IMMUNISATION AND IMMUNOTHERAPY

Adjuvant modulation of T-cell reactivity to 30-kDa secretory protein of *Mycobacterium tuberculosis* H₃₇Rv and its protective efficacy against experimental tuberculosis

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The immunoprotective behaviour of the 30-kDa secretory glycoprotein of *Mycobacterium tuberculosis* H₃₇Rv has been investigated in different adjuvant systems in two mouse strains, NMR1 and C₅₇BL/6J. In comparison with Freund's incomplete adjuvant (FIA) and dimethyldioctadecyl ammonium bromide (DDA), the 30-kDa glycoprotein complexed with polylactide-co-glycolide microparticles (PLG-MPs) induced maximum immunoreactivity in the two mouse strains. As compared with controls, immunisation with 30-kDa-PLG-MPs resulted in significantly greater protection in animals challenged with $1 \times \text{LD}_{50}$ of *M. tuberculosis* H₃₇Rv on the basis of survival rates and number of cfu in the infected organs 30 days after challenge. The degree of protection provided by 30-kDa-PLG-MPs was similar to that obtained with 30-kDa-FIA and higher than BCG immunisation. These findings suggest that biodegradable PLG microparticles can be used as an efficient carrier system for the key immunoprotective 30-kDa secretory protein antigen of *M. tuberculosis* H₃₇Rv.

Introduction

The recent increase in the number of tuberculosis cases worldwide has evoked a strong interest in the identification of new mycobacterial antigens as vaccination candidates, particularly as vaccination with *Mycobacterium bovis* BCG has been shown to have variable efficacy (0–80%) [1–3]. The characterisation of new antigens may identify candidates for improved vaccines, particularly in the context of current investigations into subunit and DNA vaccines to protect against tuberculosis. The fact that only live organisms provide protection in animal models has put emphasis on secreted protein antigens [4–6]. These secretory proteins are released into mycobacterial culture medium during the early log phase of growth and are collectively known as culture-filtrate proteins [7]. These proteins are reported to be major targets of T cells in mice and human patients infected with mycobacteria [8, 9]. Hence, these proteins have been found to be potentially important in inducing protective immunity

against tuberculosis by expanding the appropriate arm of the T-cell response at an early stage of infection [9–12]. In recent years several reports have indicated the role of culture-filtrate proteins and purified secretory proteins used alone or in combination with suitable carrier systems in inducing protection against tuberculosis [7, 10, 13, 14]. Recently, a 30-kDa secretory protein derived from culture filtrates of *M. tuberculosis* in log phase growth has been reported to induce protective immunity in mice against challenge with *M. tuberculosis* H₃₇Rv with Freund's incomplete adjuvant [15]. However, no studies have been conducted into the development of an effective antituberculous vaccine with a suitable biodegradable adjuvant formulation which may impart better immunoreactivity and protective efficacy. In recent years, the delivery of antigens in polymeric materials such as polylactide-co-glycolide (PLG microparticles) which are biodegradable and biocompatible, has attracted considerable attention [16–18]. Furthermore, the release of antigen can be controlled in these carrier systems [16]. Therefore, the immunoreactivity and protective efficacy of a 30-kDa secretory protein antigen of *M. tuberculosis* H₃₇Rv entrapped in PLG microspheres has been investigated in comparison with other adjuvant systems in two different strains of mouse.

Received 11 June 1998; revised version accepted 24 Nov. 1998.

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Materials and methods

Animals

Two strains of mouse (NMRI, C₅₇BL/6J) both male and female) weighing 15–20 g were obtained from CRI, Kasauli (H.P.) and IMTECH, Chandigarh, India. The animals were kept in appropriate animal facilities and given mouse chow and water *ad libitum*.

Mycobacterial culture

M. tuberculosis H₃₇Rv (originally obtained from the National Collection of Type Cultures, 61 Colindale Avenue, London) was maintained on Lowenstein-Jensen medium or in modified Youmans's agar medium [19]. It was subcultured in liquid Youmans's medium and grown at 37°C on an orbital shaker for 8 days to produce culture filtrate protein.

Culture-filtrate protein

M. tuberculosis H₃₇Rv was cultured in modified Youmans's medium (as above) and cells were removed by centrifugation at 16 270 *g* for 20 min at 4°C. The supernates were filter-sterilised with 0.45- μ m pore size membrane filters and concentrated 100 times by ultrafiltration on an Amicon PM 10 membrane (Amicon, Danvers, MA, USA). The concentrated proteins were de-salted by extensive washings in the ultrafiltration chamber with distilled water and were designated as culture-filtrate proteins. The protein content in the culture filtrate was determined by the modified Lowry's method [20].

Purification of 30-kDa protein

The 30-kDa protein of *M. tuberculosis* H₃₇Rv was separated from other culture-filtrate proteins by the method of Sinha *et al.* [15]. Briefly, the sample was passed through a DEAE-Sepharose CL-6B (Sigma) anion-exchange column equilibrated with 30 mM Tris-Cl buffer (pH 8.7) containing methyl cellosolve 3%. The protein was eluted with a 50–300 mM NaCl gradient. The protein content in each fraction was determined by measuring the absorbance at 280 nm. The pooled fractions were analysed by SDS-PAGE to determine the mol. wt. The 30-kDa protein was purified further with a DEAE-sepharose CL-6B and sephacryl S-200 HR or phenyl sepharose CL-4B column, or both, and the corresponding pooled fractions were analysed by SDS-PAGE by separation on a 12% gel under denaturing conditions. The gel was stained with silver nitrate to determine the apparent mol. wt of the protein. The purified protein was stored at –20°C until used.

Characterisation of the protein

The carbohydrate content in the protein was determined by the phenol sulphuric acid assay as described by Chaplin *et al.* [21]. N-terminal amino acid sequencing

was performed with a gas phase sequencer Model 476A; Applied Biosystems, ABI-PE) following transfer on to PVDF membranes at the Institute of Microbial Technology, Chandigarh, India.

Immunoreactivity of 30-kDa protein

Adjuvants. The following adjuvants were used: Freund's incomplete adjuvant (FIA; Sigma), poly(lactide-co-glycolide) microparticles (PLG-MPs; Boehringer Ingelheim, Germany); and dimethyldioctadecylammoniumbromide (DDA; Sigma). The PLG formulation was prepared by dissolving co-polymer of PLG in dichloromethane 1:1 w:w. The primary emulsion was made with the antigen, which was added at a ratio of 1:1 by sonicating the material for short intervals, suspended in polyvinyl alcohol (88% hydrolysed) and stirred overnight to form a secondary emulsion, centrifuged and the pellet was dissolved in phosphate-buffered saline (PBS). Protein entrapment was checked by the method of Lees and Paxman [22].

The DDA formulation was made by the method of Lindblad *et al.* [16]. DDA was dissolved in PBS and heated at 80°C until it dissolved. The concentration of DDA taken was 10-fold the concentration of antigen used for inoculating the animals.

Immunisation of animals. Groups of 20 or 25 inbred NMRI and C₅₇BL/6J mice were immunised subcutaneously with the 30-kDa protein antigen of *M. tuberculosis* H₃₇Rv in FIA, PLG-MPs or DDA. The animals were given three equal doses of 25 μ g at weekly intervals, to study both the immune responses and protection. Control animals were inoculated with saline in adjuvant only.

Immune response. Each week three animals were killed and their spleens were removed aseptically. The T-cell responses were evaluated by a T-cell proliferation assay [9] with spleen cells from immunised and control mice. The lymphocyte count was adjusted to a final concentration of 2×10^5 cells/ml in RPMI 1640 medium containing penicillin 100 IU/ml, streptomycin 100 U/ml, fetal calf serum 10% and 5×10^{-5} M 2-mercaptoethanol and incubated with or without antigen (100 μ l/well: 6 μ g/ml) at 37°C for 4 days in an atmosphere of CO₂ 5%, air 95% and 100% humidity. Cell proliferation was observed by pulsing the culture with 0.5 μ Ci of [³H]-thymidine (BARC, India) and incubated for a further 18 h. Cells were harvested on glass fibre filters and the radioactivity incorporated was counted in a liquid scintillation counter. The results were expressed as a stimulation index (SI), i.e., cpm of test/cpm of control.

Protective immunity against virulent challenge

Immunised and control animals were challenged intravenously with 1 $\times$ LD₅₀ (3×10^7 cfu/animal) of *M.*

tuberculosis H₃₇Rv 8 weeks after immunisation. Challenged animals were observed continuously for 30 days for mortality, and percentage survival in each group was determined as an index of protective immunity. The numbers of bacteria in the organs (lungs, liver, spleen) of mice, 30 days after infection, were determined by plating serial 10-fold dilutions of organ homogenate on to modified Youman's agar supplemented with BSA 1% w/v. Colonies were counted after 5–6 weeks and were expressed as mean counts from three animals in each group.

Statistical analysis

The lymphocyte proliferation and cfu data were evaluated by Student's *t* test. The protective efficacy was compared by the χ^2 test.

Results

Purification of 30-kDa secretory protein

Combinations of columns were used to isolate the 30-kDa secretory protein and purify it to homogeneity. The purified protein yielded a single band when analysed by SDS-PAGE under denaturing conditions and had a mol. wt of 30 kDa (Fig. 1). The carbohydrate content of the protein was 18%. The tentative N-terminal amino acid sequence of the first 15 residues was (A?) PKDNTLYLGATKLL.

Comparative immunoreactivity in the two strains of mice immunised with 30-kDa secretory protein entrapped in different adjuvant systems

The immunoreactivity of the 30-kDa protein was evaluated in PLG-MPs and DDA, and compared with that obtained in FIA. Both NMRI and C₅₇ BL/6J mice exhibited an increasing proliferation response from the first to the third week after immunisation with 30-kDa-FIA and up to the fifth week after immunisation with 30-kDa-PLG and 30-kDa-DDA. As compared with the group immunised with 30-kDa-FIA, peak responses obtained with 30-kDa-PLG were significantly higher ($p < 0.01$) and sustained until the eighth week after immunisation, although the response obtained with 30-kDa-DDA-immunised groups was significantly less (Fig. 2a, b).

Protective efficacy of 30-kDa secretory protein

In the initial experiments, groups of NMRI mice were immunised with different concentrations (18.75, 37.5 or 75 μ g) of 30-kDa-FIA. A dose-dependent protection was observed against the i.v. challenge with $1 \times \text{LD}_{50}$ of *M. tuberculosis* H₃₇Rv 8 weeks after immunisation, with maximum protection at 75 μ g of the 30-kDa protein (Fig. 3). Hence, this concentration of the antigen was used for further experiments. Later, the protective efficacy of the 30-kDa-FIA complex, 30-kDa-PLG microparticles and BCG vaccination against

Fig. 1. SDS-PAGE analysis (12%) of 30-kDa (purified) protein. Lane A, marker (29–205 kDa); B, purified 30-kDa protein.

experimental tuberculosis was compared by challenging the animals with $1 \times \text{LD}_{50}$ of *M. tuberculosis* H₃₇Rv 8 weeks after immunisation. The animals were observed for mortality up to 30 days after challenge. The percentage survival observed was 83.3% in animals immunised with 30-kDa-PLG, which was better than that for animals treated with 30-kDa-FIA (80%, statistically non-significant) and BCG-vaccinated animals (73.0%, $p < 0.01$) (Fig. 4). Furthermore, at the end of the observation period (4 weeks), cfu counts in infected lungs, liver and spleen of animals immunised with 30-kDa-PLG were significantly lower ($p < 0.001$) in the lungs and spleen than in control animals. The decrease in cfu in the infected organs of immunised animals was 50-fold less when compared with saline-treated controls, indicating significant clearance of bacilli (Table 1). The clearance of bacilli in mice immunised with 30-kDa-PLG was more than that in BCG-vaccinated mice (statistically non-significant).

Discussion

Mycobacterial secretory proteins contain the immuno-protective epitopes that are recognised by T lymphocytes at an early stage of infection [6, 10, 13]. Recently, the 30-kDa secretory protein of *M. tuberculosis* H₃₇Ra has been reported to be highly protective against experimental tuberculosis with FIA as an adjuvant [15]. However, until now there has been no report of the effects of different adjuvants on the efficacy of the 30-kDa secretory protein of a virulent strain, i.e., *M. tuberculosis* H₃₇Rv. In the present study,

Fig. 2. Comparative T-cell proliferative response to 30-kDa antigen of *M. tuberculosis* H₃₇Rv in mice immunised with 30 kDa-PLG/DDA/FIA: **a**, NMRI mice; **b**, C₅₇BL/BJ mice. The results are presented as stimulation index, cpm test/cpm control. Mice (in groups of 20 each) were immunised and three animals were exsanguinated and their spleens removed 1, 3, 5 and 8 weeks after immunisation for T-cell proliferation assays. ▨, control PLG; ▩, PLG; ▤, control DDA; ▥, DDA; ▦, control FIA; ▧, FIA.

a series of well-characterised adjuvant systems were used to stimulate the immune response to purified 30-kDa secretory protein of *M. tuberculosis* H₃₇Rv in two mouse strains. The protein was isolated from mid-log

phase of *M. tuberculosis* H₃₇Rv cultures when there was minimum autolysis of the cells, as reported earlier on the basis of isocitrate dehydrogenase (ICD) activity in the culture media [15]. The N-terminal amino acid

Fig. 3. Percentage survival of NMRI mice immunised (in groups of 25 each) with 30 kDa-FIA in three different doses (▲, 18.75 µg; ◆, 37.5 µg; ●, 75.0 µg; □, control) and challenged 8 weeks after immunisation with the median lethal dose (LD50; 3×10^7 cfu/mouse) of *M. tuberculosis* H₃₇Rv.

Fig. 4. Percent survival of NMRI mice immunised (in groups of 25 each) with 30 kDa-PLG/FIA/BCG and challenged 8 weeks after immunisation with the median lethal dose (LD50, 3×10^7 cfu/mouse) of *M. tuberculosis* H₃₇Rv (□, control; ■, BCG; ▲, FIA; ●, PLG).

sequence (first 15 residues) of the 30-kDa protein does not have any N-terminal sequence homology with previously cloned and sequenced secretory proteins of the antigen 85 (Ag85) complex from *M. tuberculosis* H₃₇Rv and *M. bovis* BCG (MPT 59, MPT 44, MPT 45), suggesting that the isolated protein is different

from the Ag85 complex, a major secretory protein of mycobacteria [10, 13, 23, 24]. Being a glycoprotein, its chemical nature also differs from the non-glycosylated proteins of Ag85 complex. Animals immunised with 30-kDa-PLG showed significantly greater T-cell proliferation than with other adjuvant systems (30-kDa-

Table 1. Viable *M. tuberculosis* H₃₇Rv obtained from immunised (30-kDa-PLG, BCG-vaccinated) and control NMRI mice challenged 8 weeks after immunisation with 1 × LD₅₀ of *M. tuberculosis* H₃₇Rv

Vaccination group	Mean (SD) cfu recovered from		
	Lung	Spleen	Liver
30-kDa-PLG	3.19 (0.22) × 10 ⁷ *	1.14 (1.05) × 10 ⁵ *	8.73 (0.51) × 10 ⁶ †
BCG-vaccinated	3.9 (0.17) × 10 ⁷ †	2.6 (0.3) × 10 ⁶ †	3.07 (0.89) × 10 ⁶ †
Control	1.38 (1.05) × 10 ⁹	1.82 (0.39) × 10 ⁶	2.1 (1.57) × 10 ⁷

Results are presented as means (SD) of three animals.

*p < 0.001 compared with control.

†p < 0.01 compared with control.

FIA and 30-kDa-DDA) and the immunoreactivity was even better than that seen with BCG vaccination, as reported earlier [25]. Thus, the finding of the present study is consistent with earlier reports regarding the use of PLG-MPs for the development of an antituberculosis vaccine [26]. PLG microparticles have also been used successfully for the delivery of tetanus toxoid and staphylococcal enterotoxin B toxoid [26, 27]. Because of their biocompatibility and controlled release of antigen, PLG microparticles are considered ideal antigen delivery systems for future vaccine development [26]. Furthermore, in two different mouse strains with H₂^d and H₂^b as their major histocompatibility complex (MHC) haplotypes, comparable immunoreactivity of 30-kDa-PLG was obtained irrespective of genetic variations. Therefore, the protective effect of immunisation with 30-kDa-PLG was studied only in the NMRI-strain (H₂^d). A high level of protective immunity against a sublethal challenge of *M. tuberculosis* H₃₇Rv induced by immunisation with 30-kDa-PLG, was similar to that produced by 30-kDa-FIA and BCG immunisation.

To further substantiate the protective effect of 30-kDa-PLG, cfu counts indicated a significantly greater clearance of bacterial load (p < 0.001) from the infected lungs and spleen than with controls. These findings are in agreement with earlier reports, in which the protective effects of secretory proteins have been demonstrated in both mice and guinea-pigs [3–5, 8]. It has also been reported that culture-filtrate proteins of *M. tuberculosis* H₃₇Rv with mol. wts of 6–12 kDa and 28–32 kDa correspond to maximum reactivity, which further substantiates the findings in the present study. [28]. Furthermore, the protection provided by the 30-kDa secretory protein of *M. tuberculosis* H₃₇Rv entrapped in PLG microparticles (Fig. 4) was better than the protective efficacy of BCG reported earlier [25], which could be due to suppression of T-cell activation by non-protein antigens present in BCG. Thus, the results of this study suggest that the 30-kDa secretory protein might be a key immunoprotective antigen for subunit vaccine development with PLG microparticle-based carrier systems.

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