Immunogenicity of ribonucleic acid-protein fraction of *Mycobacterium tuberculosis* encapsulated in liposomes

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Summary. Immunologically potent RNA-protein extracted from *Mycobacterium tuberculosis* strain H37Ra, when entrapped in phosphatidylcholine multilamellar liposomes and injected into mice, induced both cellular and humoral immune responses. Significant protection against infection with *M. tuberculosis* H37Rv was also induced in the immunised mice, as monitored by (i) higher survival rates, (ii) decreased viable counts of *M. tuberculosis* H37Rv in lungs, livers and spleens, (iii) lower lung density, and (iv) lower root specific lung weight, in comparison with a control group of unimmunised mice.

Introduction

Killed mycobacteria or their cell constituents, such as ribonucleic acid-protein fractions or ribosomal preparations, can induce immunity against experimental tuberculosis when administered with Freund's incomplete adjuvant (FIA) (for review see Youmans, 1979). However, because of the formation of focal granuloma and other related side effects associated with the use of Freund's adjuvant (Freund, 1956), efforts have been made to find alternative antigen carriers (Youmans and Youmans, 1967, 1971). Attempts to substitute FIA with various polybasic amines and several emulsified and non-emulsified adjuvants were unsuccessful (Youmans and Youmans, 1972). In recent years, phosphatidylcholine (PC) multilamellar liposomes, which lack any immunogenic activity of their own, have shown promising results as carriers of protein antigens (Alving, 1987) and of anti-tubercular and other antimicrobial drugs (Orozco et al., 1986; Lopez-Berestein, 1987). However, the use of liposomes as carriers for mycobacterial RNA-protein fraction (Myc RNA-P) has not been examined. Therefore, we have attempted to replace FIA with PC liposomes and to study the immune responses and host protection against experimental tuberculosis induced by such liposome-entrapped Myc RNA-P in mice.

Materials and methods

Bacterial cultures

*Mycobacterium tuberculosis* strains H37Ra and H37Rv were obtained from the National Collection of Type Cultures (NCTC), Colindale Avenue, London and maintained on either Lowenstein Jensen's medium or on modified Youmans's medium (Khuller et al., 1984).

Mice

Mice of the 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 (Hindustan Lever Ltd, Bombay, India) and given water ad libitum.

Isolation of mycobacterial RNA-protein fraction

Myc RNA-P fraction was extracted from *M. tuberculosis* H37Ra cells with phenol. Briefly, mycobacterial cells suspended (2 g/ml) in buffer containing 0.1 M Tris-HCl, 10 mM Na₂EDTA (pH 7-4) were disrupted by intermittent sonication in a 'Sonifier' cell disruptor (Branson Sonic Power Co.) for 1 h in ice. Immediately after sonication, sodium dodecyl sulphate 10% w/v was added, to a final concentration of 0-5% in the cell suspension, which was then centrifuged at 6000 g for 10 min at 4°C, in a refrigerated centrifuge (Sorvall RC-20). The supernate was then processed further for RNA extraction as described by Sripati and Warner (1978) with a mixture of phenol:chloroform:isoamyl alcohol (50:48:2, v:v:v) as the deproteinising agent. DNA from isolated Myc RNA-P fraction was removed by centrifugation at 2000 g after drop-by-drop addition of isopropyl...
alcohol (Marmur, 1961). The supernate was then flash rotary vacuum evaporated at 37°C to remove iso-propyl alcohol. All Myc RNA-P fraction extraction buffers and glassware were made free of RNAase by autoclaving and the addition of diethyl pyrocarbonate 0·1% v/v.

Chemical analysis and characterisation of Myc RNA-P

RNA was quantitated by direct ultraviolet absorption at 260 nm, and protein and DNA contents were estimated by the methods of Lowry et al., (1951) and Burton (1956) respectively. Spectral studies from 220 nm to 320 nm of native (untreated) Myc RNA and of Myc RNA after RNAase treatment (Hotchkiss, 1957) were performed to determine the hyperchromic increase associated with the isolated Myc RNA. The percentage hyperchromic increase was calculated from the formula:

\[
\text{OD increment of Myc RNA after RNAase treatment} \times 100
\]

\[
\frac{\text{OD}_{260} \text{ of native Myc RNA} - \text{OD}_{260} \text{ of native Myc RNA after RNAase addition}}{\text{OD}_{260} \text{ of native Myc RNA}} \times 100
\]

Preparation of liposomes

Multilamellar phosphatidylcholine liposomes (PCML) were prepared by the method of Van Rooijen and Van Nieuwmeegen (1980) as modified by Vinayak and Sharma (1986). Briefly, 150 mg of phosphatidylcholine (Council of Scientific and Industrial Research Centre for Biochemicals, New Delhi, India) was dissolved in 25 ml of chloroform and evaporated in a round-bottom flask by rotary vacuum evaporation at 37°C. The thin film on the walls of the flask was dispersed by gentle shaking for 10 min in 3·3 mm phosphate-buffered saline, pH 7·2 (PBS 7·2) containing 15–20 mg of Myc RNA-P in 20 ml of PBS 7·2 (for empty liposomes, PBS buffer alone was added instead of antigen). The suspension was kept at room temperature for 2 h and was then subjected to ultrasonic disintegration (MSE Scientific Instruments, Crawley, Sussex), at 23 kHz for 45 s in ice. The liposomes formed thereby were centrifuged at 100 000 g at 4°C and washed three times in PBS 7·2. The multilamellar character of the liposomes was confirmed by phase contrast microscopy. The amount (percentage) of antigen-entrapped in the liposomes was calculated as:

\[
\text{Amount of RNA-P present in supernates and washings of liposomal pellet} \times 100
\]

\[
\frac{\text{Total amount of RNA-P originally added}}{\text{Amount of RNA-P present in supernates and washings of liposomal pellet}} \times 100
\]

Immunisation protocol

Mice were divided into two groups of 35 animals each. Group I was immunised intramuscularly with a total of 150 µg of Myc RNA-P entrapped in 0·3 ml of PCML, in three equal doses of 50 µg each on days 0, 5 and 10. Group II received 0·3 ml of empty PCML on each of the days and served as a control group.

Measures of humoral immune responses

Enzyme-linked immunosorbent assay (ELISA) was employed for detection of anti-RNA-P antibodies. For ELISA, Myc RNA-P at an optimal concentration of 100 µg/ml (as determined by checker-board titrations) was used for coating microtitration plates (Dynatech Laboratories, Alexandria, VA, USA). Goat antimouse IgG coupled to alkaline phosphatase (Sigma), diluted 1 in 1000 (v/v), was used as enzyme label and p-nitrophosphosphate 1 mg/ml (in diethanol amine buffer pH 9·8) as substrate. Test plates were read at 405 nm on a Titertek Multiskan Micro-ELISA reader and results were expressed as log, serum antibody titres.

The plaque-forming cell (PFC) technique of Jerne et al. (1974) was employed to study the number of actively antibody producing cells in vitro. Briefly, 5–6 mice (control and immunised groups) were inoculated intraperitoneally with 10⁸ sheep red blood cells (SRBC) in 0·5 ml of 0·1 M PBS, pH 7·2. Four days later, the mice were bled to death and the spleen cells taken for primary PFC assay. Direct (IgM) haemolytic PFC were determined by plating 10⁶ spleenocytes mixed with 0·1 ml of 20%, SRBC in 2 ml of Bacto-Agar (0·7% containing 0·1 ml of DEAE-dextran at a concentration of 10 mg/ml) on petri plates containing 12 ml of solidified Bacto-Agar (1–4% v/w in PBS, pH 7·2). Plates were incubated at 37°C for 2 h after which they were flooded with fresh guinea-pig serum (diluted 1 in 10, v/v as a source of complement). The plaques thus formed after incubation at 37°C for 1 h were counted macroscopically against a light and expressed as the number of IgM haemolytic antibody titres against SRBC (which measures the overall increase in IgG and IgM serum antibody titres).

Measures of cell-mediated immune responses (CMI)

Cell-mediated immune (CMI) responses were studied by the in-vitro leucocyte migration inhibition (LMI) test (Falk and Zabriskie, 1971) in the presence of specific Myc RNA-P antigen (175 µg/ml) and other non-specific antigens, viz. yeast RNA (y RNA—50 µg/ml) and mycobacterial crude protein extract (CPE—25 µg/ml). These antigen concentrations were the highest that gave no significant (i.e., ≤ 25%) inhibition of migration with leucocytes from unimmunised animals. The study of in-vivo delayed-type hypersensitive (DTH) response was performed according to the method of Collins and Mackaness (1970). This entailed injection of Myc RNA-P (100–200 µg/0-03 ml) or CPE (20–50 µg/0-03 ml) test antigens intradermally into the right hind footpads of a group of 5–6 immunised or control mice, while an equal volume of sterile normal saline (0-85%) was injected into the left hind footpads of these mice as controls. Footpad thickness was measured with Schnelltaster dial-gauze.
callipers (Syiten Kroplin, West Germany) at 0, 24, 48 and 72 h after injection. Saline-control values were subtracted from the test-antigen values and the differences in both control and immunised groups evaluated statistically.

**Determination of LD50 value for M. tuberculosis H37Rv**

*M. tuberculosis* H37Rv cells, grown as shake cultures in Youmans's medium containing Tween-80 (0.05%, v/v) were harvested by centrifugation at 1000 g for 10 min. The cell pellet was washed after resuspending twice in sterile saline and again suspended in the saline at a concentration of 10⁹ cfu/ml. Ten-fold serial dilutions of the suspension were made in sterile saline. Groups of 8–11 mice were inoculated intravenously with 0.1 ml of each dilution. Mortality and morbidity were then followed up to a period of 30 days post-challenge, at the end of which the LD50 dose was calculated (Karber, 1931). The reproducibility of the experiment was checked by repeating it four times, each time with a different cell population and results were expressed as colony-forming units (cfu) per mouse.

**Protection studies**

To assess the protection induced by immunisation with liposome-entrapped Myc RNA-P, separate batches (groups I and II), comprising 80–85 animals each, were immunised as described in the previous section under *Immunisation protocol*. Control mice similarly received an equal volume (0.3 ml) of empty liposome preparation. One week after complete immunisation, all animals were challenged with an LD50 dose of *M. tuberculosis* H37Rv. The protection offered by the immunisation was evaluated by the following parameters.

**Percentage mortality and survival.** Survival rates (alive vs total) in the immunised as well as control animals were noted for a period of 30 days after challenge.

**Lung density determination.** The lung density (specific gravity) of control and immunised animals challenged with *M. tuberculosis* H37Rv was determined at varying post-challenge periods on the basis of the tendency of the excised lung either to sink or to float in standard test baths (varying in densities from 0-68 to 1-0), prepared by mixing various organic solvents as described by Crowle (1958)—an increase in lung density values depicted an enhanced rate of tuberculous infection in intravenously infected mice.

**Evaluation of “root specific lung weight” (RSLW).** RSLW of control and immunised animals challenged with *M. tuberculosis* H37Rv was determined at varying periods after challenge, as detailed by Orozco et al. (1986), and was calculated as follows:

\[
\text{RSLW} = \frac{\text{Lung weight (mg)}}{\sqrt{\text{Body weight (g)}}} \times 10
\]

**Statistical**

Mortality and survival rate data were evaluated by Fisher’s *χ²* test and other parameters (immune responses and protection studies) were evaluated by Student’s *t* test.

**Results**

Chemical analysis of the isolated Myc RNA-P fraction revealed a RNA:protein ratio of 7:2:1 and an RNA:DNA ratio of 68:1. A hyperchromic increase of 30% was observed when isolated Myc RNA-P was treated with RNAase, indicating that the isolated Myc RNA-P fraction was native.

**Immune responses**

Myc RNA-P entrapped in PCML (percentage entrapment was 21–25%) induced significant humoral (figure) as well as cell-mediated immune (table I) responses. Elevated humoral immune responses were shown by increased serum ELISA antibody titres to Myc RNA-P, anti-SRBC agglutination titres (which measures the overall increase of IgG and IgM response), and by enhanced PFC response (which measures the IgM direct haemolytic plaque-forming cell response), in mice immunised with liposomally-entrapped RNA-P (lip-RNA-P) (figure).

**CMI responses**

As shown in table I, maximum cellular sensitisation in animals immunised with lip-RNA-P was observed by LMI in the first week after complete immunisation with Myc RNA-P as the sensitising antigen. The percentage (%) LMI of immunised animals with Myc RNA-P antigen was significantly higher (p < 0.01) than the mean value for the control group over the different time periods, i.e., from 1–4 weeks after immunisation. Yeast RNA (yRNA) as sensitising antigen did not reveal any significant (p > 0.05) cellular sensitisation of immune leucocytes in mice immunised with lip-RNA-P during different periods as compared to LMI (%) observed.
Table I. Effects of immunisation with liposome-entrapped Myc RNA-P (lip-RNA-P) on cellular immune responses to various antigens in mice, measured by the LMI test

<table>
<thead>
<tr>
<th>Week after immunisation</th>
<th>Control (empty liposome-immunised)</th>
<th>Lip-RNA-P immunised</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myc RNA-P (yRNA)</td>
<td>CPE</td>
</tr>
<tr>
<td></td>
<td>20.54 (3.67)</td>
<td>55.49 (4.76)</td>
</tr>
<tr>
<td>1</td>
<td>20.75 (2.90)</td>
<td>21.23 (3.58)</td>
</tr>
<tr>
<td>2</td>
<td>28.96 (0.77)</td>
<td>21-68 (1.32)</td>
</tr>
<tr>
<td>3</td>
<td>20.88 (3.20)</td>
<td>27.91 (3.24)</td>
</tr>
<tr>
<td>4</td>
<td>24.21 (3.45)</td>
<td>28.0 (2.50)</td>
</tr>
<tr>
<td></td>
<td>15.0 (1.32)</td>
<td>20.47 (4.39)</td>
</tr>
</tbody>
</table>

yRNA = Yeast RNA; CPE = crude mycobacterial protein extract.
* Five mice were tested in each set.

with control leucocytes with the same antigen. The LMI (%) observed with leucocytes from immunised mice with CPE antigen did not differ significantly (p > 0.05) from the values observed in control mice, except at the third week after immunisation, when the mean LMI of animals immunised with lip-RNA-P revealed significantly higher (p < 0.01) LMI of 28.0% (SE 2.50) compared with LMI of control animals of 15.0% (SE 1.32).

DTH monitored during varying periods after immunisation revealed no significant DTH reaction (p > 0.05) to test antigens, Myc RNA and CPE in mice immunised with lip-RNA-P compared with control (empty-liposome) mice (table I). Only results obtained during the first week after immunisation are presented in this table, because maximum cellular sensitisation by LMI index was observed during this period.

Protection experiments

The mean LD50 value of M. tuberculosis H37Rv obtained was 3.2 × 10^7 cfu/mouse. The mice immunised with lip-RNA-P were challenged with this dose of strain H37Rv one week after immunisation, when maximum immune responses (as checked by ELISA and LMI assays before challenge) were observed (figure; table I).

Percentage survival. Survival rates of 75–80% were observed in mice immunised with lip-RNA-P, compared with a 40% rate observed in control mice (immunised with empty-liposome) (table III), representing nearly a two-fold increase for the former group.

Lung density and RSLW value. In control groups, lung densities and RSLW values increased markedly with the duration of infection, as recorded between day 7 and day 30 after challenge, whereas in the mice immunised with lip-RNA-P, the increase in each was significantly lower (p < 0.05–<0.01) than for the control mice during this period (table IV).

Enumeration of bacterial load in infected organs. Viable counts of lungs, livers and spleens from control mice (immunised with empty liposomes) revealed an increasing bacterial load between 7 and 30 days after challenge (table V). However, livers of mice immunised with lip-RNA-P showed significantly (56-fold) lower (p < 0.001) bacterial counts 30 days after challenge, as compared with those of the control group. Similarly, spleens of the test group animals had 36-fold lower (p < 0.01) bacterial counts than those of control groups at 30 days after challenge.

Table II. Effect of immunisation with liposomal-entrapped Myc RNA-P (lip-RNA-P) on delayed-type hypersensitivity responses in mice, 24 h after foot-pad challenge with mycobacterial antigens

<table>
<thead>
<tr>
<th>Group†</th>
<th>Antigen employed (amount injected)</th>
<th>Foot pad thickness‡ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (empty liposome-immunised)</td>
<td>Myc RNA (200 μg)</td>
<td>0.06 (0.03)</td>
</tr>
<tr>
<td></td>
<td>CPE (20 μg)</td>
<td>0.08 (0.04)</td>
</tr>
<tr>
<td>Lip-RNA-immunised</td>
<td>Myc RNA (200 μg)</td>
<td>0.10 (0.005)*</td>
</tr>
<tr>
<td></td>
<td>CPE (20 μg)</td>
<td>0.11 (0.006)*</td>
</tr>
</tbody>
</table>

† A group of five animals was used for testing each different antigen.
‡ Values are expressed as mean increase in foot-pad thickness after subtracting the saline-control values. Values in parentheses represent standard errors for each set of five mice.
* p > 0.05 compared with control (empty liposome-immunised) group.
Table III. Protective effects of immunisation of mice with liposomal-entrapped Myc RNA-P (lip-RNA-P), measured by survival rate after challenge with *M. tuberculosis* H37Rv (LD50)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Days after challenge</th>
<th>Control (empty-liposome-immunised)</th>
<th>Lip-RNA-P-immunised</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (n = 20)</td>
<td>7</td>
<td>19/20 (95)</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>17/20 (85)</td>
<td>19/20 (95)</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>15/20 (75)</td>
<td>18/20 (90)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>8/20 (40)</td>
<td>15/20 (75)</td>
</tr>
<tr>
<td>II (n = 10)</td>
<td>7</td>
<td>10/10 (100)</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>9/10 (90)</td>
<td>9/10 (90)</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>8/10 (80)</td>
<td>9/10 (90)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4/10 (40)</td>
<td>8/10 (80)</td>
</tr>
</tbody>
</table>

Table IV. Protective effects of immunisation of mice with liposomal-entrapped Myc RNA-P (lip-RNA-P), measured by lung density and root specific lung weight (RSLW) after challenge with *M. tuberculosis* H37Rv (LD50)

<table>
<thead>
<tr>
<th>Group</th>
<th>Days after challenge</th>
<th>Lung density†</th>
<th>RSLW†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (empty-immunised)</td>
<td>7</td>
<td>0·70 (0·01)</td>
<td>14·60 (0·37)</td>
</tr>
<tr>
<td>lipsosome-immunised</td>
<td>14</td>
<td>0·74 (0·02)</td>
<td>16·36 (0·58)</td>
</tr>
<tr>
<td>Lip-RNA-P-immunised</td>
<td>21</td>
<td>0·82 (0·02)</td>
<td>18·54 (0·50)</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0·89 (0·01)</td>
<td>18·74 (0·13)</td>
</tr>
</tbody>
</table>

† Each value represents mean (SE) for 5–6 animals.
* *p < 0·05.
† ‡ p < 0·01.

challenge (table V). With lung samples, such differences were only slight and statistically nonsignificant.

**Discussion**

The present investigation demonstrates that *M. tuberculosis* RNA-P complex entrapped in PCML is immunogenic for mice, because it induced both humoral and CMI responses (table I; figure). Other studies with RNA-P fractions from a variety of micro-organisms have also shown that such com-
Table V. Protective effects of immunisation of mice with liposomal-entrapped Myc RNA-P (lip-RNA-P), measured by viable counts of tubercle bacilli recovered from lungs, livers and spleens after challenge with *M. tuberculosis* H37Rv

<table>
<thead>
<tr>
<th>Organ</th>
<th>Group</th>
<th>7 days after challenge</th>
<th>30 days after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Control (empty-liposome-immunised)</td>
<td>$1.60 \times 10^7$ ($0.95 \times 10^7$)</td>
<td>$3.63 \times 10^9$ ($1.05 \times 10^9$)</td>
</tr>
<tr>
<td></td>
<td>Lip-RNA-P-immunised</td>
<td>$9.51 \times 10^6$ ($4.61 \times 10^6$)</td>
<td>$9.42 \times 10^6$ ($8.12 \times 10^6$)</td>
</tr>
<tr>
<td>Liver</td>
<td>Control (empty-liposome-immunised)</td>
<td>$3.84 \times 10^7$ ($1.44 \times 10^7$)</td>
<td>$3.77 \times 10^9$ ($0.16 \times 10^9$)</td>
</tr>
<tr>
<td></td>
<td>Lip-RNA-P-immunised</td>
<td>$9.42 \times 10^6$ ($8.12 \times 10^6$)</td>
<td>$6.67 \times 10^7$ ($4.32 \times 10^7$)</td>
</tr>
<tr>
<td>Spleen</td>
<td>Control (empty-liposome-immunised)</td>
<td>$4.90 \times 10^6$ ($2.54 \times 10^6$)</td>
<td>$2.26 \times 10^7$ ($0.60 \times 10^7$)</td>
</tr>
<tr>
<td></td>
<td>Lip-RNA-P-immunised</td>
<td>$5.37 \times 10^6$ ($2.38 \times 10^6$)</td>
<td>$6.16 \times 10^5$ ($1.62 \times 10^5$)</td>
</tr>
</tbody>
</table>

† Each value represents mean (SE) of viable counts for 4–5 animals.

* $p<0.01$.

†† $p<0.001$.

components, in association with Freund's adjuvant, evoke both humoral (Leon et al., 1980; Pancholi et al., unpublished observations) and CMI responses (Youmans and Youmans, 1967; Likhite and Sehon, 1972; Sundararaj and Agarwal, 1977). However, in those studies the RNA:protein ratio varied from 1:1 to 2:1, whereas in the present study it was much higher at 7:1, thereby indicating that a more pure preparation of Myc RNA was obtained, this being the active principle (component) in inducing anti-tuberculous protection (Youmans and Youmans, 1966a, b, 1967). The quality of RNA (purity as well as degree of native RNA) is one of the critical factors for high immunogenic activity (Youmans and Youmans, 1966a). Hence, our observations, depicting a 30% increase in hyperchromicity of Myc RNA after RNAase treatment over that of untreated RNA (Pancholi et al., unpublished observations)—a figure close to the maximum of 33% (Hotchkiss, 1957)—suggests that the isolated Myc RNA is mostly native and, therefore, more efficient in inducing immune responses. Although evidence for the generation of a CMI response specific to Myc RNA (as revealed by the LMI index) was observed with leucocytes harvested from the group immunised with lip-RNA-P (table I), no DTH could be demonstrated in these mice (table II). However, DTH to RNA from several micro-organisms has been reported for mice immunised with RNA-Freund's adjuvant (complete or incomplete) (Bondevik and Mannick, 1968; Sundararaj and Agarwal, 1977; Pancholi et al., unpublished observations). The inability of liposomal-entrapped Myc RNA-P to induce DTH may relate to the fact that liposomal encapsulation of antigens protects the host against undesirable allergic reactions, as demonstrated by Gregoriadis and Allison (1974) for diphtheria toxoid antigen.

Mice immunised with liposomal-entrapped RNA-P fraction were significantly protected, in terms of increased survival rates, against challenge with *M. tuberculosis* H37Rv. These observations are comparable with the earlier findings of Youmans and Youmans (1966b, 1967, 1972) who obtained 70–72% protection in mice immunised with RNA-Freund's (incomplete) adjuvant and challenged with strain H37Rv. Increased survival time presumably reflects a retardation in the multiplication rate of the challenge organisms (Youmans, 1979) and this was confirmed by the viable counts of tubercle bacilli in infected organs, which showed significantly reduced levels in both livers and spleens of the test group of immunised mice (table V). The liver and spleen are the major tissues of the reticuloendothelial system for the sequestration of bacteria and particulate antigens that enter the circulation (Bennacerraf et al., 1959; Schulkind et al., 1967). Both parenchyma cells and fixed macrophages (Kupffer cells) in the liver are responsible for greater liposomal uptake (Gregoriadis and Neerunjn, 1974). The relatively insignificant restraint on growth of *M. tuberculosis* in the lungs of mice immunised with lip-RNA-P after challenge (table V) could have been due to poor uptake of liposomes by this organ (Vladimirsky and Ladigina, 1983).

The protective efficacy of liposomal-entrapped RNA-P fraction was further substantiated by decreased lung density and RSLW values in immunised mice (table IV). Both of these criteria have been used to evaluate the pathogenesis of tuberculous disease in mice infected intravenously.
(Portelance et al., 1974; Morrison and Collins, 1976; Mehta and Khuller, 1988), reduced values indicating a low degree of tuberculous infection. PC liposomes have also proved to be an effective replacement for Freund's adjuvant as immunopotentiators in the case of some parasitic infections (Vinayak and Sharma, 1986; Purnima et al., 1987; Vinayak et al., 1987) and, as confirmed in the present study, with mycobacteria.

In conclusion, this study demonstrates that PC liposomes provide an effective replacement for Freund's adjuvant for immunisation with mycobacterial RNA-P fraction, and that such a liposomally-encapsulated antigen imparts effective anti-tuberculous protection, as monitored by various relevant criteria.

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