11
V	0	0	0	0	0	0	0	0
IV	0	0	0	0	0	0	0	0

*Significant at $P<0.05$, **significant at $P<0.01$, ***significant at $P<0.001$

It could be seen also from the data presented in Table 1 that levamisole treatment in addition to vaccination with RB_{51} vaccine either 7-day postvaccination or 0 and 7 postvaccination (Groups III and IV) had an immune-potentiating effect where the mean antibody levels as measured by ELISA all over the experimental period were higher than those in the levamisole-untreated vaccinated mice (Group I). Mice treated with levamisole simultaneously with vaccination (Group II) showed mild elevation in antibody titer compared with vaccinated (Group I). Results of cell-mediated immunity by DTH elicited in mice vaccinated with RB_{51} strain are illustrated in Table 2. The use of BSA as an eliciting antigen resulted in developing of DTH 14-day postvaccination (Group I). The highest reaction was observed 48 h postelection.

Table 2: DTH elicited by examined mice groups

Mice groups	Elicitation (hours post soluble antigen inoculation) 14-day postimmunization		
	24 h	48 h	72 h
I	6.15±0.2	6.91±0.3	5.28±0.2
II	6.91±0.8	8.16*±0.7	5.51±0.5
III	10.22**±0.9	11.38**±0.6	8.43**±0.4
IV	15.29***±0.6	16.94***±0.8	11.56***±0.6
V	3.14*±0.3	3.65*±0.6	2.08*±0.2
VI	2.15±0.1	2.14±0.2	2.09±0.2

The mean represented of the footpad thickness (0.1 mm unit after BSA antigen inoculation). *Significant at $P<0.05$, **significant at $P<0.01$, ***significant at $P<0.001$. DTH: Delayed-type hypersensitivity

Group IV treated with levamisole 0 and 7 days postvaccination revealed the highest level of DTH followed by mice group treated with levamisole 7-day postvaccination (Group III), and then, mice in Group II treated with levamisole simultaneously with vaccination. The isolation percentage of *B. melitensis* was recovered from 100% of non-vaccinated challenged mice (Groups V and VI); the highest isolation rate was recovered from spleen. Meanwhile, the vaccinated groups revealed various percentage of *B. melitensis* biovar 3 isolation, Group II simultaneously treated with levamisole at 0 day, Group III treated with levamisole 7-day postvaccination, and Group IV treated with levamisole 0 and 7 days postvaccination resulted in 30%, 10%, and 10%; isolation rate, respectively.

DISCUSSION

B. abortus strain RB_{51} is a rough strain that was derived after multiple passages of virulent strain 2308 on rifampin containing soy agar plate. Strain RB_{51} is stable, attenuated rough variant that is essentially devoid of OPS, therefore, vaccination with RB_{51} does not induce antibody to the OPS of *Brucella* species that are detected by the use of standard serodiagnostic tests for brucellosis.[18] Therefore, the elicited antibodies can be detected only using rough antigen derived from the same vaccinal strain.[19] The application of ELISA using rough antigen on serum of vaccinated animals revealed a detection of elevated rough antibodies titer which persisted up to 8th week postvaccination.

This result confirms previous studies[20,21] that used a dot blot or indirect ELISA techniques based on extraction of rough soluble antigen from RB_{51} vaccinal strain. In these previous studies, the dot blot or indirect ELISA using rough antigen detected antibodies from RB_{51} vaccinated mice for 8-week postvaccination.

The using of rough antigen in ELISA is considered by many workers to be the only tool to detect humoral immune responses induced by RB_{51} vaccine. The antibody detected using rough RB_{51} antigen in the current study was probably directed primarily to outer membrane proteins but not to the lipopolysaccharides (LPS) O antigen.[7,22]

Levamisole which has been used extensively as anthelmintic in man and domestic animals, recently attracted attention because it has immune-potentiating properties.[23] The mechanism by which levamisole could enhance serum antibody responses to the infective agent is not clearly known.[24]

In the present study, the most enhanced antibody responses could be seen in mice group treated with levamisole 7-day postvaccination (Group III) and mice treated with levamisole

at 0 and 7 days postvaccination (Group IV) while mice group given levamisole simultaneously with vaccine showed the lowest have significantly enhanced antibody response. The immunomodulation is most likely due to the direct effects of levamisole on the immune system and to time at which it was administrated levamisole enhances macrophage and T lymphocytes function and reduces suppressor T cell function.[25]

Because antibody formation to most bacterial antigens is B lymphocyte dependent, the augmentation of the helper functions of T cells could enhance antibody production. The stimulatory effects of levamisole on macrophages may be particularly important because *Brucella* species tend to be an intracellular pathogen and macrophages are thought to be important in host defense in bovine brucellosis.[26] The positive effect of levamisole given on day 7 may be due to the fact that helper cells function is maximal at that time. DTH is *in vivo* assay of cell-mediated immune function and directly reflects the manifestation of function of the type T effector cell.[27]

DTH is a measure of antigen-specific T cell recall reaction at the site of rechallenging antigen in antigen-sensitized animals.[15]

In the present study, we evaluated the effect of administration of levamisole at different intervals post-RB_{51} vaccination on the generation of DTH. The highest DTH response was observed in the group administrated levamisole 0 and 7 days postvaccination followed by group administrated with levamisole 7-day postvaccination. These results agree with[28] who suggested that levamisole being the most effective adjuvant modulates effectors T cells. Furthermore, Kimbell and Fisher[29] found that coinoculation of levamisole with plasmid not only preferentially increase the IgG_2 production but also prolongs the production. This may be due to its indirect effect on through activation of T lymphocytes to produce more interferon which in turn mediate for observed Th_1 type responses.[29] Suggested that levamisole could be a multifunctional regulator for both of Th_1 and Th_2 functions.

In the present study, following challenge with *B. melitensis* biovar 3, the bacteriological examination of sacrificed animal revealed that the challenge strain isolated from 30% of mice vaccinated with RB_{51} alone (Group I), and the highest isolation rate was observed in spleen.

The level of protection offered by RB_{51} vaccine may be due to the fact that this vaccine induces its immunity against infection mainly through cell-mediated immunity.[21] The immunity mediated by RB_{51} against *B. melitensis* infections are primarily directed against outer membrane proteins which are prominently exposed on cell surface in the absence of LPS O side chain in RB_{51}.[20] Furthermore, Stevens and Olsen[22] proved by western immune-blot analysis that RB_{51} vaccine induces small amount of IgG, but not IgM. The IgG had a role in protection of cattle and mice against virulent *Brucella* strains. It is clear from Table 3 that lower rate of isolation of *B. melitensis* biovar 3 was associated with mice groups vaccinated and treated with levamisole either 7 days or at 0 and 7 days postvaccination.

The previous increase of protection levels against infection with *B. melitensis* biovar 3 in levamisole-treated vaccinated groups is attributed to an augmentation of lymphocyte and macrophage proliferation, phagocytosis and increase of lysozyme enzymes released with increase of intracellular killing.[30]

Furthermore, levamisole stimulates cytotoxic T cells associated with enhancement of lymphokines production. The previous concept leads to an increase in the rate of clearance of infective agents.[24]

The results of the present investigation are in agreement with those obtained by[31] who found that the postvaccination injection of levamisole in guinea pigs caused a higher protection and survival rates than control animals.

CONCLUSION

Under the conditions of the present study, levamisole given 7 days or 0 and 7 days postvaccination caused enhanced humoral and cell-mediated immune responses to RB_{51} strain. Whether this procedure could be used in the field to enhance resistance to bovine brucellosis, this requires further study.

Table 3: Results of bacteriological examination of challenged mice

Mice groups	Organ subjected to bacteriological examination				
	Spleen (%)	Liver (%)	Lung (%)	Kidney (%)	Positive mice (%)
I	30	10	20	20	30
II	30	0	10	0	30
III	10	0	20	10	10
IV	10	0	0	0	10
V	100	70	40	60	100
VI	100	80	60	40	100

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How to cite this article: Mansour AMA. Effect of levamisole administration on immunogenic and protective capacity of *Brucella abortus* RB_{51}. Natl J Physiol Pharm Pharmacol 2018;8(5):635-639.

Source of Support: Nil, **Conflict of Interest:** None declared.