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Immunoreactivity of Mycobacterial Strain ICRC and *Mycobacterium leprae* Antigens with Polyclonal and Monoclonal Antibodies[1]

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The inability to cultivate *Mycobacterium leprae in vitro* has posed major problems in defining the *M. leprae* antigens which are involved in specific host immune responses. The use of murine monoclonal antibodies and recombinant DNA expression libraries have led to the identification and characterization of species-specific and crossreactive epitopes on protein and polysaccharide antigens of *M. leprae* ([4, 5, 10, 11, 14, 20]). Of these, *M. leprae* proteins of 70, 65, 35, 18 and 12 kDa were identified as major target antigens for humoral immune responses. Despite several efforts, it has not been possible to identify *M. leprae* proteins involved in protective immunity. These reports have prompted investigators to look at antigens present on other cultivable mycobacteria that may share crossreactive epitopes with *M. leprae* ([6, 9, 16]).

ICRC, a group of cultivable, leprosy-derived mycobacteria, are undergoing clinical trials as an antileprosy vaccine in India ([7]). The vaccine brings about persistent lepromin conversion and upgrading of skin lesions in vaccinated lepromatous leprosy (LL) patients ([7]). Several laboratory investigations have demonstrated antigenic relatedness between ICRC and *M. leprae* antigens ([3, 8]). Recently, we have reported that sera from leprosy patients across the clinical spectrum, healthy contacts of leprosy patients, tuberculosis patients and healthy individuals were able to immunoprecipitate several common antigens of ICRC and *M. leprae* ([2]).

Using *M. leprae* antigens, it was not possible to differentiate among the reactivity patterns of sera from leprosy patients, contacts, or healthy individuals since all of these sera identified *M. leprae* antigens with molecular masses of 47, 36, 21 and 14 kDa. When the same sera were tested with ICRC antigens, it was observed that the 21-kDa protein of ICRC was precipitated exclusively by sera from all LL patients while the 14-kDa protein of ICRC was precipitated by sera from a few LL patients (5 of 26) but all of their contacts tested. These results suggest that the 21-kDa and 14-kDa proteins of ICRC may be useful in the serodiagnosis of leprosy.

In the present investigation, we have further established the crossreactivity between the antigens of ICRC and *M. leprae* using anti-ICRC and anti-*M. leprae* polyclonal antibodies as well as monoclonal antibodies specific to *M. leprae* in radioimmunoprecipitation assays. Our studies demonstrate that immunodominant antigens of 21 kDa and 14 kDa of ICRC share crossreactive antigenic determinants with the 14-kDa antigen of *M. leprae*. These studies provide an impetus to delineate and to analyze T- and B-cell epitopes present on the 21-kDa and 14-kDa proteins of ICRC. Such immunodominant proteins may provide important reagents for designing subunit vaccines and skin-test reagents.

MATERIALS AND METHODS

Antigens. ICRC bacilli, isolate C-44, were maintained in Dubos' modified medium as described by Chirmule, *et al.* ([3]). A sonicate of ICRC bacilli was prepared as previously described by Chiplunkar, *et al.* ([2]). The ICRC bacilli were sonicated at 80W on ice for 60 min at 50% duty cycle in a Branson sonifier (Branson Ultrasonics Corp., Danbury, Connecticut, U.S.A.). The bacterial extract was centrifuged at 218,200

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× g × 1 hr at 4°C. The supernatant, which contained soluble proteins, referred to as the sonicate of ICRC, was collected. After determination of the protein concentration ([17]), aliquots of the sonicate were lyophilized and stored at −20°C.

The sonicate of armadillo-derived and irradiated *M. leprae* (batch CD122) was kindly provided by Dr. R. J. W. Rees through the World Health Organization/Immunology of Leprosy program.

Polyclonal antibodies. Anti-*M. leprae* polyclonal antibodies raised in rabbits were a kind gift from Dr. P. Brennan, Colorado State University, Fort Collins, Colorado, U.S.A.

Anti-ICRC polyclonal antibodies were raised in rabbits as described by Harboe, *et al.* ([13]). Young female rabbits were obtained from the Haffkine Institute, Bombay, India. The rabbits were immunized with ICRC sonicate (500 μg) in 0.5 ml of phosphate buffered saline (PBS; 10 mM, pH 7.5), emulsified thoroughly with an equal volume of Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, Missouri, U.S.A.). Booster immunizations consisting of the same dose were given on days 2, 16, 30, 44, 72 and 100, and thereafter every fourth week for a period of 8 months. The antigen was administered at multiple sites: a) subcutaneously in the foot pad, b) intradermally in the scapular region, and c) intramuscularly in the thigh. Serum was obtained before the initial injection and at 8–10 days after the last immunization, aliquoted, and stored at −80°C.

Monoclonal antibodies. *M. leprae*-specific monoclonal antibodies (MAb) WML06 (recognizing the 12-kDa protein of *M. leprae*), WML03, and WML04 (recognizing the 35-kDa protein of *M. leprae*) were a kind gift from Dr. J. Ivanyi, Medical Research Council, London, U.K.

Immunoprecipitation assay. The sonicates of ICRC and *M. leprae* were labeled with Na ^{125}I (Amersham, England) by using the chloramine-T method described earlier by Chiplunkar, *et al.* ([2]). Radiolabeled mycobacterial antigens (4 × 10^6 cpm) in Tris-HCl buffer (50 mM, pH 8.3) containing 0.6 M NaCl and 0.5% Triton X-100 precleared by incubating them with 10 μl of 1:10 diluted normal BALB/c mouse serum (for MAb) or normal rabbit serum (for anti-ICRC/anti-*M. leprae* polyclonal antibodies raised in rabbits), for 2 hr on ice. The nonspecific immune complexes formed were precipitated by incubation for 30 min on ice with 25 μl of a 10% (w/v) suspension of *Staphylococcus aureus* Cowan (Sigma). This mixture was centrifuged at 10,000 × g × 5 min. The preclearing step was repeated. The supernatant containing precleared ^{125}I-labeled mycobacterial sonicate was incubated with 15 μl of *M. leprae*-specific MAb, or anti-ICRC polyclonal antibodies, or anti-*M. leprae* polyclonal antibodies for 2 hr on ice. The immune complexes were precipitated by incubation for 30 min on ice with 25 μl of a suspension of *S. aureus* Cowan. The precipitates were washed three times with Tris-HCl buffer (50 mM, pH 8.3), with a change of microtube for the last wash. Bound antigen was eluted by boiling the pellet in 30 μl of the electrophoresis sample buffer consisting of 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, and 0.0001% bromophenol blue in 62.5 mM Tris HCl, pH 6.8.

Electrophoresis and autoradiography. The immunoprecipitated antigens were separated by using 5% to 20% gradient SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli ([15]). For visualization of protein bands, the dried gels were exposed to Fuji X-ray films (Fuji Photo Film Co. Ltd., Tokyo, Japan) for 24 hr to 72 hr at −70°C.

Molecular mass estimation. The molecular masses of proteins were determined as described by Hames ([12]). For all of the proteins, the relative mobility (R_f) was measured with reference to a tracking dye. Using linear regression analysis, a standard curve of the molecular mass versus the R_f values of the standard marker proteins was plotted. Molecular masses of the unknown proteins were estimated from the standard curve.

RESULTS

Immunoprecipitation of ICRC and *M. leprae* antigens with *M. leprae*-specific MAb. An autoradiogram of ^{125}I-labeled *M. leprae* sonicate, separated on the gradient SDS-PAGE, revealed that proteins of molecular mass 63, 57, 36, 23, 21 and 14 kDa were radiolabeled (Fig. 1B, Lane a). Of these, the 14-kDa protein was intensely labeled. As

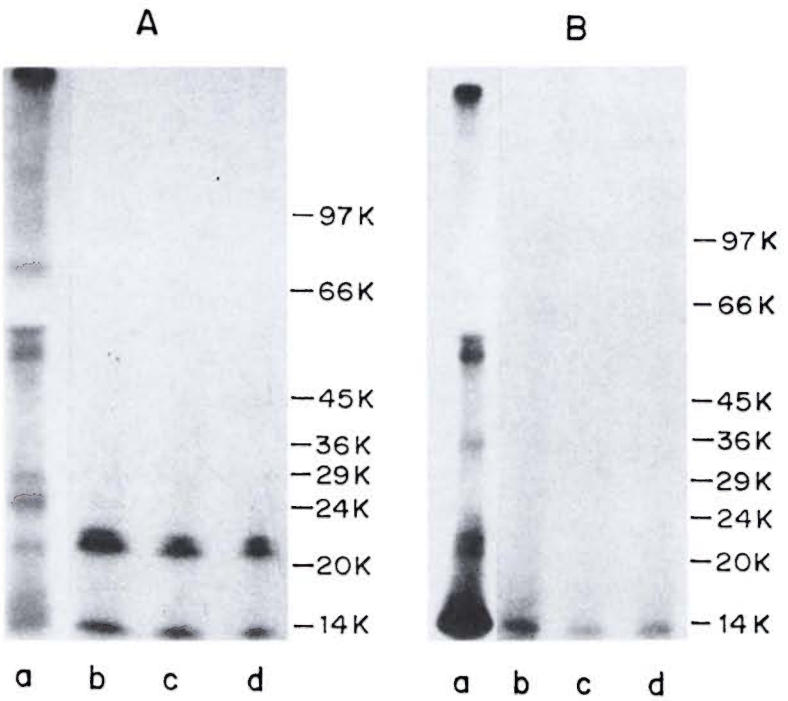

FIG. 1. Immunoprecipitation profiles of ^{125}I-labeled ICRC (**A**) and *M. leprae* (**B**) antigens with MAb WML06 at various dilutions (Lane b = 1:100; Lane c = 1:500; Lane d = 1:1000). Lane a in each panel represents autoradiogram of ^{125}I-labeled sonicate. Molecular mass markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; phenylmethylsulfonyl fluoride-treated trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.2 kDa) are shown on right of each panel.

seen in Figure 1B, Lanes b–d, MAb WML06 immunoprecipitated one protein of *M. leprae* of apparent molecular mass of 14-kDa at all three different dilutions tested. Although this MAb is known to recognize the 12-kDa protein of *M. leprae,* in our hands it showed reactivity with the 14-kDa protein of *M. leprae* as calculated from the R_f values.

On radioiodination, the ICRC sonicate exhibited protein bands in the molecular mass range of 81, 60, 55, 29, 25, 21 and 14 kDa (Fig. 1A, Lane a). When ^{125}I-labeled ICRC antigens were immunoprecipitated with *M. leprae*-specific MAb WML06, the MAb recognized the ICRC proteins of molecular mass 21 kDa and 14 kDa at all the dilutions tested (Fig. 1A, Lanes b–d).

M. leprae-specific MAb WML03 and WML04, known to react with the 35-kDa protein of *M. leprae* ([14]), showed intense reactivity with the 35-kDa protein of *M. leprae* and also demonstrated crossreactivity to *M. leprae* protein of 14 kDa to some extent (Fig. 2, Lanes b and d). When the same MAb (WML03 and WML04) were tested for their reactivity with ^{125}I-labeled ICRC antigens, it was observed that these antibodies were unable to precipitate any ICRC antigen, (Fig. 2, Lanes a and c).

Immunoprecipitation of ICRC and *M. leprae* antigens with anti-ICRC and anti-*M. leprae* polyclonal antibodies. Immunoprecipitation of ^{125}I-labeled ICRC and *M. leprae* sonicates with anti-ICRC and anti-*M. leprae* polyclonal antibodies showed the presence of crossreactive antigens on these mycobacteria. As seen in Figure 3, Lane a, anti-ICRC polyclonal antibodies identified ICRC proteins of 66, 42, 29 and 21 kDa. Of these, the 21-kDa ICRC protein was precipitated by anti-ICRC polyclonal antibodies with very high intensity. When the same serum was tested with ^{125}I-labeled *M. leprae* antigens, it was observed that only two proteins of molecular mass 21 kDa and 14 kDa were strongly immunoprecipitated (Fig. 3, Lane b).

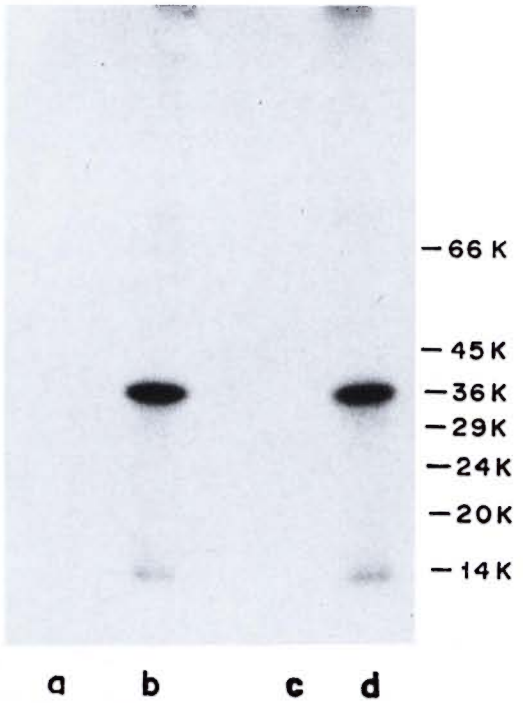

FIG. 2. Immunoprecipitation profiles of ^{125}I-labeled ICRC (Lanes a and c) and *M. leprae* (Lanes b and d) with MAb WML03 (Lanes a and b) and MAb WML04 (Lanes c and d) at 1:100 dilution. Molecular mass markers are shown on right.

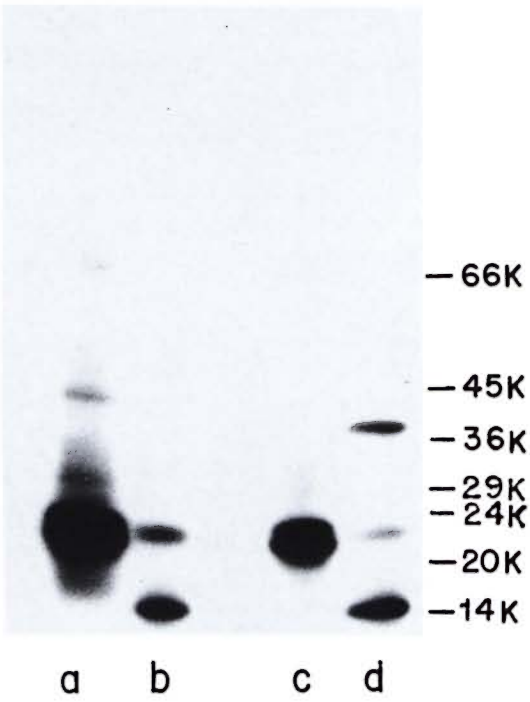

FIG. 3. Immunoprecipitation profiles of ^{125}I-labeled ICRC (Lanes a and c) and *M. leprae* (Lanes b and d) with polyclonal anti-ICRC (Lanes a and b) and anti-*M. leprae* (Lanes c and d) antibodies at 1:100 dilution. Molecular mass markers are shown on right.

A similar pattern of reactivity was observed when ^{125}I-labeled ICRC and *M. leprae* were tested with anti-*M. leprae* polyclonal antibodies (Fig. 3, Lanes c and d). Three major proteins of *M. leprae* of molecular mass 36, 21 and 14 kDa were identified by anti-*M. leprae* polyclonal antibodies, of which the 14-kDa protein showed intense reactivity (Fig. 3, Lane d). The cross-reactivity between the antigens of ICRC and *M. leprae* was confirmed further when anti-*M. leprae* polyclonal antibodies immunoprecipitated the 21-kDa ICRC protein with an intensity comparable to that observed with anti-ICRC polyclonal antibodies (Fig. 3, Lanes a and c).

DISCUSSION

The importance of studying antigens present on cultivable mycobacteria has emerged from studies by several workers ([1, 18]). They suggest that protective antigens of *M. leprae* are those that are shared by mycobacterial species rather than species-specific antigens.

In the present investigations, we have analyzed antigens present on a cultivable mycobacterium, ICRC, used as an antileprosy vaccine in India. Our earlier studies using crossed-immunoelectrophoresis have demonstrated antigenic relatedness between ICRC and *M. leprae* ([3]). The present data confirm and extend these findings, using a more-sensitive technique and specific probes such as anti-ICRC and anti-*M. leprae* polyclonal antibodies and *M. leprae*-specific monoclonal antibodies.

Our results demonstrate that the polyclonal anti-ICRC sera immunoprecipitated ICRC antigens of 66, 42, 29 and 21 kDa. Of these, the 21-kDa protein of ICRC was precipitated with very high intensity. Anti-*M. leprae* polyclonal antibodies also precipitated this antigen with equal intensity, thereby establishing the immunodominance of the 21-kDa ICRC protein and the presence of crossreactive epitopes on this

protein. Exclusive reactivity of the 21-kDa protein of ICRC with sera of LL patients and LL patients in ENL reaction has been reported earlier [2]. In the present study, we observed that MAb WML06, known to recognize the 12-kDa protein of *M. leprae* [14], immunoprecipitated the 14-kDa protein of *M. leprae* when tested in our laboratory using a 5%–20% gradient SDS-PAGE. The difference in the molecular mass of the protein recognized by MAb WML06 could be due to personal variations in experiments performed. We wish to emphasize that the 12-kDa protein of *M. leprae* reported by others and the 14-kDa protein of *M. leprae* identified by us are identical. We observed that the *M. leprae*-specific MAb WML06 reacted with the 21-kDa and 14-kDa proteins of ICRC. We have earlier shown that the 14-kDa protein of ICRC was identified by sera from all contacts of leprosy patients [2].

Crossreactivity of *M. leprae*-specific MAb with other cultivable mycobacteria such as *M. habana* [16] and *Mycobacterium w* [9] has also been reported. Lamb, *et al.* [16] demonstrated that the 18-kDa antigen of *M. habana* shares crossreactive epitopes with a *M. leprae* protein of a similar mass when analyzed with *M. leprae*-specific MAb L5. Ganju, *et al.* [9] demonstrated that *M. leprae*-specific MAb WML06 reacted with the 10-, 23- and 35-kDa proteins of *Mycobacterium w* when analyzed using Western blotting.

We observed that anti-*M. leprae* polyclonal antibodies immuno-precipitated the 36-, 21-, and 14-kDa antigens of *M. leprae.* Of these, the 21-kDa and 14-kDa proteins of *M. leprae* also were recognized by polyclonal anti-ICRC antibodies. These data again indicate that the 21-kDa and 14-kDa proteins of *M. leprae* share crossreactive epitopes with antigens of ICRC.

Another interesting observation was that ICRC antigens could not be immunoprecipitated with other *M. leprae*-specific MAb, such as WML03 and WML04 which bind to an epitope expressed by the 35-kDa protein of *M. leprae* and have similar paratope specificities. On the other hand, we observed that MAb WML06, which recognizes the 14-kDa protein of *M. leprae,* showed reactivity with the 21-and 14-kDa proteins of ICRC. These results indicate that the epitopes present on the 21-and 14-kDa proteins of ICRC are shared only with the 14-kDa protein of *M. leprae* and not with the 35-kDa protein of *M. leprae.*

Ottenhoff, *et al.* [19] have reported that the 12-kDa and 36-kDa proteins of *M. leprae,* recognized by MAb WML06 and WML03, respectively, were able to stimulate T cells of TT patients. Using SDS-PAGE separated nitrocellulose-blotted antigens of ICRC, we were able to demonstrate that the 21-kDa and 14-kDa proteins of ICRC stimulated T-cell responses in tuberculoid (TT) leprosy patients (unpublished observations), indicating that these proteins may harbor T-cell epitopes.

In conclusion, the present studies provide an impetus to explore further T- and B-cell epitopes present on the 21-kDa and 14-kDa antigens of ICRC. It would be interesting to dissect the T-cell reactivity patterns of these proteins using lymphocytes from patients at the polar ends of the leprosy spectrum. We believe that the epitope mapping and sequencing of the 21-kDa and 14-kDa proteins of ICRC would reveal important immunogenic domains of these proteins that may be useful for designing diagnostic reagents and, possibly, for immunotherapy.

SUMMARY

ICRC, a cultivable mycobacterium, is undergoing clinical trials as an antileprosy vaccine in India. In the present study, we have investigated the crossreactivity between antigens of the mycobacterial strains of ICRC and *Mycobacterium leprae* using polyclonal and monoclonal antibodies in a radioimmunoprecipitation assay. It was observed that polyclonal anti-ICRC and anti-*M. leprae* antibodies showed predominant reactivity to a 21-kDa protein of the mycobacterial strain ICRC and the 21- and 14-kDa proteins of *M. leprae.* Crossreactivity between the antigens of the mycobacterial strains ICRC and *M. leprae* was established further by using *M. leprae*-specific monoclonal antibody WML06 (reacting with the 14-kDa protein of *M. leprae*), which identified the 21- and 14-kDa proteins of the mycobacterial strain ICRC. Thus, our studies demonstrate that the 14-kDa protein of *M. leprae,* which is known to harbor T- and B-cell epitopes, shares crossreactive antigenic determinants with the 21- and 14-kDa

proteins of the mycobacterial strain ICRC. We believe that such proteins may provide important reagents for designing subunit vaccines and for determining skin-test reagents.

RESUMEN

En la India se llevan a cabo estudios de campo para valorar la eficiencia de ICRC, una microbacteria cultivable, como vacuna antileprosa. En este estudio, utilizando anticuerpos monoclonales y policlonales, y un ensayo de radioinmunoprecipitación, investigamos la reactividad cruzada entre los antígenos de ICRC y de *Mycobacterium leprae*. Observamos que los anticuerpos policlonales anti-ICRC y anti-*M. leprae* mostraron una reactividad predominante contra una proteína de 21 kDa de la cepa ICRC y contra las proteínas de 21- y de 14-kDa del *M. leprae*. La reactividad cruzada entre los antígenos de las cepas ICRC y *M. leprae* se confirmó usando el anticuerpo monoclonal WML06 (específico para la proteína de 14 kDa del *M. leprae*) el cual identificó a las proteínas de 21-y de 14-kDa de la cepa ICRC. Así, nuestros estudios demuestran que la proteína de 14 kDa del *M. leprae*, la cual se sabe contiene epítopes para células T y B, comparte determinantes antigénicos con las proteínas de 21-y 14-kDa de la cepa micobacteriana ICRC. Pensamos que tales proteínas pueden ser importantes para el diseño de vacunas a base de subunidades y para la elaboración de reactivos para pruebas en piel.

RÉSUMÉ

L'ICRC, une mycobacterie cultivable, subit actuellement des essais cliniques en Inde en tant que vaccin anti-lèpre. Nous avons analysé dans la présente étude la réactivité croisée entre des antigènes des souches mycobactériennes d'ICRC et de *Mycobacterium leprae* à l'aide d'anticorps polyclonaux et monoclonaux dans un test de radio-immunoprécipitation. On a observé que les anticorps polyclonaux anti-ICRC et anti-*M. leprae* montraient une réactivité prédominante vis-à-vis d'une protéine de 21 kDa de la souche mycobactérienne d'ICRC et des protéines de 21 et 14 kDa de *M. leprae*. L'existence d'une réactivité croisée entre les antigènes des souches mycobactériennes d'ICRC et *M. leprae* a été confirmée par l'utilisation de l'anticorps monoclonal WML06 spécifique de *M. leprae* (réagissant avec la protéine de 14 kDa de *M. leprae*); cet anticorps identifia les protéines de 21 et 14 kDa de la souche mycobactérienne ICRC. En conséquence, nos études démontrent que la protéine de 14 kDa de *M. leprae*, qui est connue comme hébergeant les épitopes des cellules T et B, partage des déterminants antigéniques réagissant de manière croisée avec les protéines de 21 et 14 kDa de la souche mycobactérienne d'ICRC. Nous croyons que de telles protéines pourraient fournir des réagents importants pour développer des sous-unités vaccinales et des réagents pour des tests cutanés.

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