

The Immunoenhancing properties of living *Rhizobium meliloti* in experimental animals

Al-Joofy K. Ikbal*

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الخلاصة

صممت هذه الدراسة لتسليط الضوء على تأثير بكتريا *Rhizobium meliloti* الحية والغير مرضية على الجهاز المناعي للفئران البالغة. ثلاثون من ذكور الفئران بعمر (6-8) اسبوع وبوزن (19-22) غم، قسمت الى مجموعتين، مجموعة الاختبار (n.=20) ومجموعة السيطرة (n.=10). جرعت مجموعة الاختبار وعن طريق الفم بـ 100 مايكروليتر (4x10⁶CFU/ml normal saline) لمدة 12 يوم، وبنفس الوقت والطريقة أعطيت مجموعة السيطرة 100 مايكروليتر من محلول ملحي وظيفي معقم. تم اختبار التأثير المناعي لهذه البكتريا ولكلا المجموعتين المدروسة من الحيوانات بعد تمنيعها بواسطة 0.2 مل من كريات الدم الحمراء للخروف SRBCs بتركيز (10%) وفي التجويف الصفاقي وفي اليومين 4 و 8 من نهاية فترة المعاملة بالبكتريا. اجري تفاعل ارثس وفرط الحساسية الاجل DTH، فحص التشكيل الزهري الثاني، تحديد عيارية الاجسام المضادة، معامل البلعمة، اختزال صبغة NBT ووزن الجسم للحيوانات الممنعة بعد مرور (11-12) يوم على المعاملة بالبكتريا. أظهرت الدراسة إن بكتريا الرايزوبيوم الحية الغير المرضية تحفز الجهاز المناعي والذي انعكس في هذه الدراسة خارج وداخل جسم الحيوان المختبري.

ABSTRACT

This study was designed to shed some light on the influence of living *Rhizobium meliloti* (non pathogenic bacteria) on the immune system of mature mice.

Thirty male mice, 6-8 weeks old, 19-22gm weight divided into two groups, test group (n.=20), orally administrated with 100µl (4x10⁶ CFU/ml normal saline) for 12 successive days, at the same time and rout control group (n.=10) received 100 µl of sterile normal saline.

Tests for immunomodulation effects of this bacteria were applied after immunization of both groups with 0.2ml, (10% sheep red blood cells) interaperitonally on day 4 and 8 of schedule. Arthus reaction, delayed type hypersensitivity, E-rosette forming test, serum antibody level, phagocytosis index and nitro blue tetrazolium reduction were assessed on day 11 and 12 of program, mean while effect of the bacteria on the body weight of each group were studied.

Interestingly, this non-pathogenic living bacteria showed immunopotentiating activities in experimental animals reflected by these in vivo & in vitro assays.

INTRODUCTION :

Nowadays focusing on herbs, fruits, vegetables and microorganisms such as bacteria, fungi and yeast that can boost, direct or restores the immune system and acts as adjuvant after and/or with other modalities of treatment.^(1,2,3)

Rhizobium meliloti is non- pathogenic, Gram negative bacteria, found in the root nodules of *Melilotus indicus* (herbaceous plant) as polymorphic forms (bacteroids), normally involved in fixing atmospheric nitrogen into combined form (ammonia) utilizable to the host plant.⁽⁴⁾

In many reports different preparations of *Rhizobium* polysaccharide are found to posses immunomodulating activities in both in vivo & in vitro assays.^(5,6)

*Biology Department, College of Science, Almustansiriya University, Baghdad – Iraq.

MATERIALS AND METHODS:

Animals :

Thirty male BALB/C mice, 6-8 weeks old, 19-22gm weight, divided into two groups, test group (n.=20) & control group (n.=10).

Preparation of *R. meliloti* suspension : The nodules were removed carefully from the root of *Melilotus indicus* (herbaceous plant), washed gently with distilled water, exposed to 95% (v/v) ethanol and immersed in acidified mercuric chloride for 4-10 minutes, washed with sterile distilled water, crushed with sterile glass rod, then the bacteria were counted by using trepan blue & number chamber, a final concentration of living rhizobium was done to contain 4×10^6 CFU /ml normal saline⁽⁴⁾.

Immunization program :

Test group received orally 100 μ l of bacterial suspension, while control group received orally 100 μ l normal saline for 12 successive days, also both groups injected interaperitonally with 0.2ml (10% sheep red blood cells) on day 4 and 8 of schedule. The weight of all animals were measured before and after immunization program.

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Arthus and delayed type hypersensitivity (DTH) :

These skin reaction were assessed by observing local reaction on day 11 and 12 after immunization.

Arthus reaction was observed as an increase in footpad swelling 4 hours following challenge with sheep RBCs, while DTH reaction peaks at 18-24 hours.

In this test each mouse was challenged by injecting the left hind footpad subcutaneously (s.c.) with 50 μ l of 10% sheep RBCs, the vehicle (50 μ l normal saline) was injected s.c. into the right hind footpad as a control. Footpad swelling measured as reported in Triolo, et al., (1989)⁽⁷⁾.

Phagocytic index :

After injection of 5ml normal saline containing 50U/ml heparin in the peritoneal cavity, gentle massage of the abdomen, then peritoneal fluid (rich with macrophages) was pooled with pasteur pipette, the cells washed twice with normal saline, then heat killed yeast (*Saccharomyces cerviciae*) was added at macrophage yeast ratio of 1:4 in PRMI1640 medium, incubated for one hour at 37°C in 5%CO₂ atmosphere, washed with normal saline, smears of these cells were dried, fixed by methanol & stained with Giemsa stain for 20 minutes, washed with tap water, dried & examined under oil immersion, the phagocytic index was calculated by the following equation⁽⁸⁾:

$$\text{Phagocytic index} = \frac{\text{No. of phagocytic cells}}{\text{Total No. of macrophages}} \times 100$$

Nitroblue titrazolium reduction test(NBT) :

This test was done as following :

One and a half ml of NBT stain was added to peritoneal macrophages in a test tube, mixed gently, incubated at 37°C for 25minute, after that a drop of cell suspension was added on slid, dried, fixed with methanol, stained with Giemsa stain, then calculate the percentage of positive cells that contain dark blue granules of formazane⁽⁹⁾.

Active E-rosette assay :

Mice were scarified after blood collection, the spleen were removed and minced by mincer. Single cell suspensions were obtained as in Brown, et al., (1978) and counted by dye exclusion method and adjusted to the desired number.

Mixture of equal volume of splenic suspended cells (200µl) and 5% sheep RBCs incubated at 4°C for on hour, after incubation, smears were made gently, fixed, stained with Giemsa stain, examined under 40x & countes the percentage of rosette forming cell (cell with three or more sheep RBCs adhered to it)⁽¹⁰⁾.

Antibody titer determination :

Samples of blood were obtained from the studied groups on day 12 from ret orbital sinus, serum samples were titrated against sheep RBCs by direct hemagglutination and complement fixation test⁽¹⁰⁾.

Statistical analysis :

The data was calculated by means of the students t-test.

RESULTS:

The weight of animals before and after administrated with living bacteria is shown in table 1. Significant increase in mean body weight of test group as compared to control group (P<0.005).

Index level of Arthus and DTH to sheep RBCs on day 11 and 12 of program is demonstrated in table 2, highly significant elevation (P<0.0005) of the mean index of both Arthus and DTH reactions in test group as compared to control group.

Highly significant increase (P<0.0005) in the means of antibody titer in the test group in comparison to control group, by using direct hemoagglutination & complement fixation tests as clearly shown in table (2).

The result of E-rosette test in table 2 showed that the mean value of T lymphocyte which isolated from the spleen of mice treated with living bacteria was significantly higher (P<0.0005) than the value of control group.

Statistical analysis of the result of nitroblue tetrazolium reduction test denoted highly significant increase (P<0.0005) in the percentage of formazan forming cells in test group as compared to control group, table (2).

Results of phagocytic activity showed highly significant elevation (P<0.0005) in phagocytic index in test group in comparison with control group as well presented in table 2.

Table 1 . Effect of orally administration of living R. meliloti on mice body weight

Study groups	Body weight (gm)		Comparison
	M±S.D.		
	Before	After	
Control group (No.=10)	17.6±1.13	19.39±1.89	Sig.
Test group (No.=20)	21.54±1.60	23.10±0.79	

Sig. = P<0.005

Table 2 . Immunomodulating effects of *R. meliloti* in immunized mice

Parameters	Study groups		Comparison
	Control group	Test group	
	No.=10	No.=20	
	M±S.D.	M±S.D.	
Arthus reaction	0.0227±0.012	0.0283±0.015	Sig.
Delayed type hypersensitivity	0.0235±0.008	0.0684±0.092	Sig.
Antibody titer			
Hemagglutination	213.33±60.34	610.10±243.24	H.S.
Complement fixation	277.33±136.59	1605.82±976.90	H.S.
E-rosette*	42.5±2.70	75.8±3.70	H.S.
Nitroblue tetrazolium reduction (NBT)**	40.49±4.30	64.9±2.77	H.S.
Phagocytic index	46.913±5.999	81.11±7.62	H.S.

* % Splenic cells,

** % cells

sig.=P<0.005, H.S. = P<0.0005

DISCUSSION:

The present results of experimental study with living *R. meliloti* indicate that, this non pathogenic bacteria have immunoenhancing activities for murine immune system. It has a marked stimulating actions on growth, function of lymphocytes and macrophages, these actions are especially shown in DTH, Arthus reaction and antibody responses that controlled by the soluble mediators of the immune system like 1L-1 and 1L-2,^(11,12) mechanisms seem to be involved in the action, stimulation of lymphocytes could be caused by an alteration of NADPH metabolism or by a change in the redox potential on the lymphocyte membrane. In antigen stimulated lymphocytes this lead to an increased release of 1L-1, 1L-2 and interferons which induce greater cell proliferation and antibody production⁽¹¹⁾.

It is not worthy that living *R. Meliloti* is considered as a T-cell and B-cell mitogen as demonstrated in this study. Mitogenicity by this bacteria is related to stimulation of macrophage, B lymphocyte and T lymphocytes, this activity may be due to stimulation of macrophages which play important role in antigen processing and monokine production⁽¹¹⁾, studies have suggested that many immunopotentiating agents are both mitogen and adjuvants because of their large polymorphic nature so that multiple receptors on lymphoid cell surfaces could be simultaneously bound, activating membrane enzymes and cyclic neucleotides, which in turn would alter cellular metabolism resulting in altered immune responsiveness⁽¹²⁾.

Finally, local immune response in the gut, that prevent mucosal attachment and systemic humoral response, with cellular immune response to eliminate interacellular microorganism were clearly shown during orally administration of living *R. meliloti* to mice (our unpublished data). So this non-pathogenic bacteria may play a role in the prevention and therapy of some disease.

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