PROTECTIVE EFFICACY OF MYCOBACTERIAL 71 KILODALTON CELL WALL ASSOCIATED PROTEIN USING POLY (DL-LACTIDE-CO-GLYCOLIDE) MICROPARTICLES AS CARRIER VEHICLES

NEELAM DHIMAN AND G.K. KHULLER

Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh-160 012 [India]

ABSTRACT

A 71 kDa cell wall associated protein of *M. tuberculosis* H₃₇Ra, on encapsulation in biodegradable microparticles composed of poly-DL-lactide-co-glycolide (DL-PLG) exhibited higher level of T-cell stimulation and cytokine release as compared to 71 kDa-FIA in mice as evaluated till sixteenth week post immunization (p.im.). Further, the protection imparted by immunization with 71 kDa-PLG microparticles (71 kDa-PLG-MPs) was significantly higher ($p < 0.5$) than the 71 kDa-FIA (70%) and BCG (65%) immunized group on the basis of survival rates, when challenged at sixteenth week p.im. The protective effect was consistent with the decreased bacterial load in the infected organs of 71 kDa-PLG primed group as compared to 71 kDa-FIA and BCG immunized groups.

KEY WORDS : Mycobacteria, 71 kilodalton protein, poly (DL-Lactide-co-glycolide); Vaccine

INTRODUCTION

In the recent years, mycobacterial proteins have gained special attention as suitable candidates for subunit antituberculous vaccine (1). A number of native and recombinant proteins have been characterized for their ability to achieve TH1 cells involved in the induction of protective immunity against tuberculosis (1). However, most of these proteins are weakly immunogenic if administered without an adjuvant. Hence, to improve vaccine efficacy, evaluation of new vaccine delivery systems based on pharmaceutically acceptable, controlled antigen delivery systems need to be identified.

Presently, synthetic, biodegradable polymers have gained considerable attention as adjuvants because of their potential for long term controlled release of proteins (2). Copolymers of lactide and glycolide such as DL-PLG have been demonstrated to have strong adjuvant activity for various antigenic proteins including ovalbumin, cholera toxoid, tetanus toxoid, malaria and pneumotrophic bacterial antigens (2).

Address for Correspondance :
Dr. G.K. Khuller at above address

In the present study, a highly immunoreactive 71 kDa cell wall associated protein of *M. tuberculosis* H₃₇Ra was entrapped in PLG-MPs to evaluate its immunogenicity and protective efficacy against tuberculosis. The protein has been previously reported to induce better protection than BCG against LD₅₀ challenge of *M. tuberculosis* H₃₇Ra in mice when complexed in FIA (3).

IMMUNE RESPONSES TO 71 kDa-PLG MICROPARTICLES

The 71 kDa protein was isolated from *M. tuberculosis* H₃₇Ra cells as described previously (3) and homogeneity of the protein was checked by silver staining on 12% SDS-PAGE. 71 kDa-PLG microparticles were prepared by double emulsion solvent evaporation technique (4).

NMRI strain mice (in groups of 30 each) were immunized with 60 µg of 71 kDa either emulsified in FIA or PLG-MPs in three equal weekly doses. Controls were sham immunized with normal saline in FIA or PLG-MPs. T cell proliferation and cytokine assays (IFN- γ and IL-2) were performed at 1,2,4,8 and 16 weeks p.im. The results of proliferative responses to 71 kDa-FIA and 71 kDa-PLG (Fig. 1) indicated significantly higher

Fig. 1 : Comparative lymphocyte proliferative responses to 71 kDa. Splenic T-lymphocytes isolated from animals immunized with 71 kDa-PLG and 71 kDa-FIA were incubated *in vitro* with 71 kDa antigen in three doses at weekly intervals. Controls were sham immunized with NS-PLG or NS-FIA. The results are presented as stimulation index (SI) as mean of two sets of independent experiments.

$$SI = \frac{\text{cpm of antigen stimulated cells}}{\text{cpm of unstimulated cells}}$$

***p<0.001 with reference to corresponding controls.
oo p<0.01; o p<0.05 with reference to 71 kDa-FIA immunized group. ns- non significant. (Data were evaluated using students 't' test)

Table 1 : *M.tuberculosis* H₃Rv enumerated from the target organs of 71 kDa-PLG-MPs, 71 kDa-FIA, BCG and control mice challenged sixteenth week p.i.m.

Group	Viable Counts		
	Lung	Liver	Spleen
71 kDa-PLG-MPs	2.10±0.39×10 ⁶ ***.###.***	2.27±0.09×10 ⁵ ****.###.***	1.42±0.21×10 ⁵ ***.###.ns
NS-PLG-MPs	3.05±0.36×10 ⁸	3.77±0.22×10 ⁷	3.96±0.25×10 ⁵
71 kDa-FIA	3.48±0.41×10 ⁷ ***.ns	3.18±0.39×10 ⁶ ***.ns	2.01±0.23×10 ⁵ ns
NS-FIA	3.55±0.65×10 ⁸	2.84±0.91×10 ⁷	2.85±0.71×10 ⁵
BCG	4.54±0.84×10 ⁷ ***	3.84±0.95×10 ⁶ ***	1.54±0.34×10 ⁵ *

Data presented as mean of 4-5 animals.

*** p<0.001;

ns = Non significant as compared to respective control.

p<0.01, ## p<0.01 as compared to 71 kDa-FIA immunized group.

●●● p<0.001 as compared to BCG immunized group.

NS - Normal Saline

LD₅₀ - Median lethal dose (3×10⁷ CFUs/mouse).

stimulation in 71 kDa-PLG immunized group at fourth (19.48 ± 3.78 , $p < 0.05$) eighth (18.68 ± 3.70 , $p < 0.05$) and sixteenth week p.im. (18.32 ± 3.29 , $p < 0.01$) as compared to 71 kDa-FIA immunized group. PMA ($1 \mu\text{g}/\text{well}$) used as positive control gave SI of 32.34 ± 4.32

The 71 kDa-PLG primed lymphocytes exhibited 30-40% higher cytokine induction as compared to 71 kDa-FIA at fourth and eighth week p.im.

The results suggest that PLG microparticles induced higher and prolonged T cell response in comparison to FIA, which were skewed towards TH1 subset, known to impart protection against tuberculosis as demonstrated previously by Vordermeir et al (4).

PROTECTIVE EFFICACY OF 71 kDa-PLG MICROPARTICLES

The protection induced by 71 kDa-FIA, 71 kDa-PLG-MPs and BCG immunization was assessed by monitoring the survival rates and colony forming units (CFUs) enumeration at 30 days post challenge (p.c. with LD_{50} of *M. tuberculosis* H₃₇Rv (3×10^7 CFUs/mouse). BCG vaccinated group was injected 1×10^6 viable *M. bovis* BCG subcutaneously.

At thirty days p.c. 71 kDa induced marginally higher percent survival (85%) as compared to 71 kDa-FIA and BCG (60-70%) when challenged at sixteenth week p.im.

The number of viable bacilli recovered from infected organs of 71 kDa-PLG-MPs and 71 kDa-FIA immunized mice exhibited significant decrease in comparison to controls as well as BCG vaccinated mice. However, the clearance of bacterial load was significantly higher in 71 kDa-PLG immunized group ($p < 0.001$) as compared to 71 kDa-FIA. (Table 1)

The highly significant and sustained protective effect of 71 kDa-PLG microparticles suggests that administration of protective antigens in appropriate adjuvant can induce a long lived protective response. The long term protective effect of 71 kDa-PLG is mediated by slow and persistent release of antigen at predetermined rates for prolonged periods, which can be achieved by choice of appropriate particle size and blending of component ratios. Previously, Anderson (5) has reported specific long term protection to soluble mixture of secretory proteins using DDA (dimethyl deoctadecyl ammonium bromide) as adjuvant at challenge 2-3 weeks p. im. However, PLG microparticles have advantage over other adjuvants like DDA and FIA due to their tissue compatibility, proven adjuvanticity when administered orally or intranasally (6) and have already been recommended for human use (6).

In conclusion, we for the first time report PLG-MPs as extremely potent delivery systems for immunoprotective mycobacterial antigens in induction of long term protective effect.

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