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Vaccinal Efficacy of Genetically Inactivated Phospholipase D Against Caseous Lymphadenitis in Small Ruminants

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Abstract: Vaccination against Caseous lymphadentitis is still a matter of dispute. Four types of vaccine for mutations were prepared in the present investigation. The first vaccine was composed of formalin-killed whole cell culture of *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) adjuvated with oil adjuvant. The second vaccine was formulated from formalin bacterin and genetically mutated recombinant phospolipase D (mrPLD) with the same adjuvant, the third vaccine was composed of Gamma irradiated whole cell culture of *C. pseudotuberculosis* combined with mrPLD and oil adjuvant; the last vaccine was prepared by combination of BCG and mrPLD without adjuvant. Single formalized whole culture of *C. pseudotuberculosis* offered 80% protection in sheep challenged with local isolate of C. *pseudotuberculosis*. Second vaccine provided full protection (100%) of vaccines. Combined δ-irradiated whole cells combined with mrPLD and adjuvant resulted in 78% protection, while combined BCG and mrPLD without adjuvant gave the least extent of protection (66%). Since there was no apparent difference in humoral immune response by vaccinated sheep, the reason for this variation in vaccine efficacy can be attributed to opsonic effect of antibodies raised against whole cell immunization; neutralization of PLD exotoxin offered by anti-PLD antibodies evoked by mrPLD, in addition to cell mediated immunity induced by mrPLD. Moreover, the oil adjuvant vaccine enhanced immune response in vaccinated animals.

Key words: Wild recombinant PLD-mutated recombinant PLD-dermonecrotic activity · Immunogencity of rPLD · Mutagenesis

INTRODUCTION

Corynebacterium pseudotuberculosis (*Cp*) is the pathogen of different diseases in different animals. It is classified into two biovars and two serotypes depending upon nitrate reduction, guinea pig pathogenicity and serological tests; serotype I (biovar 1) and serotype II (biovar 2) [1]. Biovars 1 and 2 are the etiological agents of a disease that is commonly called caseous lymphadenitis (CLA). The disease is found in the entire world's major sheep and goat production areas, causing significant economic losses [2]. Type 2 affects horses causing ulcerative lymphangitis [3] and Egyptian buffaloes causing Oedematous Skin Disease (OSD).

The phospholipase D (PLD) produced by *CP* has been shown to be an essential virulence determinant that contributes to the spread of the bacteria from the initial site of infection to secondary site within the host [4]. Vaccination against diseases caused by *CP* presents a challenge to producers and veterinarians. Previous attempts to develop an effective vaccine used inactivated whole cells, cell wall extracts or inactivated exotoxin resulted in partial protection, but did not provide solid immunity [5 - 8].

CLA vaccines are currently produced from formalin-inactivated PLD-rich *C. pseudotuberculosis* culture supernatants in which PLD is considered the major protective antigen [9]. Evidence of toxoid vaccine efficacy in prophylaxis against CLA is confusing. A new approach for preparation of toxoid vaccines is achieved by genetic inactivation of PLD instead of chemical inactivation to avoid the drastic effect of chemicals on the protective efficacy of PLD antigen. This approach has been demonstrated with other bacterial toxins; such as diphtheria and pertussis toxins, where changing one or two amino acid at specific sites has been shown to

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produce nontoxic molecules which are still immunogenic [10]. Haynes *et al.* [11] induced inactivation of PLD through changing the codon for Histidine 20 Tyrosine. Tachedjian *et al.* [12] achieved inactivated PLD through site directed mutagenesis of His20 with Tyr. Ghoneim *et al.* [13] induced mutagenesis of *pld* gene through substitute of His20 to Tyr20 by using PCR site directed mutagenesis.

The biologically inactivated PLD (toxoid) proteins are not investigated as vaccinal preparation under field conditions. Hodgson *et al.* [14] compared protective efficacy of vaccine formulated using formalin-inactivated culture supernatants that are rich in the Cp PLD exotoxin and other vaccine prepared by genetic inactivation of pld gene. The authors reported that genetically inactivated PLD protected 44% of sheep against Cp challenge compared with 95% protection offered by the formalin-inactivated preparation. On the other hand, Fontaine *et al.* [15] designed a vaccine composed of recombinant PLD combined with formalin-killed bacterin of *C. pseudotuberculosis*. The vaccine provided high protection for sheep exposed to challenge and the efficacy was higher than a commercially available inactivated –PLD vaccine (Glenvae-3™, Austeria).

The present investigation was undertaken to investigate protective efficacy of 4 different vaccine preparations to protect sheep against challenge with *Cp* virulent strains locally isolated from sheep suffered from CLA. The first vaccine was formulated of formalin-killed whole cell (bacterin) mixed with oil adjuvant. The second vaccine was formulated from genetically inactivated *pld* and expressed in *E. coli* to obtain mutated recombinant PLD (mrPLD) combined with formalin-killed whole *Cp* adjuvanted with the same oil adjuvant. The third vaccine was formulated from mrPLD combined with whole cells of *Cp* killed with Gamma irradiation and the fourth formulation was composed of mrPLD combined with BCG.

MATERIALS AND METHODS

Media and Strains: *Corynebacterium pseudotuberculosis* were grown on Brain heart infusion agar (Oxoid-UK) supplemented with Fosfomycin and malidix acid. Lowenstein Jensen media were used for sustaining BCG and Sauton media for propagation of BCG. Local strains of *C. pseudotuberculosis* were isolated and completely identified by authors in BCVSR. BCG was kindly obtained from the department of BCG vaccine preparation in Veterinary Vaccine and Serum Research Institute.

Mutated Recombinant PLD Protein: Mutagenesis of *pld* gene with PCR-Site directed mutagenesis kits (Stratagene) and expression in *E. coli* and purification of recombinant protein was processed by authors in BCVSR.

Vaccine Formations:

Formalin killed *C. pseudotuberculosis* [5]: A single colony of completely identified strain of *Cp*, was inoculated into 250 ml of brain heart infusion broth containing 1% tween 80 and incubated at 37°C with shaking for 18-24 hours.The broth culture was then centrifuged for 15 minutes at 6000 rpm at 4°C and the supernatant was decanted and bacterial cells were washed twice with acetone and twice with diethyl ether.

The bacterial mass was weighed and resuspended in 1% formalin saline solution in the concentration that each ml of suspension contained 20 mg of washed bacterial cells. Tween 80 was added to suspension till the final concentration of 3-4%. Each dose of 2 ml is composed of 1 ml contains 2 mg bacterin mixed with 1 ml of oil adjuvant.

Formalin-killed *C. pseudotuberculosis* Combined with Mutated Recombinant PLD: Each dose of 2 ml was composed of 1 ml 2 mg bacterin mixed with 5 μg of mrPLD and adjuvated with 1 ml of oil adjuvant.

Gamma Irradiated *C. pseudotuberculosis* and mr PLD: One single colony of the *C. pseudotuberculosis* local sheep isolate was inoculated into 5 ml brain heart infusion broth containing 0.1% Tween 80. Inoculated flask was incubated at 37°C for 18-24 hours in shaking incubator. The broth culture then centrifuged at 6000 rpm in 4°C for 15 minutes. The supernatant was decanted and pellet of cells was washed twice with phosphate buffer saline (PBS) then weighed and resuspended in PBS so that each 1 ml contained 30 mg washed cells. The cell suspension was distributed into bottles so that each bottle contained 1 ml of cell suspension. The bottles were lyophilized and subjected to 100, 65, 50 and 25 Gray doses of gamma irradiation in National Center for Radiation Research and Technology-Egypt. Killing activity of different irradiation dose was assayed by cultivation on BHI agar media. Abolishing of *Cp* growth was obtained in media exposed to 100 Gray dose which is considered as the minimum irradiating dose that can kill *Cp* cells. Each dose of vaccine was composed of 2 ml containing 2 mg killed irradiated cells and 50 μg mrPLD and 1 ml of oil adjuvant.

BCG combined with mr PLD: BCG was cultivated in Sauton media and centrifuged, the pellet was weighed and

resuspended in PBS so that each 1 ml of BCG contained 0.1 mg of cells. Then 50 μg of mrPLD was added.

Control Mixture: Each 2 ml contained sterile saline mixture with the adjuvant in equal volumes.

Vaccination of Challenge: Five groups of approximately 6 months local sheep breeds (Balady) were used. Each group was composed of 3 animals.

Group A: Constituted of 3 animals (A_1, A_2, A_3) and were subcutaneously vaccinated with 2 ml of vaccine A in the left axillary region. The same dose was repeated after 3 weeks.

Group B: Constituted of 3 animals (B_1, B_2, B_3) and were vaccinated with 2 ml of vaccine B subcutaneously in the left axillary region. The same dose was repeated after 3 weeks.

Group C: Composed of 3 animals (C_1, C_2, C_3). Each animal was vaccinated with 2 ml of vaccine C subcutaneously in the left axillary region. The same dose was repeated after 3 weeks.

Group D: Composed of 3 animals (D_1, D_2, D_3). Each animal was immged with vaccine D as 1 ml subcutaneously in the left axillary region. The same dose was repeated after 3 weeks.

Group E: Composed of 3 animals (E_1, E_2, E_3). Each animal was inoculated with 2 ml of saline-oil adjuvant subcutaneously in the left axillary region. The same dose was repeated after 3 weeks.

Challenge of Vaccinated Animals [16]: One single colony of *C. pseudotuberculosis* was used to inoculate a flask containing 250 ml of BHI broth, incubated at 37°C for 48 hours. Contents of the flask were centrifuged and pellets were washed 3 times with saline and finally resuspended to the original volume with saline. Viable counts were regulated to be 10^6 colony units /mg with the help of prepared standard curve. Three weeks after the second vaccination each animal was challenged with 2 ml of challenging inoculum, 1 ml as a subcutaneous infection in the neck region, approximately 20 cm cranial to the prescuplar lymph node and 1 ml as a S/C injection in the hind region, approximately 20 cm dorsal to the prefemoral lymph node.

All animals were examined every 2 weeks to observe the appearance of any enlargement of external lymph nodes or of sites of challenge inoculation. All sheep were euthanized and necropsied 12-14 weeks post last vaccination.

Scar Evaluation of Developed Lesions: Beside sites of challenge inoculation and internal organs, 10 lymph nodes (LN), were examined for abscesses and samples were collected from abscesses for reisolation of *C. pseudotuberculosis*.

Examined LN were divided into 5 groups, 4 externals (Right and left prescapular and poputeal and 5th group contains all the internal lymph nodes (bronchial, thoracic, inguinal and mesenteric). Each group is considered as one unit in case of scare evaluation, scare of infection in each unit was calculated according to size, shape and appearance of LN [17, 18].

The following scores were used:

- Enlarged and caseated LN = 3/3
- Enlarged, oedmatous or congested = 2/3
- Enlarged LN = 1/3
- Normal LN = 0/3

Therefore, the score calculation of each group (contain 3 animals) was calculated as follows:

- Maximum score for each unit = 3/3
- Maximum score for the 3 animals = 3x 3= 9
- Maximum score of all units of LN in each group = 9x5= 45

Measurement of Sheep Antibody Responses: Blood samples were taken prior to vaccination (zero time) and after one week post vaccination, then at weekly intervals all over the experiment.

Antibodies responses to *C. pseudotuberculosis* and PLD proteins were measured with an indirect enzyme-linked immunosorbent assay (ELISA) using 0.5 μg of recombinant PLD per well as coating antigen, subsequently. Plates were incubated in the presence of experimental serum samples diluted (Y50) in PBS pH 7.4. Plates were washed 5 times with PBS-Tween 0.05% and bound anti rPLD IgG was detected by use of alkaline phosphate confugated goat-anti-rabbit Ig (Sigma) and P-nitrophenyl phosphate (PNPP) (Sigma). Calorimetric reactions were stopped with 3M Na OH and absorbance of each sample at 405 nm was subsequently determined by ELISA reader (EL340 Microplate-Bio-TER instruments USA).

Statistical Analysis: Statistical analysis was preformed by SPSS 16 and the data were analyzed by one way ANOVA [19].

RESULTS

Optimisation of Experimental Condition: All sheep used in the experiment were tested by ELISA using wrPLD as coating antigens and all had no antibodies titers against wrPLD. The dose of challenging *C. pseudotuberculosis* was determined depending upon previous investigation in the present project and proved to be 2x 10^6 viable *C. pseudotuberculosis* counts. Sheep selected for vaccinal experiments were clinically examined and blood samples were collected for detection of antibodies using 0.5 µg/well wrPLD (0.5 µg/well); protein as coating antigens for ELISA test; thus the absence of natural infection within the flock was ensured. All animal were free from signs of infection and lacked circulating anti-PLD IgG.

Vaccination of Experimental Animals: Four groups of sheep (each 3 animals) were vaccinated with 4 types of vaccines. The first group (A) was vaccinated with formalin-killed whole *C. pseudotuberculosis* (bacterin). The second group (B) was vaccinated with combined bacterin plus mrPLD, the third group (C) with killed whole *C. pseudotuberculosis* cells killed with δ-irradiation (bacterin) to avoid killing the drastic effect of formalin in killing C. *pseudotuberculosis* in combination with mrPLD. The fourth group (D) was vaccinated with a combined BCG with mrPLD. The fifth group was used as a control group and was injected with the oil adjuvant.

All vaccines were revaccinated 3 weeks after the first close of vaccination. Three weeks after second vaccination, all vaccines in addition to control groups were exposed to challenge by inoculation 2x10^6 viable *C. pseudotuberculosis* cells. Prior to experimental challenge serology was performed to monitor the response to vaccination in each animal, using wrPLD-ELISA.

To compare immune response among treatment groups, data is presented as average OD_{450nm} values per group (Fig. 1).

All vaccinated groups showed increase in antibody level (as the absorbance values of 45 nm) after 3 weeks of the first dose and high levels were noticed after 3 weeks of the last dose of vaccine. After 3 weeks of challenge, the

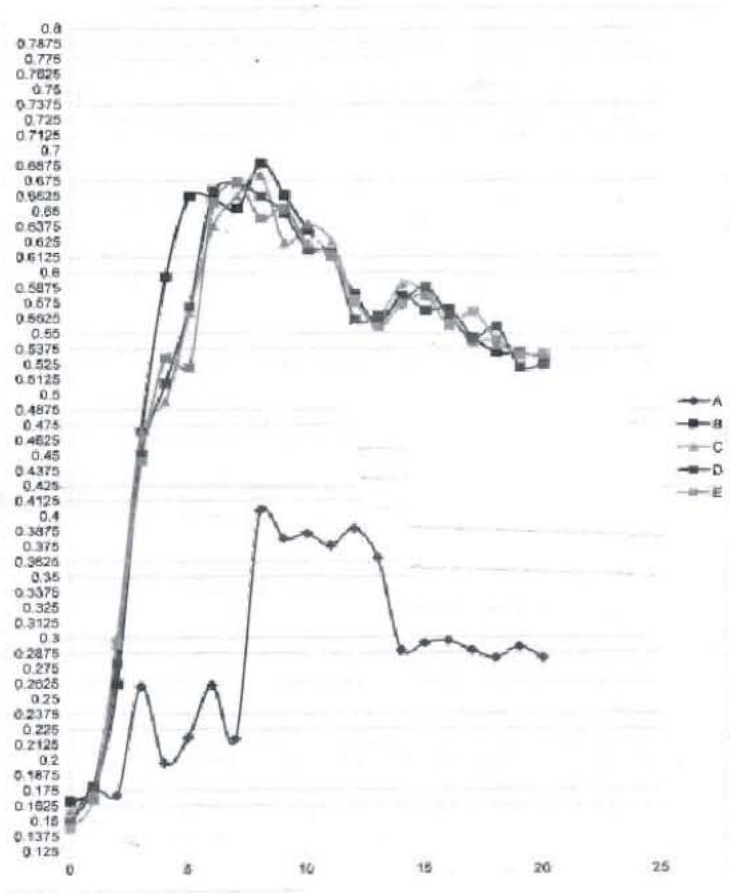

Fig. 1: The average OD 45 nm values per group.

Table 1: Score of lesions detected during post mortem examination of external and internal lymph nodes of sheep post challenge

	Lesions							
	External lymph node							
Groups of sheep	RPS	LPS	RPF	LPF	Total scores of internal lymph nodes	Total scores of each group	% of protection	% of infection
Control group								
E_1	+++(3/3)	+++(3/3)	+++(3/3)	-(0/3)	+++(3/3)	24/30	20	80
E_2	+++(3/3)	+++(3/3)	+++(3/3)	-(0/3)	+++(3/3)			
E_3	Died before challenge							
Formalized group								
A_1	++(2/3)	++(2/3)	-(0/3)	-(0/3)	+++(3/3)	9/45	80	20
A_2	-(0/3)	-(0/3)	-(0/3)	-(0/3)	-(0/3)			
A_3	++(2/3)	-(0/3)	-(0/3)	-(0/3)	-(0/3)			
Formalized mutated PLD								
B_1	-(0/3)	-(0/3)	-(0/3)	-(0/3)	-(0/3)	0/45	100	0
B_2	-(0/3)	-(0/3)	-(0/3)	-(0/3)	-(0/3)			
B_3	-(0/3)	-(0/3)	-(0/3)	-(0/3)	-(0/3)			
Irradiated *Coryne* + mutated PLD								
C_1	-(0/3)	-(0/3)	-(0/3)	-(0/3)	-(0/3)	13/45	72	28
C_2	++(2/3)	++(2/3)	-(0/3)	-(0/3)	++(2/3)			
C_3	++(2/3)	++(2/3)	-(0/3)	-(0/3)	+++(3/3)			
BBG+ mutated PLD								
D_1	++(2/3)	++(2/3)	-(0/3)	-(0/3)	++(2/3)	20/45	66	34
D_2	+++(3/3)	+++(3/3)	-(0/3)	-(0/3)	+++(3/3)			
D_3	+++(3/3)	-(0/3)	+(1/3)	+(1/3)	-(0/3)			

Score:
Category 0 : Normal or slightly enlarged LN; score 0/3
Category I : Enlarged LN; Score l/3
Category II : Enlarged and congested or hemorrhagic; score 2/3
Category III: Enlarged with focal abscesses or caseation; score 3/3
RPS: Right Prescapular lymph node LPS: Left Pre scapular lymph node
RPF: Right Prefemoral lymph node LPF: Left Prefemoral lymph node
Internal lymph nodes: (lnguinal, tracheobronchial, thoracic, mesenteric, 2 popliteal).

level of antibodies decreased in comparison with those developed after the second dose of vaccination. In case of control (unvaccinated) group antibodies increased after 1 week of challenge reversed to normal levels after 4 weeks of challenge; while all other vaccinated groups decreased in OD values till the end of experiment.

It can be observed that addition of mrPLD to bacterin did not increase the level of antibody response in vaccinated animals. In case of BCG combined with wrPLD, it gave the same levels of antibodies against mrPLD. At the same time, single bacterin vaccine stimulated the same level of antibodies. These results indicated that whole cell *C. pseudotuberculosis* include PLD proteins which indicate that antigenicity was not affected exposure to formalin or δ-irradiation. Mean anti- *C. pseudotuberculosis* IgG levels in the control group rising slightly after challenge, but not reaching an equivalent level to that of other vaccinated groups.

Post Mortem Analysis: The present work revealed that experimental infection with $2x10^6$ viable *C. pseudotuberculosis* resulted mainly in development of CLA lesions in the prescapular, perfemoral lymph nodes and rarely in bronchial lymph nodes. No lesion was observed in the internal organ including lungs. For that reason evaluation for protection was restricted to the 2 prescapular and 2 prefemoral lymph nodes and all internal lymph nodes and organs were scored as one unit. The efficacy of protection was evaluated depending upon the extent of lesions developed in the 5 score units. Extent of lesions were classified into 4 categories. 0 category represents normal or slightly enlarged LN with score 0/3; category 1 represents enlarged LN with score 1/3; category 2 represents enlarged and congested haemorrhagic LN with score 2/3 and category 3 represents enlarged with focal as caseated LN having score 3/3.

Results of protection extent of each vaccinated and control unvaccinated challenged groups are shown in Table 1.

Results in table 1 indicate a strong evidence ($p<001$) that the mean percentage of lymph nodes infected was different among the different treatment groups. The mean

of scores in the control unvaccinated group revealed 20% of protection. The highest level of protection (100%) was developed in group vaccinated with bacterin (formalin-killed whole *C. pseudotuberculosis* cells) combined with mrPLD, to a lesser extent (80%) protection developed in group (A) vaccinated with bacterin (formalin killed) only. Vaccinated group (C) with bacterin (δ-irradiated whole cells) in combination with mrPLD provided 72% protection. In case of group (D) vaccinated with a combined BCG and mr vaccine, the protection was 66%.

DISCUSSION

The results of the present investigation indicated that sheep are capable of mounting an immune response which can successfully limit the development of both internal abscesses as well as abscessation in the draining lymph nodes, or even at site of inoculation whereas all of the non vaccinated sheep from control group developed abscess in the prescapular, prefemoral (draining LN) and popliteal, bronchial thoracic, inguinal and mesenteric lymph nodes in addition to site of inoculation. These lesions appeared in varying degrees in different lymph nodes and could be achieved by S/C inoculation of animals with challenging dose composed of 2 ml saline suspension of washed *C. pseudotuberculosis* (local isolate) in a concentration of 2.0×10^6 colony units per ml. Challenge dose was distributed in two inoculation sites, one ml in the neck region near the prescapular LN and 1 ml in the hind region near the prefemoral LN. Full protection (100%) could be observed in group of sheep vaccinated with a combined vaccine composed of formalin killed (bacterin) with mutated recombinant PLD (mr PLD). These results coincide with full protection achieved by Fontaine *et al.* [15] using a vaccine composed of bacterins and recombinant PLD. The only difference between the two vaccines is the using of mr PLD in our study, while these authors used non mutated rPLD. In a comparative study in our laboratory between mutated rPLD ND RPLD proved that there was no difference in immunogenicity or antigenicity of both rPLD (unpublished data). To elucidate the possible drastic effect of formalin used for killing *C. pseudotuberculosis*, a group of sheep was vaccinated with oil adjuvated formalin-killed whole cells of *C. pseudotuberculosis*. Results in table 1 revealed that bacterin vaccine could provide 80% protection of vaccinated sheep, that confirm the previous reports about the partial protection provided by bacterin vaccine [20, 21]. On the other hand, some authors suggested that treatment of *C. pseudotuberculosis* with formalin for the purpose of inactivation, may result in some undesirable effects that may abolish some important protective antigens included in *C. pseudotuberculosis* cultures [11,12].

To declare this suggestion, bacterin was prepared by gamma irradiation inactivation of *C. pseudotuberculosis* cultures. Bacterial cells were exposed to the minimal dose of δ-irradiation that could inactivate 30 mg washed cells. The dose was 100 rad. A combined vaccine that was composed of 2 ml vaccine dose contains 40 mg δ-irradiation cells (bacterin) mixed with 50 μg mrPLD adjuvated with the same oil adjuvant which were used for vaccination of a third group of sheep with the same schedule of vaccination described in other groups of vaccinated and challenged animals in the present study. Surprising results were obtained where protection was 72%, less than combined formalin killed mrPLD vaccine (100%) or even formalin bacterin vaccine (80%). This result indicated that δ-irradiation had more drastic effect on immunogenicity of *C. pseudotuberculosis* in comparison to formalin inactivation which is more suitable for inactivation of bacteria than δ-irradiation prepared for vaccine preparation.

In relation to immune responses developed during vaccination, it is well documented that any form of CLA vaccines could induce humoral immune response, which in opinion of many authors play the major role in protection against CLA [9, 14, 22]. Meanwhile, it is well known that *C. pseudotuberculosis* is a facultative intracellular microorganism and cell-mediated immunity must share humoral immune response in providing adequate protection against CLA. To compensate the deficiency of providing cell- mediated immunity not available during vaccination with rPLD [15] a combined vaccine composed mrPLD (50 μg) plus 0.1 mg/ml without adjuvant was used for vaccination a fourth group of sheep. Schedule of immunization and challenge used as previously described in the previous vaccine formulas. BCG-rPLD developed protection in 66% of vaccinated animals.

The reason for the decrease of recombinant PLD protection activity in presence of BCG is not clear, although BCG is known as a nonspecific cell mediated immune-ostimulant and even used by some authors in vaccination of sheep against CLA [1].

Fontaine *et al.* [15] reported that complete protection provided by a combined rPLD-toxoid vaccine may be attributed to a stimulation of a strong antibody mediated response, combined with priming of cell mediated immune system through subcutaneous vaccination with the killed

whole cells. Some authors confirmed the development of cell mediated immune response after the vaccination of Balb/c mice with combined bacterin-toxoid vaccine. El-Enbaawy *et al.* [23] could assay humoral immune response by ELISA and cell-mediated immune response by assaying the level of IFN-δ in vaccinated mice. IFN-B is considered one of the potent cytokines that activate macrophages. Depending upon this information, it can be proposed that vaccination with combined rPLD-bacterin vaccine may provide humoral immune response characterized by the production of antibodies against bacterial and PLD antigens, in addition to cell-mediated immunity represented by the induction of highly activated macrophages by IFN-δ. In case of non activated macrophages *C. pseudotuberculosis* bacteria resist digestion macrinside macrophages by secretion of certain enzymes including PLD [24, 25]. This resistance of digestion of phagocytosed bacteria decreases the presentation of corynebacterial antigens to T helper cells, which is the main source of macrophages activator cytokines as IL-1 and IFN-δ. IFN- δ, TNF-a and IL-1 are required for the development of protective immunity to secondary *C. pseudotuberculosis* infection [26]. In another publication, Lan *et al.* [27] reported that CD4 and CD8 T cells play an essential role in the establishment of protective immunity against a primary infection caused by *C. pseudotuberculosis*. On murine model, the authors reported that in secondary exposure of already infected murine to formalin killed bacteria, IFN-δ and TNF-a are produced rapidly after secondary exposure. These cytokines may participate in the establishment of protective immunity through the activation of marcophages, since their capacity to activate the bactericidal function of macrophages is well established [28].

It can be concluded that protection against CLA depends upon the development of both humoral and cell mediated immune responses. Antibodies neutralize PLD that may be produced by invasive *C. pseudotuberculosis*. This rapidly produced antibodies in vaccinated animals which would presumably abrogate vascular permealisation to some extent that limiting the spread of bacteria out of the entrance site. Moreover, antibodies can epitomize bacteria that enhance their killing by non activated marcophages [15], while cell mediated response resulted from activation of T helper and cytotoxic T cell [26] and production of IL-1, IFN-δ and TNF-a. The major activators of macrophages that can kill bacteria were already restricted to the site of invasion.

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