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Immunogenic "Subunit" of the ICRC Antileprosy Vaccine[1]

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For the past 7 years, an antileprosy vaccine containing the ICRC bacilli, a group of leprosy-derived mycobacteria belonging to the *Mycobacterium avium-intracellulare* complex, has been undergoing clinical trials in lepromatous leprosy (LL) patients and lepromin-negative healthy subjects ([5, 9, 10]) who represent a high-risk group in leprosy-endemic areas ([27]). The vaccine has brought about lepromin conversion in 55% of the patients and in 95% of healthy lepromin-negative individuals, associated with upgrading of the tissue response in the former ([2, 9]).

Recently, we have shown that monkeys exhibit a pattern of Mitsuda reactivity similar to that seen in man. Further, the ICRC vaccine is able to induce lepromin conversion in the lepromin-negative animals ([6]). These, as well as other laboratory studies, indicate that the ICRC bacilli show antigen crossreactivity with *M. leprae* ([14, 16, 18]).

To further isolate the immunogenic component(s), the ICRC sonicate was fractionated using high-pressure liquid chromatography (HPLC) and gel permeation columns. The sonicate yielded a number of fractions, the most dominant being a high molecular weight glycolipoprotein with an apparent molecular weight of 10^6 daltons. The glycoprotein, which accounts for 70% of the protein of the sonicate, carries antigenic epitopes for both B and T cells. *M. leprae* sonicate also yields a similar high molecular weight glycolipoprotein. In this communication, we will show that the high molecular weight fractions from the two organisms exhibit antigenic crossreactivity, both at B- and T-cell levels.

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MATERIALS AND METHODS

Sources of mycobacteria

ICRC bacilli. Strain C-44 of the ICRC bacilli, isolated from a biopsy of a lepromatous leprosy (LL) patient in 1969, is in the 110th passage in Dubos' modified medium. The medium essentially contains Dubos' phosphate-buffered base, amino acids and vitamin mixtures of Dulbecco, supplemented with minerals (ferric ammonium citrate, magnesium sulfate, copper sulfate, calcium chloride, and zinc sulfate) and 10% human AB serum. To each liter of the medium, 0.5 ml of 10% Tween 80 was added as a dispersing agent, and 0.5% tetradecane and 5% dimethylsulfoxide were added as recommended by Kato ([24]). The cultures were harvested between 13–15 days, and the mycobacteria washed extensively with normal saline.

M. leprae. Human-derived *M. leprae* were isolated from skin biopsies of untreated LL patients with a bacterial index (BI) of >5 from the Acworth Leprosy Hospital, Wadala, Bombay, India. After removal of the epidermis, the biopsies were homogenized in a Kinematic Polytron Homogenizer (Brinkmann Instruments, Inc., Westbury, New York, U.S.A.), and the mycobacteria isolated essentially by the World Health Organization (WHO) protocol 1/79 ([12]), using the aqueous two-phase system (PEG 6000, Dextran).

Sonication. Soluble proteins of the ICRC bacilli and *M. leprae* were prepared by sonicating the organisms in a Biosonik III sonicator (Bronwill Scientific Co., Inc., Rochester, New York, U.S.A.) at 80 W intensity for 2 hr at 4°C to obtain the maximum yield of extractable proteins. The time of sonication was determined by preliminary studies in which sonicates obtained at different time intervals were tested for protein content. The sonicate was centrifuged at 50,000 rpm in a Kontron Ultracentrifuge for 1 hr

at 4°C, and the supernatant filtered through a 0.22 μ Millipore filter.

Fraction of the sonicates

HPLC. Fractionation of the sonicates was carried out by HPLC (Waters Associates, Milford, Massachusetts, U.S.A.). The buffer system consisted of 0.02 M Tris acetate, pH 7.2, and the flow rate was 1 ml/min. Optical density (OD) was noted at 280 nm. The soluble proteins were separated on a Protein Pak 300 SW gel permeation column (exclusion limit 4 K). The high molecular weight component, eluted in the void volume of this column, was further purified on a U Bondagel E-1000 (exclusion limit 2000 K) and called PP-I (purified protein-I).

Studies in human volunteers. A comparative study of the response to intradermal administration of the PP-I fractions was carried out on healthy residents of Malwani, Malad, Bombay. Ten adult males between 20 and 45 years of age participated in this study. Leprosy is endemic in Malwani. In the second study, 100 healthy household contacts of LL patients were tested for lepromin (Mitsuda) reactivity. Of the 16 who were lepromin negative, 10 were administered 100 μg (50 μg in each forearm) of PP-I of the ICRC bacilli intradermally (Group I); the other 6, who received saline, served as controls (Group II). A lepromin test was repeated at 10 weeks.

Immunological tests

Immunodiffusion. Antigenic profiles of the PP-I fractions of the ICRC bacilli and *M. leprae* against pooled lepromatous serum (PLS) and rabbit anti-ICRC serum (RAS) were studied, using the Ouchterlony double-diffusion technique with 1% agarose in 0.5 M barbitone buffer, pH 8.6. The positions of the antigens and antibodies are shown in Figure 2. The precipitin lines were photographed under illumination.

Enzyme-linked immunosorbent assay (ELISA). The assay was performed essentially by the method of Young, *et al.* ([34]). One hundred μl of 20 μg/ml ICRC sonicates or PP-I protein were coated onto ELISA plates (Nunc, Denmark) overnight at 4°C. The antigens were reacted with 1:50 dilutions of PLS at 37°C for 2 hr. After washing, the plates were incubated with the second antibody, peroxidase-labeled goat anti-human immunoglobulin (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), for 1 hr at 37°C. The plates were again washed extensively, 100 μl (0.1 mg/ml) of o-phenylenediamine and 0.005% hydrogen peroxide were added, and the plates were incubated at room temperature for 30 min. The reaction was terminated by the addition of 20 μl 8 N HCl, and readings were taken on an automatic ELISA reader at 492 nm. In another parallel study, the test was repeated using PLS adsorbed with 100 μg PP-I of *M. leprae.*

Sources of antisera

Pooled lepromatous sera (PLS). Serum was collected from 10 untreated LL patients with a high BI (>5+) from the Acworth Leprosy Hospital. The pooled sera contained 2 ml of serum from each patient, and was stored at −20°C.

Rabbit anti-ICRC serum (RAS). Rabbits were immunized by the method of Harboe, *et al.* ([19]). Briefly, intradermal, subcutaneous, and intramuscular injections of the ICRC sonicates with Freund's incomplete adjuvant were administered at weekly, and later at monthly, intervals. The animals were bled through the ear vein 8 days after each immunization; the serum was separated and then stored at −20°C.

Skin tests

Skin test with PP-I. Twenty μg PP-I, each of ICRC and *M. leprae,* were injected intradermally into the left and right forearms, respectively, of healthy human volunteers. Erythema and/or induration was read at 48 hr and 3 weeks. In a few volunteers, elliptical biopsies of the reaction sites were taken, fixed in 10% formol saline, and 5 μm sections were stained with hematoxylin and eosin (H&E).

Lepromin test. The lepromin test was carried out using lepromin provided by Dr. W. F. Kirchheimer through the WHO. One tenth ml of Mitsuda antigen containing 4×10^7 armadillo heat-killed *M. leprae* per ml was injected intradermally. The local reaction (Mitsuda type) was read at 3 weeks, and an induration of 3 mm or more was considered positive.

Biochemical tests

Isoelectric focusing (IEF). Since the PP-I-proteins were of very high molecular weight,

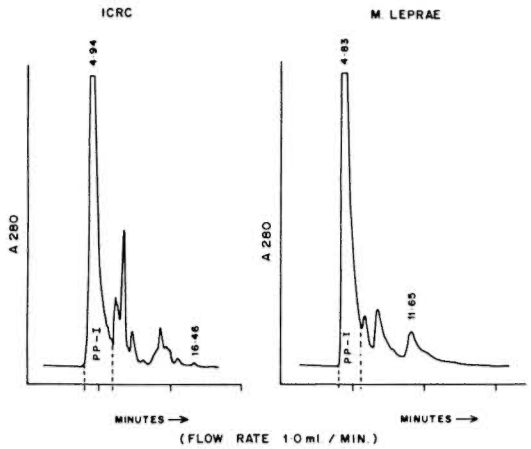

FIG. 1. HPLC profiles of sonicates of ICRC and human-derived *M. leprae* showing 5 and 4 peaks, respectively. The major peaks, elution time 4.94 and 4.83 min, eluted in the void volume.

IEF in polyacrylamide gels was not possible, even when a very low concentration gel was used. Thus, an alternative method using a sucrose density gradient as the supporting medium was used. IEF was done on a 110 ml LKB type 8101 column at 4°C with a pH gradient generated using physiological buffers HEPES and BICINE. The sample containing 500 μg of PP-I of ICRC and 500 μg of PP-I of *M. leprae,* either separately or in a single sample, was added in the center of a 20–50% sucrose density gradient. To focus the proteins, 1100 volts was applied for 3 hr. One ml fractions were collected, and the pH and OD at 280 nm noted to compute the pI values.

Analytical methods

The techniques employed in estimating components of the HPLC fractions of the PP-Is were as follows: Protein, Hartree's method ([20]) with bovine serum albumin (BSA) as standard; carbohydrate, Dubois' ([13]) method with glucose as standard; lipids, Saito and Sato technique ([30]) with palmitic and linoleic acid as standards. The values of protein, carbohydrate, and lipid were expressed as relative values, considering the values of protein as 100.

Amino acid analysis. Amino acid analysis of the PP-Is was determined on an LKB Alpha plus amino acid analyzer (LKB, Biochrome, U.K.) using the sodium citrate buffer system. One hundred μg of the sample was hydrolyzed with 6 N HCl at 110°C

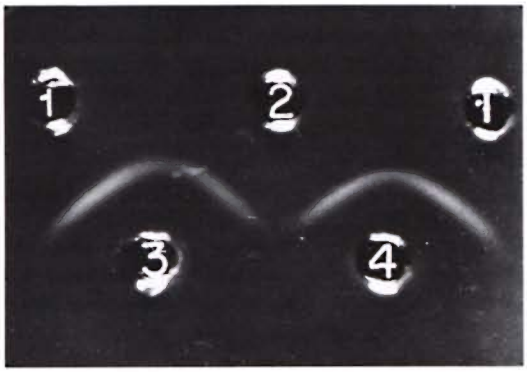

FIG. 2. Immunodiffusion showing lines of identity between ICRC PP-I and *M. leprae* PP-I. Well 1 = ICRC PP-I; well 2 = *M. leprae* PP-I; well 3 = RAS; well 4 = PLS.

in vacuo for 24 hr. The sample was then lyophilized and reconstituted in 0.2 N sodium citrate buffer, pH 2.2. The relative ratios of the amino acids were calculated, considering the value of histidine as 1.0. The results were not corrected for losses due to hydrolyses.

RESULTS

When the ICRC sonicate was run on HPLC with a Protein Pak 300 SW column, five peaks were observed (Fig. 1). The high molecular weight fraction, which eluted in the void volume of this column, was separated and run on another column, U Bondagel E-1000. Here it separated into a single peak between blue dextran and IgM, indicating that it had an approximate molecular weight of 1 million daltons. This fraction, which was named PP-I, accounted for 70% of the protein of the sonicate. A similar high molecular weight fraction was also isolated from the sonicate of *M. leprae* which, however, yielded only four peaks (Fig. 1) on the protein-pak column.

Immunodiffusion. The PP-I of both ICRC and *M. leprae* gave single precipitin lines. Further, a complete line of identity was observed between the two, against both RAS and PLS (Fig. 2).

ELISA. In the ELISA, the ICRC sonicate gave an absorbance of 0.842 ± 0.089 (mean of 6 experiments). With the PP-I, the reading was 0.698 ± 0.106 (mean of 6 experiments). The reactivity was completely abolished when PLS was pre-adsorbed with PP-I of *M. leprae.*

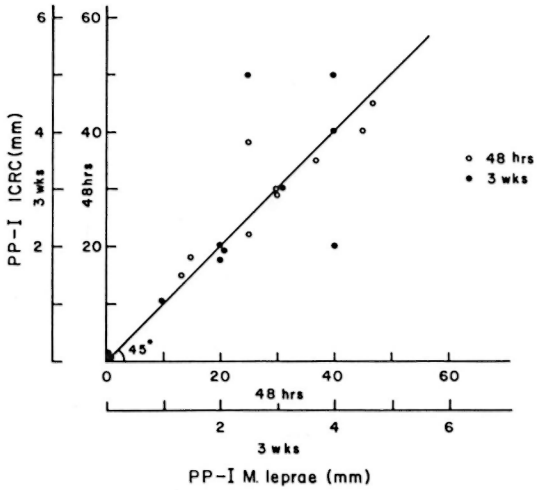

FIG. 3. Comparison of skin responses (both 48 hr and 3 week) to PP-I fractions of ICRC and *M. leprae* in healthy human volunteers, showing an excellent correlation between the responses to the fractions of the two organisms.

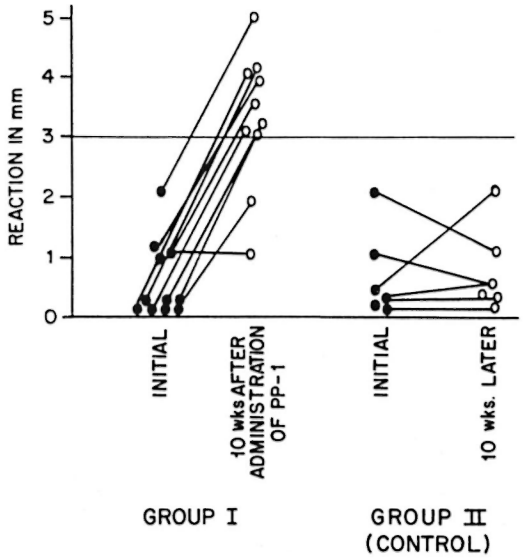

FIG. 4. Lepromin reaction before and 10 weeks after administration of ICRC PP-I (Group I). Lepromin conversion (reading of 3 mm and above) was seen in 8 out of 10 individuals (80%).

Skin response to PP-I. In healthy human volunteers, the PP-I of ICRC and *M. leprae* showed similar skin reactions at both 48 hr and 3 weeks (Fig. 3). Biopsies of the positive reactions showed granulomatous inflammation with the presence of lymphocytes, macrophages, and ill-formed giant cells.

Lepromin conversion. Eight weeks after administration of the ICRC PP-I, 8 of the 10 individuals showed lepromin conversion (Fig. 4). The controls did not show any change in their lepromin reactivity during the same period.

Biochemical parameters. The PP-I of both ICRC and *M. leprae,* when subjected to IEF separately, focused as a single peak, ICRC PP-I at pH 5 and *M. leprae* PP-I at pH 4.76 (Fig. 5, A and B). The difference in the pI values of the two was confirmed by running them as a single sample where they separated into two different peaks (Fig. 6).

Protein, carbohydrate, and lipid analysis showed that, as compared to ICRC PP-I, *M. leprae* PP-I contained ½ the amount of carbohydrate and ⅙ the amount of lipid. However, the amino acid composition of the two was quite similar (The Table). PP-Is from both ICRC and *M. leprae* contained a high number of Glu, Gly, and Ala residues.

DISCUSSION

Sonicates of both the ICRC bacilli and *M. leprae* yield a high molecular weight glycolipoprotein (PP-I) when separated on HPLC using gel permeation columns. The PP-I fractions from the two organisms show a line of identity in the double-immunodiffusion test with both RAS and PLS, and give comparable skin responses in human volunteers. Biopsies taken from representative volunteers 3 weeks after intradermal administration of the PP-I fractions exhibited ill-formed, lymphocyte-rich granulomas. PP-I, which is a rather large molecule, is likely to be a polymer of smaller units. Yet, surprisingly, it behaves like a single homogeneous protein which focuses as one band in IEF and gives a single line in immunodiffusion. Attempts are being made to cleave the molecule.

Our data show that the PP-I fractions of the ICRC bacilli and *M. leprae* contain epitopes for both B and T cells, but exhibit close antigenic relatedness with each other. The two PP-I fractions carry different charges, and also differ in their chemical composition. Since the amino acid composition of the two is almost identical, the differences in the pI may be related to the

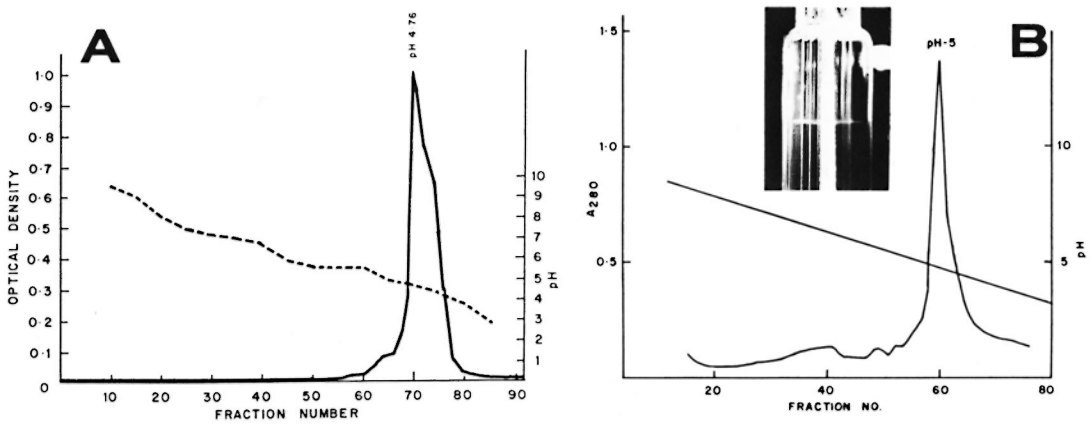

FIG. 5. Isoelectric focusing (IEF) of PP-I fractions: **A** = *M. leprae*; **B** = ICRC. Inset in **B** shows a sharply focused single band of ICRC PP-I in the IEF column.

degree of glycosylation or to qualitative differences in their carbohydrate and lipid moieties. Reitan, *et al.* ([28]) have shown that *M. leprae* antigen 7, which is a high molecular weight protein, is also a bifunctional molecule that carries both B- and T-cell epitopes. Hunter, *et al.* ([22]) have isolated a high molecular weight lipopolysaccharide antigen, LAM-B, that appears to be one of the major immunogens of *M. leprae*. It has recently been shown that antigen 60 of BCG is a very high molecular weight lipopeptidoglycan (molecular weight of 10^6–10^7). The antigen is a powerful immunogen for both B and T cells ([7, 15]). The relationship of PP-I-fractions to their antigens is being elucidated.

We have shown elsewhere that the ICRC vaccine is able to induce persistent lepromin conversion in LL patients as well as lepromin-negative healthy subjects ([5, 9]). The data from this study show that the ICRC PP-I could be the immunogenic "subunit" responsible for the lepromin conversion. There is strong clinical, epidemiologic and laboratory evidence which indicates that the late (Mitsuda) lepromin reaction correlates well with host resistance to *M. leprae* ([27]). Thus, in LL patients, who represent one end of the leprosy spectrum, the lepromin reaction is consistently negative but the tissues are laden with *M. leprae*. On the other hand, in the paucibacillary, tuberculoid form of leprosy, the lepromin reaction is strongly positive ([29]). The pioneering work of Dharmendra and Chatterjee has shown that lepromin-negative individuals in leprosy-endemic areas run a very high risk of contracting the multibacillary forms of the disease ([11]).

According to Job, *et al.* ([23]), Mitsuda-positive armadillos are relatively resistant to the disease. Leprosy has recently been induced in monkeys, with the disease showing a pattern of tissue involvement akin to that seen in man ([33]). Further, the lepromatous variety is seen in lepromin-negative animals ([1]). On the other hand, according to Bjune ([3]), the lymphocyte transformation test (LTT) using *M. leprae* antigens, the other popularly used test for T-cell functions, is not a good indicator of protective immunity. It correlates well with cellular or de-

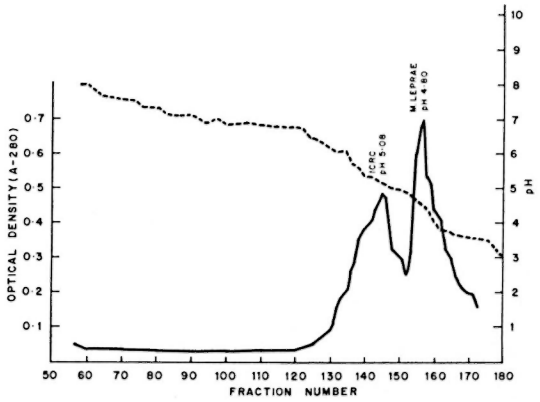

FIG. 6. Isoelectric focusing of PP-I fractions when run together as a mixture of the two, showing two distinct bands focused at pH 5.08 (ICRC) and 4.80 (*M. leprae*).

THE TABLE. *Chemical composition of PP-I fractions.*

PP-I	Protein[a]	Carbohydrate[a]	Lipid[a]	Amino acid composition[b]													
				Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
ICRC	100	47	25	5	3	5	10	3	7	7	4	1	2	4	1	3	1	4	2
M. leprae	100	25	4	5	3	5	9	3	7	7	4	1	2	4	1	2	1	4	2

[a] The protein, carbohydrate, and lipid contents are expressed as relative ratios. The results are expressed as the mean of three individual estimations, each carried out in triplicate.

[b] The values are an average of six determinations.

layed hypersensitivity. Somewhat similar conclusions could be drawn from the studies by Convit, *et al.* ([8]). In their vaccinated patients, although skin responsiveness was persistent over the years, the LTT was only occasionally positive. In monkeys, vaccination with ICRC bacilli induces lepromin conversion without affecting the *in vitro* response of lymphocytes to *M. leprae* ([6]). These observations indicate that the antigens involved in the two tests are different. Further, in terms of protective immunity, the lepromin (Mitsuda) reaction is a better indicator. For these reasons, any antigen that brings about lepromin conversion is likely to contain protective antigenic epitopes.

Global attempts are being made to develop a subunit antileprosy vaccine. The *M. leprae* genome has been recently cloned in *Escherichia coli* by Young, *et al.* ([35]). The protective antigens will have to be identified among the five demonstrated proteins before they are used in vaccine preparation. It may be mentioned that if the protective antigens turn out to be nonprotein in nature, the cloning technique may not yield the desired components for preparation of a subunit vaccine. In this study, we have adopted an alternate strategy of fractionation of the ICRC sonicate, and have isolated a high molecular weight glycolipoprotein (PP-I) which, for the reasons mentioned above, appears to be the dominant immunogen for T-cell immunity, the main defense against *M. leprae.* ICRC PP-I also meets the other desirable requirements as a candidate for preparation of a subunit vaccine, namely, it is the major constituent of a cultivable organism, hence easily available in large quantities. Further, its high lipid content would provide the necessary intrinsic adjuvant action.

The immunogenicity of the ICRC PP-I could reside in its protein, lipid, and/or carbohydrate moieties. There is extensive antigenic crossreactivity among mycobacteria ([17, 25, 31]). However, it appears that mycobacterial specificity resides in the chemical nature of their glycolipids, and a phenolic glycolipid which is a specific antigen for *M. leprae* has been identified ([21]). As mentioned above, the PP-I fractions of the ICRC bacilli and *M. leprae* have almost identical amino acid compositions. The nature of the

carbohydrates and lipids in these fractions is under investigation.

Although patients may show individual variations, in general, in the five-stage classification of Ridley and Jopling there is an inverse relationship between cell-mediated immunity (CMI) and humoral immunity. In the highly bacilliferous lepromatous patients, high levels of circulating anti-*M. leprae* antibodies are observed, but the patients show specific anergy to the antigens of the organism. The reverse is the case with the tuberculoid variety of the disease, wherein the patients exhibit strong CMI but the antibodies are present in very low concentrations.

A major challenge in leprosy vaccinology is how to correct the T-cell defect/tolerance in the LL patients. One of the approaches could be to modify the antigen(s) in such a manner that the unwanted and harmful epitopes are removed through molecular engineering. However, before this could be done it would be essential to know the nature and molecular basis of the immune imbalance, on which there is as yet no unanimous opinion. Using the LTT as the assay system in LL patients, Bloom and Mehra ([4]) have reported stimulation of T-suppressor cells with *M. leprae* antigens and have further identified the phenolic glycolipid as the responsible antigen. On the other hand, other workers have demonstrated stimulation of the T-suppressor cell in TT patients ([26, 32]). Their data suggest that the increased suppression in TT is directed toward the B-cell response which is abrogated, promoting the T-cell response. It is very essential to resolve this question of segregation of antigenic moieties responsible for the stimulation of B and T cells and the interaction of immunoregulatory T cells with other cells of the immune system in order to provide a rational basis for molecular engineering. Depending on the cellular and molecular basis of the imbalance, the harmful moieties could be removed, giving a molecule that would selectively induce protective immunity. For the reasons mentioned above, the ICRC PP-I contains not only protective antigens but also the epitopes for both types of immune responses. It would be, therefore, a good candidate for such molecular engineering.

SUMMARY

The administration of a vaccine containing ICRC bacilli, which is currently undergoing clinical trials in India, induces persistent lepromin conversion in lepromatous leprosy (LL) patients and lepromin-negative healthy subjects, with "upgrading" of tissue response in the former. A sonicate of ICRC bacilli, when subjected to gel-filtration chromatography using high-pressure liquid chromatography (HPLC), yields a high molecular weight glycolipoprotein (PP-I) with an apparent molecular weight of 10^6 daltons. PP-I, which brings about lepromin conversion in lepromin-negative healthy subjects, is a major immunogen of the organism, and carries epitopes for both B and T cells. A similar high molecular weight glycolipoprotein (PP-I *Mycobacterium leprae*) has been isolated from the sonicate of *M. leprae.* The two PP-I fractions exhibit a close antigenic relatedness at both B- and T-cell levels. However, they differ in their chemical composition and carry different charges.

PP-I of ICRC is not only a good immunogen. Its high lipid content provides the necessary built-in adjuvant that would make it a good candidate for a "subunit" antileprosy vaccine. Also, since it carries epitopes for both B and T cells, PP-I ICRC could be used for "molecular engineering" to obtain molecules which selectively stimulate T-cell immunity which is the dominant host defense against *M. leprae.*

RESUMEN

La administración de una vacuna conteniendo bacilos ICRC, la cual se usa actualmente en ensayos clínicos en la India, induce una conversión a la lepromina persistente en los pacientes con lepra lepromatosa y en sujetos sanos lepromino-negativos, con una "mejoría" de la respuesta tisular en los primeros. Cuando se fracciona un sonicado de bacilos ICRC por cromatografía de líquidos de alta presión, se aisla una glicoproteína (PP-I) con un peso molecular de 10^6 daltones. La PP-I, responsable de la conversión en los sujetos sanos lepromina-negativos, es un inmunógeno "mayor" del microorganismo y porta epitopes tanto para linfocitos B como para linfocitos T. Una glicoproteína de alto peso molecular similar a la PP-I, se ha aislado de sonicados de *M. leprae* (PP-I *Mycobacterium leprae*). Las 2 fracciones PP-I exhiben una cercana relación antigénica en cuanto a la respuesta que inducen sobre las

células T y B, sin embargo, difieren en su composición química y portan diferentes cargas.

La PP-I de ICRC no solo es un buen inmunógeno sino que además, su alto contenido en lípidos le proporciona la adyuvanticidad necesaria para considerarla como un buen candidato de una vacuna antileprosa. También, puesto que porta epitopes para las células B y T, la PP-I ICRC podría usarse en "ingeniería molecular" para obtener moléculas capaces de estimular la inmunidad mediada por T, la cual representa la defensa predominante del huésped contra el *M. leprae.*

RÉSUMÉ

L'administration d'un vaccin contenant le bacille ICRC, qui fait actuellement l'objet d'essais cliniques en Inde, a entraîné un virage permanent de la réaction à la lépromine chez des malades atteints de lèpre lépromateuse (LL) et chez des individus sains négatifs à la lépromine. On a également observé une amélioration (upgrading) de la réponse tissulaire dans la lèpre lépromateuse. Un sonicat de bacilles ICRC, lorsqu'on le soumet à la chromatographie sur gel avec un liquide à haute pression (HPLC), permet d'isoler une glycolipoprotéine de poids moléculaire élevé (PP-I), d'approximativement 10^6 daltons. Cette glypolipoprotéine PP-I, responsable du virage de la réaction à la lépromine chez les individus sains négatifs à cet antigène, est un immunogène important pour l'organisme et il porte des épitopes à la fois pour les cellules B et pour les cellules T. Une glypolipoprotéine semblable de poids moléculaire élevé (PP-I *Mycobacterium leprae*) a été isolée du sonicat de *M. leprae.* Les deux fractions PP-I témoignent d'une relation antigénique étroite en ce qui concerne tant les cellules B que les cellules T. Néanmoins, elles diffèrent en leur composition chimique et portent des charges différentes.

Le PP-I de ICRC n'est pas seulement un bon immunogène. Son contenu lipidique élevé inclut l'adjuvant nécessaire qui pourrait en faire un bon candidat pour un vaccin antilépreux constitué de sous-unités. Cette protéine, du fait qu'elle port également des épitopes pour les cellules B et T, elle pourrait être utilisée pour des manipulations moléculaires en vue d'obtenir des molécules qui simuleraient de manière sélective l'immunité aux cellules T, qui constitue la défense principale de l'hôte contre *M. leprae.*

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