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Immunoprotective behaviour of liposome entrapped cell wall subunit of *Mycobacterium tuberculosis* against experimental tuberculous infection in mice

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Immunoprotective behaviour of liposome entrapped cell wall subunit of Mycobacterium tuberculosis against experimental tuberculous infection in mice. I.B. Chugh, G.K. Khuller. ©ERS Journals Ltd 1993.

ABSTRACT: We wanted to determine the immunoprotective behaviour of cell wall protein peptidoglycan complex (CW-PPC) of *Mycobacterium tuberculosis* $H_{37}Ra$, using liposomes as adjuvant, in an experimental animal model.

Immunization of mice with CW-PPC entrapped in liposomes induced both humoral response, as measured by enzyme-linked immunosorbent assay (ELISA), and cell-mediated immune responses, as seen by delayed type hypersensitivity (DTH) and leucocyte migration inhibition (LMI) techniques. Ten days after complete immunization, the animals were challenged with median lethal dose (LD_{50}) of *M. tuberculosis* $H_{37}Rv$. The animals exhibited significant protection, as evident by 72% survival after 30 days of infection, compared to 38% survival in control animals. Protective effect of immunization with liposome entrapped CW-PPC was further substantiated by significant decrease in the number of viable bacilli in lungs, liver and spleen of immunized animals, as compared to control animals.

These results indicate that immunization with liposome-entrapped mycobacterial cell wall protein peptidoglycan complex induces protection against experimental tuberculosis.

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Tuberculosis caused by *Mycobacterium tuberculosis* continues to be a major health problem, especially in the developing countries. Since the discovery of *M. tuberculosis,* intensive research has been carried out to develop a suitable antituberculous vaccine. Amongst various mycobacterial constitutents, protoplasmic antigens have been shown to confer no protection against experimental tuberculosis [1]. A number of studies have reported the protective efficacy of the mycobacterial cell wall [2], and its components, *e.g.* cord factor [3], mannosides [4], *etc.* using Freund's incomplete adjuvant (FIA).

BCG is, globally, the most widely used live vaccine against tuberculosis. However, due to controversial reports on its protective efficacy [5], attempts are being made to develop immunoprotective agents as alternatives to BCG. Most recently, attention has been focused on the proteins released by mycobacteria into their surrounding medium during the early phase of growth. These proteins, known as "secretory proteins", have been suggested as protective antigens responsible for the rapid recognition of bacilli by host lymphocytes [6]. Some of these proteins (Mr 10 KDa, 23 KDa and 30 KDa) have been reported to be part of a highly immunogenic protein peptidoglycan complex isolated from *M. tuberculosis* cell walls [7]. Moreover, these peptidoglycan-associated proteins of mycobacterial cell wall are also known to stimulate T-lymphocytes in tuberculous patients [7]. These findings suggest the potential role of cell wall protein peptidoglycan complex (CW-PPC) in inducing protective immunity against tuberculosis, using some suitable adjuvant. Although, FIA is the most commonly used adjuvant, it is not acceptable for human use, due to some associated side-effects. In recent years, phosphatidylcholine (PC) liposomes, which lack any immunological activity, have shown promising results as carriers of protein antigens [8], which is due to their natural targeting to liposomes [9]. The present investigation has, therefore, been carried out to assess the protective efficacy of CW-PPC, isolated from *M. tuberculosis* $H_{37}Ra$, against experimental tuberculosis, using PC liposomes as adjuvant.

Materials and methods

Bacterial cultures

Mycobacterium tuberculosis strains $H_{37}Ra$ and $H_{37}Rv$ were obtained from the National Collection of Type Cultures (NCTC), London, and maintained on either Lowenstein Jensen's medium or on modified Youman's medium [10].

Animals

Mice of NMRI strain, of either sex (4–5 weeks old), were obtained from the Central Research Institute, Kasauli, India. The mice were fed on a standard pellet diet, and given water *ad libitum*.

Isolation and chemical analysis of cell wall protein peptidoglycan complex of M. tuberculosis $H_{37}Ra$

CW-PPC from *M. tuberculosis* $H_{37}Ra$ was isolated, using the method of HUNTER *et al.* [11]. Briefly, cells were extracted with chloroform: methanol (2:1) mixture to remove the lipids. Delipidated cells were refluxed in 70% ethanol, to remove lipoarabinomannans and lipomannans. In order to remove residual soluble proteins, the resulting residue was further extracted with 2% sodium dodecyl sulphate (SDS) at 56°C, to yield a completely insoluble cell wall containing fraction, "cell wall core" (CWC). Subsequent selective removal of bound arabinogalactans with periodate oxidation followed by borohydride reduction, as described by HUNTER *et al.* [11], yielded the final CW-PPC which constituted 25 $mg \cdot gm^{-1}$ dry wt of cells. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of CW-PPC, carried out by the method of LAEMMLI [12], yielded a band at the junction of 7% stacking and 12.5% resolving gel. Chemical analysis of CW-PPC was carried out on acid hydrolysate. Glucosamine was quantitated as described by ROONDLE and MORGAN [13], which represented 6.9% (w/w) of CW-PPC. Diaminopimelic acid was qualitatively identified by paper chromatography [14].

Solubilization of CW-PPC

Complete solubilization of CW-PPC was achieved by direct sonication in 6 M urea solution [11]. Briefly, 5 mg of CW-PPC was sonicated in 1 ml of 6 M urea, followed by centrifugation at 15,000×g. The pellet was resonicated twice, and supernatants from all of the centrifugations were pooled. Urea from the pooled supernatants containing solubilized CW-PPC was removed by dialysis against buffer (50 mM Tris HCl, pH 7.5, 1 mM dithiothreitol, 0.2% Triton X-100 and 1 M NaCl). Protein estimation of CW-PPC was carried out, using the method of LOWRY *et al.* [15].

Preparation of liposomes

Phosphatidylcholine (PC) liposomes were prepared as described previously [16]. Briefly, a thin film of PC was dispersed in buffer containing CW-PPC (1 $mg \cdot ml^{-1}$), or without CW-PPC (empty liposomes), followed by brief sonication for 45 s at 4°C. The liposomal pellet was obtained by centrifugation at 100,000×g for 1 h. Protein in the liposomal pellet and supernatant was estimated by the method of LEES and PAXMAN [17] and percentage entrapment was calculated as follows:

$$\% \text{ entrapment} = \frac{\text{Protein in the pellet}}{\text{Protein in the pellet+ protein in supernatant}} \times 100$$

Immunization

A group of 30 animals was immunized subcutaneously on day 0 and 7, followed by an intramuscular injection on day 14. Each animal received 150 μg of liposome entrapped CW-PPC, divided into three equal doses. Control animals received equal volumes of empty liposomes.

Immune responses

At weekly intervals, 4–5 animals were exsanguinated and their spleens were removed aseptically. The sera were stored at -20°C until further use.

Assay of humoral immune responses

Enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies to CW-PPC. The optimum concentration of CW-PPC (25 $\mu g \cdot ml^{-1}$) as determined by checkerboard titration, was used for coating the microtitration plates. Goat anti-mouse immunoglobulin G (IgG) alkaline phosphatase (Sigma), diluted 1:1000 (v/v), was used as enzyme label and 4-nitrophenyl phosphate (1 $mg \cdot ml^{-1}$ in diethanolamine, pH 9.8) as substrate. Test plates were read at 405 nm using a micro-ELISA autoreader. The threshold value was calculated by adding twice the SEM to the absorbence of the control serum. All those values of immune sera which were higher than threshold value were considered to be significant.

Measures of cell-mediated immune responses

Aseptically removed spleens were used to study cellular immune response by leucocyte migration inhibition technique [18]. Briefly, the capillary tubes (length 75 mm, internal diameter 1.1 mm) were filled with the spleen cell suspension at a concentration of 5×10^7 $cells \cdot ml^{-1}$, centrifuged (200×g; 10 min) and cut at the cell fluid interphase. The capillaries were placed in the leucocyte migration chambers, in the presence and absence of optimum concentration of CW-PPC (35 $\mu g \cdot ml^{-1}$). The optimum concentration was the highest concentration that gave no significant (≤25%) inhibition of migration with leucocytes from control animals. The percentage migration inhibition was calculated by the formula: (C-T/C)×100, where C is the migration in the absence of antigen, and T is the migration in the presence of antigen.

The study of *in vivo* delayed type hypersensitivity (DTH) was performed, according to the method of COLLINS and MACKANESS [19]. Immunized and control mice were injected with CW-PPC at an optimal concentration (30 $\mu g \cdot 30\ \mu l^{-1}$ per animal) into the right hind footpad. The same volume of sterile saline was injected into

the left hind footpad, and served as control. Footpad thickness was measured with a gauge, just before injection and after 24 h. The mean net increase in foot pad thickness was recorded by subtracting the control values from the test values. All values greater than 0.18 mm were considered significant at the level of $p \leq 0.01$ [20].

Phagocytosis assay

Phagocytic activity of peritoneal macrophages was investigated as described previously [21], with some modifications. Briefly, peritoneal macrophages isolated from control and immunized mice were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), for 24 h at 37°C in an atmosphere of 5% CO_2. These macrophages were then used as effector cells, and incubated with labelled *M. smegmatis* cells (target cells) in a ratio of 1:10 for 2 h. To examine the phagocytosis of *M. smegmatis* cells, macrophages were lysed with lysing solution (0.25% sodium dodecyl sulphate) and radioactivity in lysate was counted. Results were expressed as percentage uptake of labelled *M. smegmatis* cells by macrophages.

Protection studies

To assess the protection induced on immunization with CW-PPC entrapped in liposomes, 10 days after complete immunization, the animals were challenged intravenously with 50% lethal dose (LD_{50}) of *M. tuberculosis* $H_{37}Rv$. The protection induced was evaluated by observing the survival rates and colony forming units (CFU) enumeration in infected organs, as described previously [16].

Statistical analysis

The data of CFU enumeration were evaluated using Student's t-test, and those of survival rates by Fisher's Chi-squared test.

Results

Immunoreactivity of mycobacterial CW-PPC was investigated by studying both humoral and cell-mediated immune responses to CW-PPC in animals immunized with liposome entrapped CW-PPC, at weekly intervals after immunization.

Humoral immune response

CW-PPC entrapped in liposomes (percentage entrapment=24%) induced significant humoral immune response. ELISA values, in terms of absorbance taken at 405 nm, were found to be significantly higher in immunized animals, as compared to that of control animals (fig. 1). Maximum antibody production was seen at first week postimmunization (pim), whereafter a gradual decline was seen up to the fourth week.

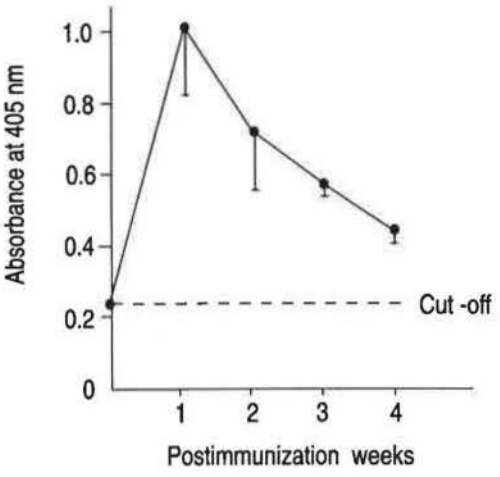

Fig. 1. – Follow-up of ELISA values of immunized animals. Dotted line represents the threshold value which is equal to value of control +2 SEM. All the values of immune sera higher than threshold value were considered significant. Y-axis represents the ELISA ±SEM values of immune sera at 405 nm, ELISA: enzyme-linked immunosorbent assay.

Cell-mediated immune responses

T-cell activation in CW-PPC immunized animals was seen by leucocyte migration inhibition (LMI) and DTH techniques. Significant inhibition in the leucocyte migration from immunized animals was seen as compared to that of control animals (fig. 2). The maximum cellular sensitization, as measured by LMI was found to be 33 (SEM 2.8)% and 45 (5.0 SEM)%, during the second and third week pim, respectively. Significantly higher DTH reaction was obtained in immunized animals as compared to control (fig. 3). Maximum DTH reaction was 0.3 (0.01 SEM) mm during the first week pim, and remained significantly higher, as compared to control, up to fourth week pim.

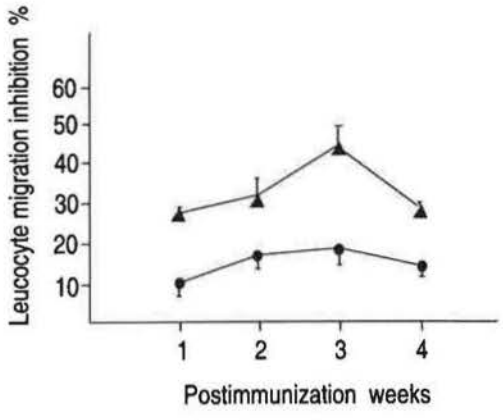

Fig. 2. – Migration inhibition of leucocytes obtained from control (●——●) and immunized (▲——▲) mice. Results expressed as mean ±SEM. All values more than 25% were taken as significant.

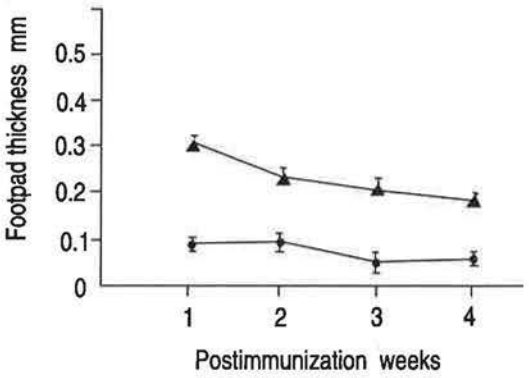

Fig. 3. – Delayed type hypersensitivity reaction in control (●—●) and immunized mice (▲—▲). Results expressed as mean ±SEM. Values more than 0.18 mm were considered significant at the level of $p \leq 0.01$.

Table 1. – Viable bacteria counts from lungs, liver and spleen of immunized and control mice after 30 days of intravenous challenge with LD_{50} dose *M. tuberculosis* $H_{37}Rv$

Group	Viable counts		
	Lung	Liver	Spleen
Control	4.3×10^8 (1.3×10^8)	1.3×10^8 (0.1×10^8)	6.7×10^6 (1.2×10^6)
Immunized	5.4×10^7 * (1.4×10^7)	2.0×10^7 ** (0.4×10^7)	1.5×10^6* (0.8×10^6)

Data are presented as mean (SEM) of 4–5 animals. *: $p<0.05$; **: $p<0.001$, significant decrease as compared to control animals. LD_{50}: median lethal dose.

Phagocytic activity

Second week pim, a significantly higher phagocytic activity of macrophages obtained from immunized animals (7.9%) was observed, as compared to that of control (3.5%), expressed in terms of percentage uptake of labelled mycobacteria by macrophages.

Protection experiments

Ten days after complete immunization, immunized/control animals were challenged with LD_{50} dose of *M. tuberculosis* $H_{37}Rv$ (2.8×10^7 CFU·mouse^{-1}), and the protection was assessed by the following parameters:

Percentage survival. Immunization with CW-PPC entrapped in liposomes resulted in increased survival (fig. 4) of immunized animals. At 30 days postinfection, percentage survival was significantly higher ($p<0.01$) in immunized animals (72%), as compared to that of control animals (38%).

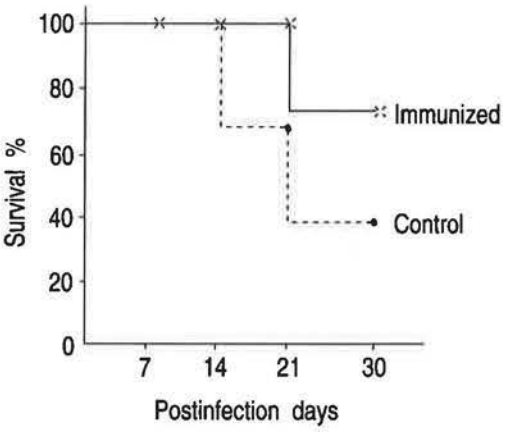

Fig. 4. – Protective efficacy of immunization with liposome entrapped CW-PPC against challenge with LD_{50} dose of *M. tuberculosis* $H_{37}Rv$ in terms of percentage survival. CW-PPC: cell wall protein peptidoglycan complex; LD_{50}: median lethal dose.

Viable counts. The number of viable bacteria recovered at 30 days post-infection from the various organs of immunized animals, as well as control animals, are shown in table 1. A significant decrease in the viable bacilli was seen in lungs ($p<0.05$), liver ($p<0.001$) and spleen ($p<0.05$) of immunized animals, as compared to control animals.

Discussion

Protective immunity to tuberculosis is thought to be mainly cell-mediated [22]. Recent studies, indicating the ability of mycobacterial cell wall associated proteins to activate T-lymphocytes from tuberculous patients, make them a suitable candidate for antituberculous vaccine [7].

We found that immunization of mice with CW-PPC encapsulated in liposomes, induced both humoral and cell-mediated immune responses, as seen by ELISA, LMI and DTH, thus indicating the activation of B- as well as T-cells by CW-PPC. These immune responses declined by the fourth week pim, possibly due to sequestration of specific antigen by activated macrophages, leading to a gradual dilution of the antigen in circulation. Furthermore, an increased phagocytic activity of macrophages was observed in immunized animals, as compared to control animals, which can be attributed to increased T-cell activity in the former. The augmentation of T- and B-cell mediated immune responses by liposomal entrapped CW-PPC is in accordance with earlier studies, in which liposomes have been used as adjuvant for mycobacterial antigens [16], membrane antigens of *Leishmania* sp. [23], and *Entamoeba histolytica* [24]. The adjuvant role of liposomes was further supported by the observation that animals immunized with CW-PPC alone (*i.e.* without liposomes) did not exhibit any DTH response (unpublished data).

As elevated cell-mediated immune responses were obtained during the first and second weeks pim with CW-PPC, its protective effect was studied by challenging the animals with LD_{50} dose of *M. tuberculosis* $H_{37}Rv$, 10 days after complete immunization. After 30 days of

infection, significantly higher percentage survival ($p<0.01$) was observed in immunized animals, as compared to control. Our findings are comparable to those reported by PANCHOLI *et al.* [16], who found increased survival rates in animals immunized with liposome entrapped mycobacterial ribonucleic acid protein (RNA-P). Similarly, in a number of other parasitic infections, the protective effect of liposome entrapped antigens has been reported, on the basis of increased survival rates in immunized animals [23, 25]. Protective efficacy of CW-PPC was further supported by significantly reduced levels of viable tubercle bacilli in infected organs (table 1) of the immunized animals, as compared to control animals, which indicated a retardation in the multiplication rate of tubercle bacilli in immunized animals.

In the present study, the reduction in the number of viable bacteria was of almost equal extent in all three organs, *i.e.* lungs, liver and spleen. Reduced bacterial load in the lungs, as observed in the present investigation, offers an advantage over the previously reported protective efficacy of mycobacterial RNA-P, which resulted in a decreased number of viable bacteria in liver and spleen and not in lungs [16]. Our findings seem to be of greater significance in relation to pulmonary tuberculosis, the most common form of tuberculosis which involves lung macrophages as the effector cells.

In conclusion, the present study suggests that the mycobacterial cell wall, progressively depleted of lipids, soluble proteins, mycolic acids and arabinogalactans, and only consisting of proteins associated with peptidoglycan, *i.e.* CW-PPC, is able to induce protective immunity when encapsulated in liposomes.

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