Lepr Rev (1993) **64,** 316–324

Transport of amino-acids across renal brush border membrane vesicles in *Mycobacterium leprae* infected Swiss albino mice—effect of Convit vaccine

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Accepted for publication 8 April 1993

Summary Brush border membrane vesicles prepared from kidneys of *Mycobacterium leprae* infected (non-vaccinated) and vaccinated-infected Swiss albino mice were used to assess the effect of Convit's combined vaccine (BCG + *M. leprae*) on amino acid transport activity across the tubular basement membrane. The protective effect of Convit's vaccine was more pronounced with respect to the uptake of L-alanine than L-aspartate. Uptake of L-lysine showed no significant difference in the different groups. Footpad counts followed characteristic growth curves in the non-vaccinated infected group but showed a lag in the development of peak levels in the vaccinated group. Further Convit's vaccine appeared to have a protective effect on renal impairment in the mouse model of leprosy in the initial stages of infection only, as indicated by the transient reversal of amino acid uptake and a diminution in the footpad counts induced by *M. leprae* infection. No significant ($P > 0{\cdot}05$) protective effect of the vaccine was found in the advanced disease state.

Introduction

Renal involvement associated with leprosy has often been observed in both humans and animals.[1,2] The disease has been shown to affect the glomerulus as well as the tubules,[3,4] both at the structural and functional levels. Tubular damage resulting from the shedding of the epithelial membrane, and consequently the brush border enzymes, has been demonstrated in human cases of lepromatous leprosy.[5] Since this occurs before any histopathological damage, these biochemical parameters of renal tubular function can serve as early markers of renal damage. However, the direct involvement of renal parenchyma by *Mycobacterium leprae* has still not been shown.

It has been demonstrated that both in field trials and in experimental animals Convit's vaccine and other mycobacteria-based vaccines achieve a quick recovery through the

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augmentation of cell-mediated immune responses.[6] In an earlier study, uptake of amino acids by vesicle preparations from *M. leprae*-infected mice has been shown to be only transient, occurring predominantly during the initial stages of infection.[7] In the present study, mice were immunized with Convit's vaccine before *M. leprae* challenge to discover if the transport of nutrients across brush border membrane vesicles (BBMV) could be reversed by vaccination. As far as we know, this is the first study to explore the role of Convit's vaccine in modulation of renal brush border membrane function in experimental leprosy using the uptake of different amino acids across BBMV preparations.

Materials and methods

We used outbred Lacca strain of Swiss albino mice, raised in the animal house of the Postgraduate Institute of Medical Education and Research, Chandigarh, India, for the study. The bacterial counts of *M. leprae*, isolated from skin biopsies of lepromatous leprosy patients (BI 4+ to 6+, MI > 1%), were adjusted to a concentration of 1×10^4 bacilli/mouse footpad for infecting mice.

VACCINE PREPARATION

The vaccine preparation was a mixture of live BCG (obtained from BCG Vaccine Laboratory, Guindy, Madras, India) and killed armadillo-derived *M. leprae* (obtained through the courtesy of Dr R. J. W. Rees, from the Immunology of Leprosy, IMMLEP Bank) at a concentration of 1×10^5 AFB and 3×10^6 AFB per mouse,[8,9] respectively.

IMMUNIZATION OF MICE

Mice were immunized with 30 μl vaccine given subcutaneously in the left hind footpad. After 21 days the animals were injected in the left hind footpad with the same freshly-prepared vaccine, which was diluted $\times 10$ to serve as the booster dose.[10] After 7 days the mice were infected with *M. leprae* in the right hind footpad, subcutaneously. The animals were thus challenged with *M. leprae* 4 weeks after the primary vaccination. This comprised the vaccinated infected group (V-I group). Mice comprising the control group were neither infected nor vaccinated in both the hind footpads. Control animals received 30 μl of normal saline.

DETECTION OF LEPROSY

M. leprae infection was calculated by the amount of bacteria harvested from the infected footpads. The bacteria were stained by the Ziehl–Nelson method and were counted by the modified pin-head method[11] between 1 and 9 months after the infection.

EXPERIMENTAL DESIGN

A total of 150 Swiss albino mice were divided into 3 groups of 50 animals each:

(i) normal controls;
(ii) infected-nonvaccinated (I-NV);
(iii) vaccinated-infected (V-I).

HOW AND WHEN THE ANIMALS WERE KILLED

At least 15 mice, 5 each from the control, I-NV and V-I groups, were killed 1, 3, 6 and 9 months after infection. The mice were lightly anaesthetized with ether and the skin overlying the abdominal surface was wiped clean with spirit. The animals were then killed by cardiac puncture, after the thoracic cavity was cut open with a pair of fine scissors. Both kidneys were removed and used for the preparation of brush border membrane vesicles (BBMV).

PREPARATION OF BRUSH BORDER MEMBRANE VESICLES (BBMV)

Brush border membrane vesicle (BBMV) preparation from the renal cortex was carried out by the method of Malathi *et al.*[12] and the quality of the vesicles was checked as described by Turner & Moran.[13] To summarize, the renal cortex was removed from both the kidneys, suspended in a homogenizing buffer (pH = 7·0) and homogenized for 10 min, using hotline mixer. The resulting homogenate was left for 10 min with $CaCl_2$ on ice and then centrifuged at 6000 g for 15 min. The supernatant was then centrifuged at 43,000 g for 20 min and then the pellet was washed twice using homogenizing buffer. The pellet was then reconstituted in reconstitution buffer (pH = 7·5) and repeatedly passed through a 25-gauge needle. Finally, the vesicle preparation was incubated at 30°C for 15–20 min. This ensured that the membranes were obtained in a vesicular form that was ideally suited for transport studies, and that the vesicles were intact.

The purity of BBMV preparation from the 3 groups of mice killed at different periods was checked by estimating the characteristic renal brush border marker enzymes.[7] Fold enrichment was found to be ranging from 8 to 11% for γ-glutamyl transpeptidase, 12–17% for leucine aminopeptidase and 15–17% for alkaline phosphatase.

TRANSPORT OF AMINO ACIDS

Uptake of L-lysine, L-aspartate and L-alanine was determined by the Millipore filtration technique of Hopfer *et al.*[14] To summarize, 10 μl of BBMV (60–100 μg protein) were incubated at 20°C in the incubation medium that was composed of 50 μM L-aspartate, L-lysine and L-alanine, respectively, and 0·1 μCi of the respective ^{14}C-labelled substrate. The reaction was stopped after 30 seconds by the addition of 5 ml stopping buffer (150 μM NaCl, 1 mM Tris Hepes; pH 7·5). The mixture was filtered through a 0·45 μM Millipore filter. The filter was air dried and radioactivity was counted in a LKB-1215 Rackbeta Liquid Scintillation Counter.

FOOTPAD COUNTS OF *M. LEPRAE*

Each time the mice were killed both the hind footpads of the I-NV group and the right hind footpad of V-I group were counted for *M. leprae* bacteria by the pin-head method.[11]

STATISTICAL ANALYSIS

The Student's *t*-test was used to compare various groups and the values were expressed as mean ± S.E.

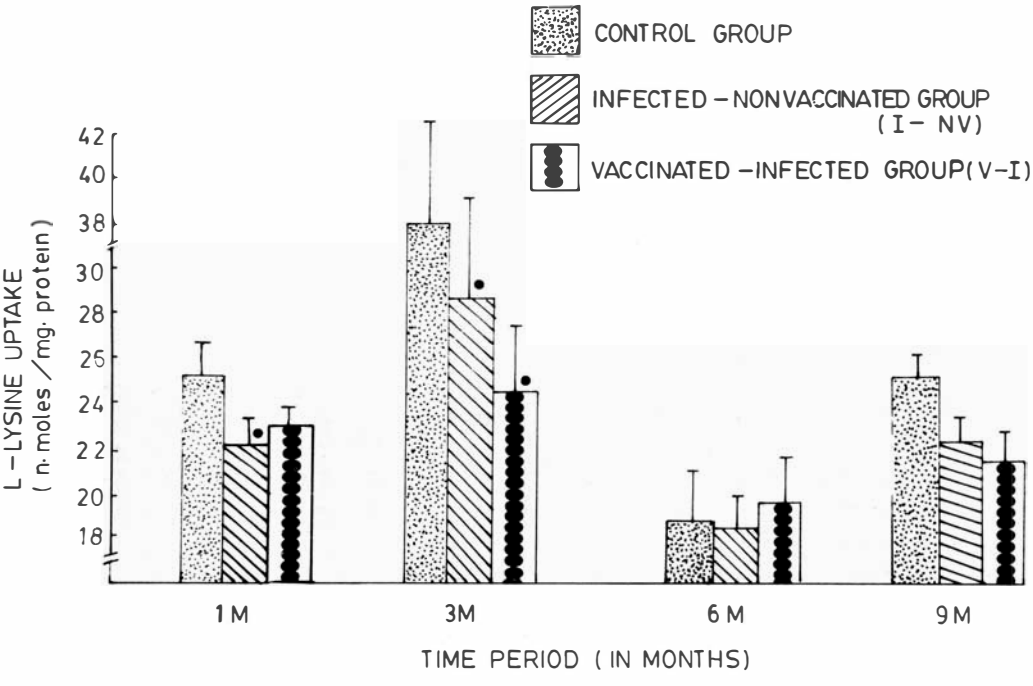

Figure 1. Uptake of L-lysine across renal BBMV in the control, infected and vaccinated groups at 1, 3, 6 and 9 months. The level of significance is: (●) $P < 0{\cdot}001$ *vs.* control group.

Results

Uptake of L-lysine, L-alanine and L-aspartate was determined in renal BBMV prepared from the control, infected and vaccinated-infected groups. Any differences in the uptake of different amino acids in the animals infected with *M. leprae* compared to controls would provide good evidence of an altered biochemical status of the renal tubules in response to *M. leprae* infection.[7]

Uptake of L-lysine across BBMV was decreased significantly ($P < 0{\cdot}001$) at 1 and 3 months post-infection in the I-NV group compared to the control group, but there was no significant difference at 6 and 9 months post-infection (Figure 1), which demonstrates the transient nature of the altered uptake of nutrients. The vaccinated group (V-I), on the other hand, showed no significant difference ($P > 0{\cdot}05$) in the uptake of L-lysine when compared to I-NV group at different periods (Figure 1). Similarly no significant difference ($P > 0{\cdot}05$) was observed in the uptake of L-aspartate across BBMV between the I-NV and V-I groups compared to the control group at different periods of study between 1 and 9 months (Figure 3). Increase in uptake of L-aspartate was observed in the V-I group between 1 and 9 months as compared to its corresponding I-NV group and between 1 and 6 months as compared to its corresponding control group. However, the increase observed in the uptake of L-aspartate was statistically insignificant.

Uptake of L-alanine across BBMV showed a significant difference ($P < 0{\cdot}001$) at 3 and 6 months post-infection between the I-NV group compared to the control group (Figure 2), although at 1 month post-infection no significant difference was observed between the

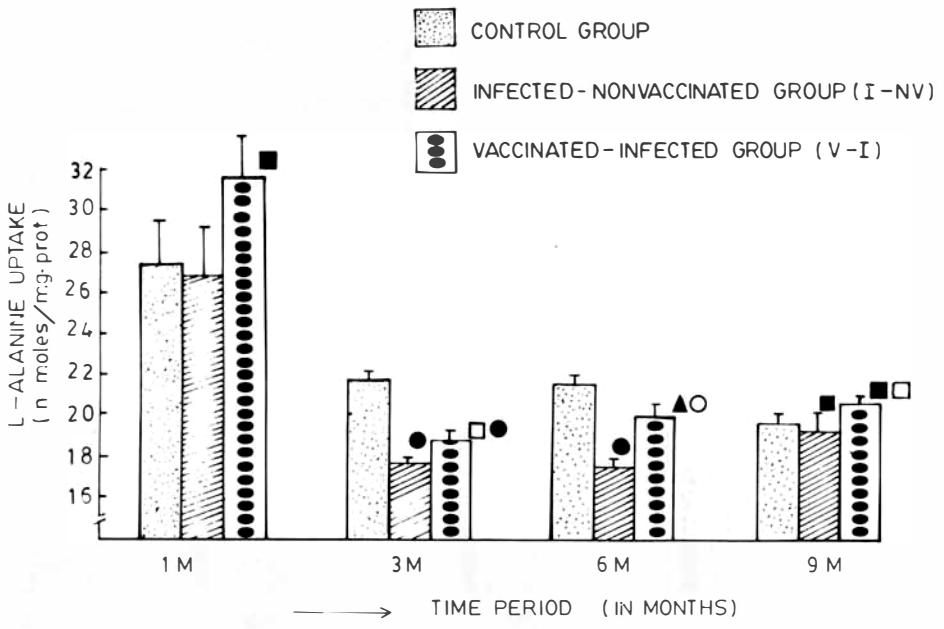

Figure 2. Uptake of L-alanine across renal BBMV in the control, infected and vaccinated groups at 1, 3, 6 and 9 months. The level of significance is: (●) $P<0{\cdot}001$ *vs.* control group; (○) ($P<0{\cdot}01$) *vs.* control group; (■) ($P<0{\cdot}05$) *vs.* control group; (□) ($P<0{\cdot}05$) *vs.* infected group; (▲) ($P<0{\cdot}001$) *vs.* infected group.

2 groups. The V-I group, on the other hand, showed a significant increase ($P<0{\cdot}05$) in the uptake of L-alanine across BBMV between 1 and 6 months post-infection, although the increase was more pronounced at 6 months post-infection ($P<0{\cdot}001$) when compared to the corresponding I-NV group. Increase in the uptake of L-alanine in V-I group compared to I-NV group, however, failed to achieve the level observed in the control group (Figure 3).

Footpad counts of *M. leprae* at different periods were found to follow the characteristic growth curve in both the I-NV and the V-I groups. This was demonstrated by the linear increase in footpad counts between 3 (ranging from $0{\cdot}56\times10^4$ AFB/ml to $0{\cdot}846\times10^4$ AFB/ml) and 6 months post-infection (ranging from $0{\cdot}98\times10^5$ AFB/ml to $1{\cdot}64\times10^5$ AFB/ml), which reached a stationary phase at 9 months post-infection with a mean count of $1{\cdot}39\times10^5$ AFB/ml (Table 1). The V-I group, on the other hand, showed a decrease in the footpad bacillary counts at different periods compared to I-NV group, though the decrease was statistically insignificant ($P>0{\cdot}05$) (Table 2). The bacterial counts in the V-I group showed a characteristic growth curve with a linear increase in the bacillary count between 3 and 6 months and reaching stationary phase at 9 months. Lag in the development of peak counts was observed in the V-I group as compared to the I-NV group.

Discussion

We carried out studies on the uptake of L-lysine, L-aspartate and L-alanine to assess the

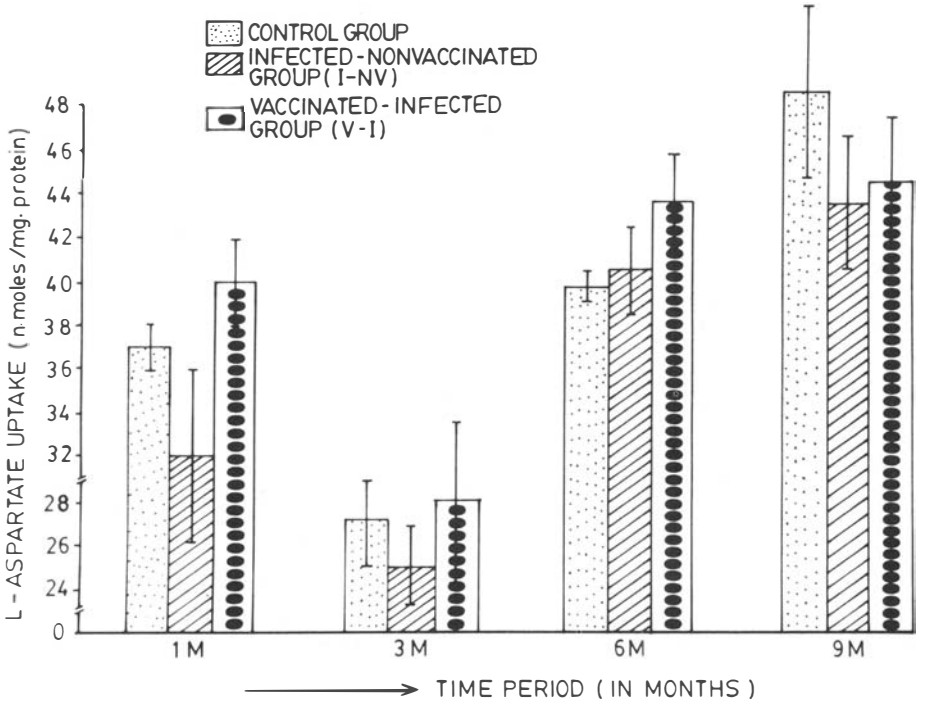

Figure 3. Uptake of L-aspartate across renal BBMV in the control, infected and vaccinated groups at 1, 3, 6 and 9 months.

biochemical functional status of the renal tubule at the brush border membrane level and to evaluate the effect of Convit's combined vaccine on leprosy in mice.

There was a transient alteration in the uptake of L-lysine, L-alanine and L-aspartate across BBMV between 3 and 6 months post-infection, which then returned to normal. These findings are in agreement with our previous observations on leprosy in mice[7] and pyelonephritis in rats.[15] The difference in the tubular uptake of different amino acids could be due to different carrier systems for different amino acids,[16] or multiple carrier systems in renal brush border membrane for handling the organic cation,[17] or to significant internephron heterogeneity for amino acid reabsorption.[18]

In the present study, the protective effect of the combined vaccine has been demonstrated by the increased uptake of L-aspartate (1–3 months post-infection) and L-alanine (1–6 months post-infection), thereby reflecting an improvement in the altered reabsorption capacity of tubular segments. However, no protective effect of vaccine could be observed in the uptake of L-lysine. Variable response of vaccine to different amino acids is possibly due to differences in the carrier systems for individual amino acids.[16] The present study demonstrated only limited arrest of infection by vaccination, as is shown by the increase in the lag phase and low bacterial footpad counts during the early phase of infection. It is during this period that the increased uptake of amino acids was observed in the vaccinated group (V-I) compared to the infected-nonvaccinated group. This finding is

Table 1. Bacterial footpad counts (I–NV group)

Month period	Counts of AFB	
	Range of AFB/ml	Mean ± S.E. (AFB/ml)
1	—	—
3	$0{\cdot}516 \times 10^4 - 0{\cdot}84 \times 10^4$	$0{\cdot}696 \times 10^4 \pm 0{\cdot}120 \times 10^4$
6	$0{\cdot}98 \times 10^5 - 1{\cdot}64 \times 10^5$	$1{\cdot}30 \times 10^5 \pm 0{\cdot}34 \times 10^5$
9	$1{\cdot}14 \times 10^5 - 1{\cdot}64 \times 10^5$	$1{\cdot}39 \times 10^5 \pm 0{\cdot}25 \times 10^5$

Table 2. Bacterial footpad counts (V–I group)

Month period	Counts of AFB	
	Range of AFB/ml	Mean ± S.E.
1	—	—
3	$0{\cdot}525 \times 10^4 - 0{\cdot}810 \times 10^4$	$0{\cdot}690 \times 10^4 \pm 0{\cdot}10 \times 10^4$
6	$0{\cdot}710 \times 10^5 - 1{\cdot}30 \times 10^5$	$1{\cdot}10 \times 10^5 \pm 0{\cdot}120 \times 10^5$
9	$0{\cdot}735 \times 10^5 - 1{\cdot}35 \times 10^5$	$1{\cdot}17 \times 10^5 \pm 0{\cdot}51 \times 10^5$

consistent with the protective effect of immunization by *M. leprae*[6] which is evident by a significant fall in footpad counts at 6 and 9 months. Our findings showed a consistent lag in bacterial footpad counts in reaching the plateau level after 6 months. The differences between the individual series of assays on the uptake of amino acids at different times could be due to certain differences in the bacterial load of micro-organisms in individual animals in a particular group that could have occurred during the course of infection. Since the animals were monitored at 1, 3, 6 and 9 months only, in-between fluctuations could have occurred which might lead to differences in individual series of assays on the uptake of amino acids.

Reversal of the transient alterations in the uptake of different amino acids seen in BBMV preparation in the vaccinated group during the early post-infection phase may have implications, viz: that biochemical changes across the renal brush border membrane were specific to *M. leprae* infection, as vaccination tends to revert the values to normal. In addition, reversal of amino-acid uptake in the vaccinated group could be related to a lag in the development of peak bacterial load and lower counts during the 3–6 month period corresponding to the infected-nonvaccinated group (I-NV). It is possible that the Convit's vaccine prevents the formation of immune-complexes by disturbing the ratio of antigen : antibody required for the formation of immune complexes. Based on these observations, it is reasonable to suggest that Convit's combined vaccine does play a role in improving the functional status of the renal brush border with respect to the amino-acid uptake, though vaccination alone fails to limit the disease. Furthermore, this study has shown that amino-acid uptake could be used as a physiological marker in experimental models to assess the effect of vaccination, and that antileprosy drugs, in conjunction with vaccination, could limit infection successfully.

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Transport des amino-acides à travers les vésicules rénales de la membrane à bordure en brosse chez des souris Swiss Albino infectées par le Mycobacterium leprae—Effet du vaccin de Convit

MANJULA KOHLI, SURINDER KAUR, NIRMAL K. GANGULY, VINOD K. SHARMA ET KIRPAL S. CHUGH

Résumé Nous avons utlisé des vésicules de la membrane à bordure en brosse préparées à partir des reins de souris Swiss Albino infectées par le *Mycobacterium leprae* mais non vaccinées et infectées et vaccinées, pour évaluer l'effet du vaccin combiné de Convit (BCG + *M. leprae*) sur l'activité de transport des amino-acides à travers la couche sous-épithéliale tubulaire. L'effet protecteur du vaccin de Convit était plus prononcé dans l'absorption de L-alanine que dans celle de L-aspartate. L'absorption de L-lysine n'a présenté aucune différence significative entre les divers groupes. Les comptes plantaires suivaient des courbes de croissance caractéristiques dans le groupe infecté non vacciné mais présentaient un retard dans le développement des concentrations-pics chez le groupe vacciné. Il semblerait d'autre part, que le vaccin de Convit n'a un effet protecteur sur le délabrement rénal chez la souris dans la lèpre, qu'aux premiers stades de l'infection comme indiqué par l'inversion passagère de l'absorption d'amino-acides et par une diminution des comptes plantaires induites par le *M. leprae*. Dans la phase avancée de la maladie, l'effet protecteur du vaccin ne s'est pas révélé significatif ($P > 0{,}05$).

Trasporte de aminoácidos a través de las vesículas de la membrana renal de borde piloso en ratones albino suizo—efecto de la vacuna Convit

MANJULA KOHLI, SURINDER KAUR, NIRMAL K. GANGULY, VINOD K. SHARMA Y KIRPAL S. CHUGH

Resumen Se utilizaron vesículas de la membrana de borde piloso tomadas de rinones de ratones albino suizo infectados con *Mycobacterium leprae* (no vacunados) y ratones infectados mediante vacuna, para evaluar el efecto de la vacuna combinada Convit (BCG + *M. leprae*) sobre la actividad de traspaso de aminoácidos a través de la membrana de base tubular. El efecto protector de la vacuna Convit fue más pronunciado en lo que se refiere al consumo de L-alanina y L-aspartato. El consumo de L-lisina no presentó diferencias significativas en los diversos grupos. Los recuentos obtenidos de las patas siguieron las curvas de aumento características en el grupo infectado no vacunado, pero observaron un retraso en el desarrollo de niveles pico en el grupo vacunado. Más aún, la vacuna Convit sólo parece tener un effecto protector sobre las deficiencias renales en el modelo de lepra de los ratones durante las etapas iniciales de infección, tal como lo indica la inversión transitoria del consumo de aminoácidos y la disminución en los recuentos de patas inducidos mediante la infección de *M. leprae*. La vacuna no demostró un efecto protector significativo ($P > 0{,}05$) en el estado avanzado de la enfermedad.