In Vitro Proliferation of Lymphocytes from Human Volunteers Vaccinated with Armadillo-derived, Killed *M. leprae*[1]

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Leprosy is caused by *Mycobacterium leprae,* an obligate intracellular parasite. Immunity against such organisms is mediated by cellular mechanisms ([8]). It is clearly important, therefore, to establish that a candidate vaccine against leprosy is able to induce this type of immunity.

Previously, we have reported the results from a trial of an armadillo-derived, heat-killed *M. leprae* vaccine in human volunteers ([2]). Briefly, this study involved four groups of individuals who were vaccinated with 1.5×10^7, 5×10^7, 1.5×10^8, and 5×10^8 armadillo-derived, heat-killed *M. leprae* intradermally (i.d.). The criteria used to assess the efficacy of this vaccine was its ability to induce delayed-type hypersensitivity (DTH) to soluble *M. leprae* skin-test antigen (MLSA). Skin tests are normally used to monitor the cell-mediated immune (CMI) response to *M. leprae* antigens in leprosy antigens ([13]). The results revealed that the individuals in the groups receiving the three highest doses of vaccine showed a significant increase in their skin-test response to purified protein derivative (PPD), which was generally high since these volunteers came from a population which had been vaccinated with BCG.

The *in vitro* lymphocyte transformation test (LTT) is an alternative method for monitoring CMI responses. It has been widely used in leprosy patients ([1, 4, 11]) and their contacts ([3, 5, 9, 10, 12]) to study their cell-mediated responses to *M. leprae* antigens. In this test, an individual's peripheral blood mononuclear cells (PBMC) are cultured with antigen and then assayed for proliferation. Proliferation in the presence of an antigen indicates that sensitization to that antigen has occurred. The LTT offers several advantages over the skin test. Firstly, it can be performed repeatedly on the same individual without affecting his response. Secondly, it offers a means of testing several doses of antigen simultaneously. And, lastly, because proliferation is assessed by thymidine incorporation, the results are quantitative and not subject to any reading bias.

In this report, we present the results obtained when the LTT was used to measure the induction of a cell-mediated response in the vaccinated volunteers of the previous study ([2]). The results show that vaccination with the higher doses (1.5×10^8 and 5×10^8) of the armadillo-derived, heat-killed *M. leprae* vaccine induces a highly significant increase in the *in vitro* proliferative response to *M. leprae* antigens. This increase lasted throughout the test period (1 year).

MATERIALS AND METHODS

Subjects and study design. The participants of this study were nursing students of Diakonhjemmet, Oslo, Norway. They were not given any incentives, and none of the trial organizers were regular teachers or examiners at their school.

The study design has been described elsewhere ([2]). In brief, 31 volunteers (19 females and 12 males) between the ages of 23 and 28 years were PPD tested before vaccination. Seven to nine subjects were randomly assigned to four groups so that an even distribution of PPD responsiveness occurred in each group. One month later, each group was re-tested with PPD and MLSA, which were provided by the World Health Organization as coded samples. The skin-test re-

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sponses were recorded at 72 hr. After the skin-test responses were recorded, the first group was vaccinated with 1.5×10^7 bacilli i.d. at three sites (forming the apices of an equilateral triangle of 3-cm length) on the deltoid region. The study was designed so that the group which was to receive the higher dose of vaccine attended the 1-month examination of the vaccination scar of the previous group to decide for themselves if they would accept the higher dose. A similar group of eight subjects, who were skin tested only, was included as controls.

The trial design was approved and prior clearance was obtained from ethical committees of the Norwegian Radium Hospital, Oslo, Norway; the National Norwegian Drug Agency (Statens Legemiddel Kontroll); and the World Health Organization Ethical Committee (SCRIHS).

Isolation of peripheral blood mononuclear cells (PBMC). Peripheral blood was obtained at day −3 before vaccination and 1, 3, 6, and 12 months after vaccination. Bleeding at day −3 and at 3 months preceded skin testing. Mononuclear cells were separated from peripheral blood by density centrifugation on a Ficoll/metrizoate gradient (Lymphoprep; Nyegaard and Co., Oslo, Norway). These cells were frozen and stored in liquid nitrogen until the cells for all the time intervals from each individual were available.

Antigens. The *M. leprae* soluble antigen, batch CD45 was kindly provided by Dr. R. J. W. Rees (Mill Hill, London). Streptokinase-streptodornase (SK-SD) was obtained from Cyanamid, Iberia SA, Madrid, Spain. Tetanus toxoid (TT) and diphtheria toxoid (DT) were obtained from the Serum Institute, Copenhagen, Denmark. Human *M. leprae* was kindly provided by Dr. R. J. W. Rees. Soluble antigens were used at three different protein concentrations of 0.1 μg, 1 μg, and 10 μg per ml. Whole *M. leprae* was used at three different concentrations: 5×10^7, 1×10^7, and 2×10^6 bacilli per ml.

***In vitro* proliferation.** PBMC from a single vaccine, taken at various time intervals, were removed from storage in liquid nitrogen, thawed, and assessed for viability. The PBMC were cultured at a concentration of 10^5 cells/well in 96-well U-bottom trays in the presence of antigens added in triplicates. The trays were incubated for 6 days at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. On day 6, the cultures were pulsed with 0.045 MBq ^{3}H-thymidine (specific activity = 185×10^3 MBq/mmole) for 4 hr, after which they were harvested with a Skatron Harvester (Norway). The radioactivity incorporated was determined by liquid scintillation spectroscopy. The results were expressed as the median value of counts per minute (cpm) of each triplicate.

RESULTS

At the beginning of this study, freshly isolated PBMC were tested for their *in vitro* responses to the various antigens. However, after testing the 1-month post-vaccination responses of the second group, it became obvious that the day-to-day variations in the LTT were too large to yield meaningful results. Thus, the PBMC from the control group and the groups which had received the two highest doses of vaccine were frozen down and stored in liquid nitrogen. At the end of the study period, each individual's cells taken at all of the time intervals were simultaneously tested for their *in vitro* antigen responses. The results presented here are based on this strategy.

The LTT responses are expressed at the optimal dose of antigens. For all groups, this was between 1 μg and 10 μg/ml for the soluble antigens, 5×10^7 bacilli/ml for armadillo *M. leprae,* and 1×10^7 bacilli for human *M. leprae.*

The kinetics of the group 4 responses (5×10^8 bacilli) is shown in Figure 1. The peak response appeared 3 months post-vaccination. The pre-vaccination response to PPD was quite high (mean ± S.E.M. = 28,630 cpm ± 12,471); while the pre-vaccination response to MLSA was much lower (mean ± S.E.M. = 8970 cpm ± 3679). There was an increase in the post-vaccination response to both MLSA and PPD, but while the difference between the pre-vaccination and post-vaccination response to MLSA was highly significant ($0.01 > p > 0.001$), the difference between the pre-vaccination and post-vaccination response to PPD was not significant. This phenomenon was observed in responses to other antigens, e.g., SK-SD, BCG, tetanus toxoid and diphtheria toxoid, indicating that killed *M. leprae* vaccine may

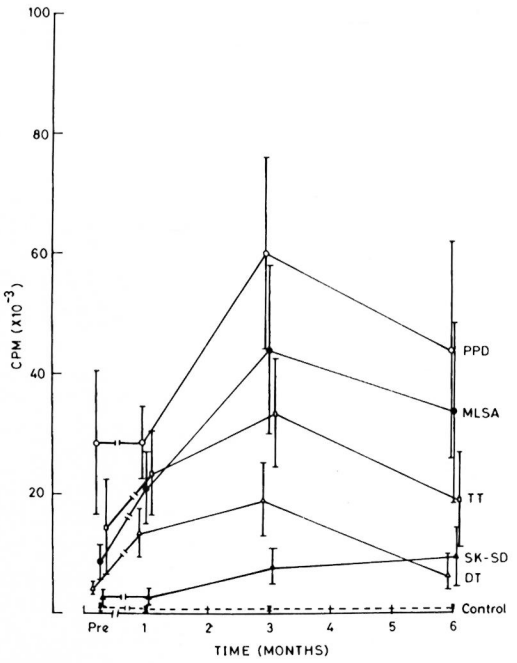

FIG. 1. Kinetics of the lymphocyte transformation responses to MLSA (●), PPD (○), SK-SD (▲), DT (△), and TT (□) in volunteers vaccinated with 5×10^8 armadillo-derived *M. leprae* (group 4). Control responses, i.e., without antigen, are also included. The results are expressed as the mean response of the groups, and the vertical bars represent the S.E.M. The difference between the pre-vaccination response and the 3-month post-vaccination response to MLSA was significant ($0.01 > p > 0.001$, Student's *t* test); the difference between the pre-vaccination response and the 3-month post-vaccination response to PPD was not significant ($p > 0.05$). The differences between the pre-vaccination responses and the 3-month post-vaccination responses to SK-SD, DT, and TT were also not significant ($p > 0.05$).

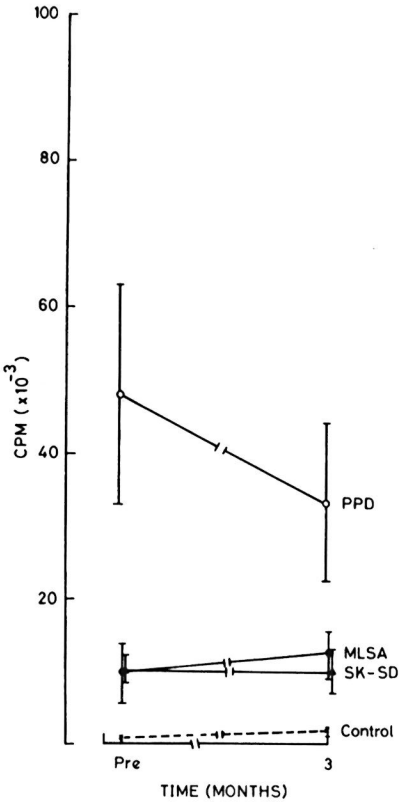

FIG. 2. Lymphocyte transformation responses to MLSA (●), PPD (○), and SK-SD (▲) in unvaccinated control volunteers. Control responses, i.e., without antigen, are also included. The differences between the pre- and post-vaccination responses to these antigens were not statistically significant ($p > 0.05$).

possess nonspecific adjuvant properties. Similar observations were also made for group 3 individuals (data not shown).

No significant changes were observed in the control group receiving skin tests only (Fig. 2). In fact, a slight decrease in their response to PPD was apparent. This pattern was also observed in their skin-test reactivity ([2]).

A separate set of PBMC samples of group 5 taken at day −3, 3 months, and 1 year were tested in order to assess the duration of the response. As shown in Figure 3, the response persisted throughout the test period.

The *in vitro* responses to various preparations of *M. leprae* were also tested. While MLSA gave the strongest responses, significant post-vaccination responses were also observed with *M. leprae* derived from human biopsies as well as the preparation of *M. leprae* used for vaccination (Fig. 4).

DISCUSSION

Leprosy is a disease with a low incidence and a long incubation period. Consequently, human trials of vaccines against this disease assessed by protection would entail the long-term (>10 years) surveillance of large populations living in endemic countries. Under these circumstances, IMMLEP ([14]) adopted the strategy of conducting the killed *M. leprae* vaccine trial in phases. Information gained at each phase would be valu-

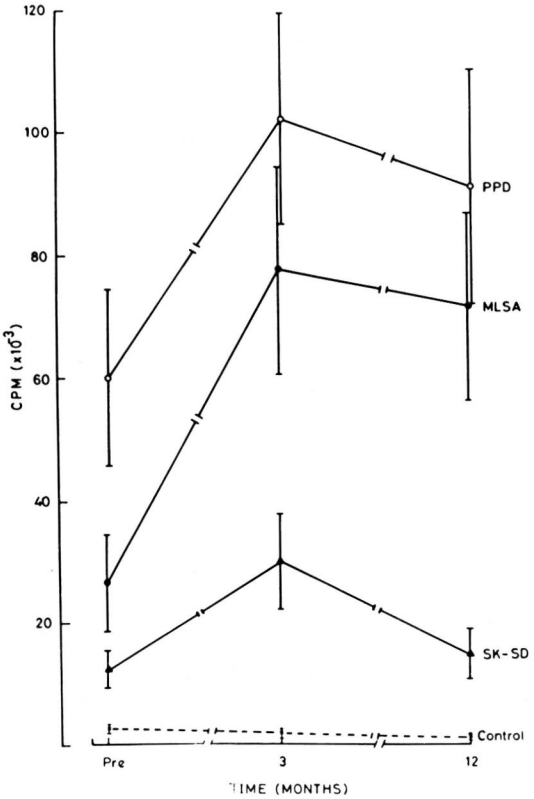

FIG. 3. Kinetics of the lymphocyte transformation responses to MLSA (●), PPD (○), and SK-SD (▲) in volunteers vaccinated with 5×10^8 bacilli. Control responses, i.e., without antigen, are also included (-----). The difference between pre- and 3-month post-vaccination responses to MLSA was significant ($0.02 > p > 0.01$) as was the difference between pre- and 1-year post-vaccination responses to MLSA ($0.05 > p > 0.002$). Difference between pre-vaccination and 3-month post-vaccination responses to SK-SD was not significant ($p > 0.05$).

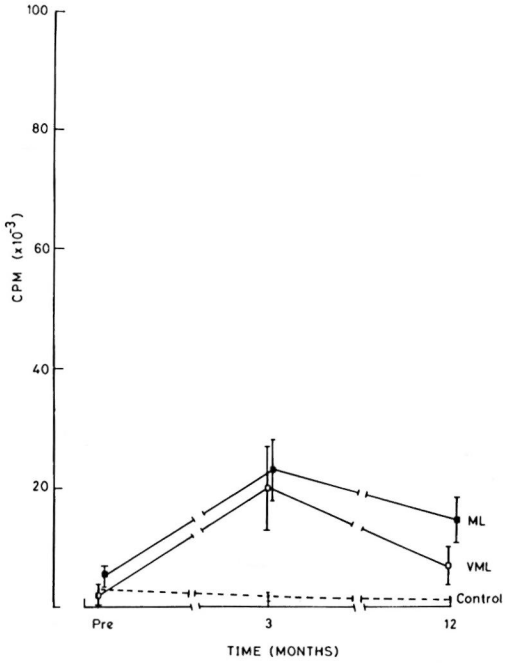

FIG. 4. Lymphocyte transformation responses to vaccine *M. leprae* (□) and human *M. leprae* (■) in volunteers vaccinated with 5×10^8 bacilli (group 4). Control responses, i.e., without antigen, are also included. Differences between pre-vaccination and 3-month post-vaccination responses to vaccine *M. leprae* (VML) and human *M. leprae* (ML) were significant ($0.05 > p > 0.02$ and $0.01 > p > 0.001$, respectively).

able for the design and the ultimate undertaking of long-term protection trials.

The phase I study reported here and previously ([2]) was conducted in healthy volunteers residing in a nonendemic country. The objective of this study was to establish the optimum dose of killed *M. leprae* which would induce a cell-mediated immune response in an individual without causing any unacceptable side effects. In the previous report, this was assessed in terms of the induction of a delayed-type hypersensitivity response by measuring skin-test conversion to a *M. leprae*-derived soluble antigen preparation in these volunteers. In this report, a second assay, the LTT, was employed on the same individuals to measure the cell-mediated immune response induced by vaccination with the killed *M. leprae* vaccine. It is important to stress that the exact relationship between these assays and resistance to infection has yet to be established. This, in itself, is a very persuasive argument for employing the two assays in assessing the ability of the killed *M. leprae* vaccine to induce a cell-mediated immune response. Furthermore, the assays complement each other in the sense that the one assay compensates for the shortcomings in the other assay. As mentioned earlier, the LTT can be performed repeatedly without affecting an individual's response. This is not the case with skin testing. While skin testing is an *in vivo* assay which is relatively simple and rapid, it has been shown that repeated skin testing can affect an individual's response ([7]). Since both the level and

duration of immunity brought about by a vaccine is crucial to its success, this is an important advantage offered by the LTT. It would appear, therefore, that the results of the two assays would facilitate a better judgment of the performance of the vaccine.

This study shows that individuals vaccinated with 1.5×10^8 and 5×10^8 killed armadillo-derived *M. leprae* develop a strong response to *M. leprae* antigens in the LTT 3 months after vaccination. The response appears to remain high during the period of study (6 months–1 year). Furthermore, the *in vitro* response to *M. leprae* antigens parallels the *in vivo* skin-test response to these antigens in that both vaccinated groups showed a marked increase in response to *M. leprae* antigens after vaccination.

The *in vitro* and *in vivo* responses to PPD, however, did not follow a parallel trend. While there was very little change in the skin-test responses before and after vaccination, the LTT response to PPD increased somewhat after vaccination. The reasons for this divergence in trends and for the boosting effect in the LTT to PPD are not altogether apparent. One possible explanation for the divergence is that the two assays do not measure identical phenomena. Insofar as the boosting is concerned, it is important to ascertain whether the boosting is specific or nonspecific in nature. Because PPD, BCG, and *M. leprae* are crossreactive ([6]), the effect may be specific. However, the slight boosting of LTT responses to SK-SD, tetanus toxoid and diphtheria toxoid may indicate a nonspecific effect. It is also quite conceivable that the boosting of the LTT response to PPD may be a result of both specific and nonspecific effects.

In conclusion, the results presented here support and extend the skin-test results reported previously from the same group of healthy volunteers vaccinated with killed *M. leprae*. Thus, it appears that in man, too, *M. leprae* is a potent inducer of cell-mediated responsiveness as assessed both *in vivo* and *in vitro*. This responsiveness is of considerable duration, remaining high up to 1 year after vaccination.

SUMMARY

A killed, armadillo-derived *Mycobacterium leprae* vaccine was examined for its ability to induce cell-mediated responsiveness in purified protein derivative (PPD)-positive volunteers residing in a nonendemic country using the lymphocyte transformation test (LTT). A marked increase in the proliferative responses to a *M. leprae*-soluble antigen preparation was observed in the two groups which were vaccinated with the highest doses of the vaccine, i.e., 1.5×10^8 and 5×10^8 bacilli. This increase was observed in both groups 3 months after vaccination, and persisted for the study period of 1 year. The *in vitro* proliferative responses to whole bacilli, of both armadillo and human origin, showed a similar but smaller increase 3 months after vaccination. Some enhancement of responses to cross-reactive antigens, such as PPD, and to unrelated antigens such as streptokinase-streptodornase, tetanus toxoid and diphtheria toxoid, was also observed. Thus, the LTT revealed that while the killed *M. leprae* vaccine induced a specific cell-mediated response to *M. leprae,* it was also responsible for a nonspecific immune-enhancement effect in healthy volunteers.

RESUMEN

Usando la prueba de transformación de linfocitos (PTL) se probó una vacuna muerta de *Mycobacterium leprae* derivada de armadillo en cuanto a su capacidad para inducir respuestas inmunes celulares en voluntarios PPD-positivos residentes de un país no endémico. En los 2 grupos que fueron vacunados con las dosis más altas de la vacuna (1.5×10^8 y 5×10^8 bacilos) se observó un marcado incremento en las respuestas proliferativas contra una preparación antigénica soluble derivada del *M. leprae.* Este incremento se observó en ambos grupos 3 meses después de la vacunación y persistió durante el periodo de estudio de 1 año. Las respuestas proliferativas *in vitro* contra los bacilos totales tanto de armadillo como de humanos, mostraron un incremento similar pero más pequeño 3 meses después de la vacunación. También se observó cierto incremento de las respuestas contra antígenos de racción cruzada (PPD) y contra antígenos no relacionados (estreptoquinasa-estreptodornasa, toxoide tetánico y toxoide diftérico). Así, la PTL reveló que mientras la vacuna de *M. leprae* muerto indujo una respuesta celular contra *M. leprae,* también fue responsable de un efecto incrementador no específico de la respuesta inmune en los voluntarios sanos.

RÉSUMÉ

On a utilisé l'épreuve de transformation lymphocytaire (LTT) pour examiner dans quelle mesure un

vaccin préparé à base de *Mycobacterium leprae* tués, dérivés du tatou, pouvait induire une réponse à médiation cellulaire chez des volontaires positifs au PPD, habitant une région non endémique. Une augmentation marquée des résponses prolifératives à un antigène soluble de *M. leprae* a été observée dans les deux groupes de vaccinés avec les doses les plus élevées de vaccin, à savoir $1{,}5 \times 10^8$ et 5×10^8 bacilles. Cette augmentation a été observée dans les deux groupes, trois mois après la vaccination; elle a persisté pendant toute la période d'étude qui s'est étendue sur un an. Les réponses prolifératives aux bacilles entiers, tant de tatous que d'origine humaine, qui ont été suivies *in vitro,* ont montré une augmentation similaire mais toutefois plus faible, trois mois après la vaccination. Un certain renforcement des réponses aux antigènes de réactions croisées, tels que le PPD, de même qu'à des antigènes sans aucune relation, tels que la streptokinase-streptodornase, la toxoïde tétanique, la toxoïde diphtérique, ont également été observées. L'épreuve de transformation lymphocytaire (LTT) permet dès lors de montrer que le vaccin à base de *M. leprae* tué, s'il entraîne une réponse à médiation cellulaire spécifique pour *M. leprae,* est également responsable d'un renforcement non spécifique de l'effet immunogène chez des volontaires en bonne santé.

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