

Recognition of *Mycobacterium leprae* Recombinant 18-kDa Proteins in Leprosy¹

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An increasing number of *Mycobacterium leprae* genes have been cloned and sequenced (¹⁸). Several of these have been shown to belong to the family of heat-shock proteins, such as the *M. leprae* 65-kDa and 18-kDa antigens (^{8, 12}), and are available through the World Health Organization IMMLEP bank. Most of the *M. leprae* proteins exhibit immunologic crossreactivity at the antibody level with proteins from other mycobacteria. However, the determinant of the 18-kDa protein recognized by the monoclonal antibody L7.15 (²), later designated as L5, seemed to be species-specific for *M. leprae* (⁶). Human T-cell recognition of the 18-kDa antigen was first reported using crude *Escherichia coli* lysates containing the antigen as the β -galactosidase fusion protein; these preparations stimulated T-cell clones from *M. leprae*-vaccinated donors (⁶). When the 18-kDa β -galactosidase (18K β gal) fusion protein was purified and used in proliferation assays with human peripheral blood mononuclear cells, there was a substantial antigen-specific response to β -galactosidase which masked T-cell responses to the 18-kDa antigen itself (¹⁵). The 18-kDa gene was, therefore, recloned and expressed fused to a short leader sequence containing six consecutive asparagine residues (18K Asn) to reduce proteolysis; this protein stimulated T-cell proliferation in a high proportion of patients with the tuber-

culoid type of leprosy and in healthy contacts of leprosy patients (³). This 18-kDa gene, originally cloned in λ gt11 (¹⁹) was found to contain only 70% of the whole 18-kDa gene as sequenced from a genomic *M. leprae* cosmid library (¹). The full-length 18-kDa antigen (18K JDW) has now been cloned, purified and tested for B- and T-cell reactivity in mice (^{1, 4, 5, 7}).

The choice of expression systems in the presence or absence of β -galactosidase (β gal) or a leader sequence and the method of purification may all affect the antigenic epitopes recognized by B and T cells to variable extents. In addition, epitopes recognized by the leprosy patients at both the B- and T-cell level may be different than those recognized by mice. We have, therefore, directly compared these three preparations of the *M. leprae* 18-kDa protein (18K Asn, 18K β gal and 18K JDW) for their immunological reactivity in patients with leprosy.

MATERIALS AND METHODS

Patients and controls. Untreated leprosy patients presenting at the Marie Adelaide Leprosy Center, Karachi, Pakistan, were recruited for our studies and have been described in detail elsewhere (^{8, 9}). Patients are diagnosed clinically as well as histologically on a 4-mm punch biopsy taken from the edge of an active lesion. Ninety-six patients across the leprosy spectrum, two healthy contacts of leprosy patients, and employees working at The Aga Khan University, Karachi, Pakistan, with no known contact with leprosy (endemic controls) were included in the study.

Antigens. The recombinant *M. leprae* 18-kDa preparations used in the study were 18-kDa β -galactosidase (18K β gal), and asparagine 18-kDa fusion proteins (18K Asn), both of which contained the carboxy-terminal two-thirds of the full 18-kDa gene (¹⁵), and the full length 18-kDa protein (¹)

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(18K JDW). All three recombinant 18K preparations were *E. coli*-derived; 18K β gal was purified by preparative SDS polyacrylamide gel electrophoresis (SDS-PAGE) and acetone precipitated; 18K Asn was purified by high pressure liquid chromatography (HPLC); 18K JDW was first ammonium sulfate precipitated and further subjected to HPLC; β -galactosidase was prepared in the same way as the 18K β gal antigen after induction of *E. coli* with isopropyl- β -D-thiogalactopyranoside (IPTG). All proteins were > 95% pure as assessed by SDS-PAGE.

Armadillo-derived, soluble, whole *M. leprae* sonicate (MLSON) (Batch CD114 and CD135) was a gift from Dr. R. J. W. Rees, National Institute of Medical Research, London, U.K., through the IMMLEP leprosy antigen bank.

Antisera. Five-ml blood samples collected from both leprosy patients and endemic controls were allowed to separate overnight at 4°C. The serum was removed and centrifuged at 2000 rpm for 15 min; the clear supernatant was distributed in small aliquots and frozen at -70°C before use.

Antibody determinations. Plates were coated with 100 μ l of antigens at 1 μ g/ml in carbonate buffer, pH 9.6, for 4 hr at 37°C and then overnight at 4°C. After washing, blocking with 200 μ l phosphate buffered saline (PBS) containing 5% bovine serum albumin (BSA) was performed for 2 hr at 37°C. After washing three times in PBS-Tween 20, 100 μ l of sera diluted in PBS-Tween containing 1.0% BSA was added and incubated for a further 2 hr at 37°C; 100 μ l alkaline phosphatase-labeled goat anti-human IgG (Fc specific; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, U.S.A.) diluted 1/1000 was used. In some assays biotinylated goat anti-human IgG diluted 1/1000 (Fc specific; Jackson) followed by streptavidin-coupled alkaline phosphatase, diluted 1/1000, was used instead of the first conjugate. In the avidin-biotin system, plates were coated with 0.1 μ g/ml of antigen due to the high sensitivity of the assay system. In both cases, the reaction was stopped with the addition of 50 μ l 3 N NaOH. Sera were initially screened at 1/100 and four sera with high levels of antibodies to 18K were pooled and used as a reference positive control in all subsequent assays. For some of the ELISAs, two additional internal con-

trols representing high and low activity to 18K were also run simultaneously. In addition, at least two control sera with no activity to 18K were repeatedly run to monitor the background binding. Correlation of antibody activity to 18K Asn and 18K β gal was carried out on 65 sera (38 LL/BL and 37 BT/TT) and 18K Asn and 18K JDW on 39 sera (29 LL/BL and 10 BT/TT) from untreated leprosy patients.

Lymphocyte transformation test. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood on Ficol-Histopaque 1077 (Sigma, Poole, U.K.). The study group consisted of 4 endemic controls, and 7 untreated leprosy patients (5 BT/TT, 1 indeterminate, 1 BL). Cells were resuspended at 2×10^5 PBMC in RPMI 1640 (Sigma) containing 10% autologous plasma. Culture conditions were as described previously (3). The results were expressed as stimulation index [mean counts per minute (cpm) of triplicate cultures with antigen divided by mean cpm of the triplicate cultures without antigen]. A stimulation index of 2.0 or greater was considered significant.

Statistical analysis. Spearman rank correlation was used to determine the association between lymphocyte proliferative responses to the different antigens of *M. leprae*.

RESULTS

Comparison of ELISAs for 18-kDa recombinant antigens. Figure 1 shows the dose-response curve obtained with the positive serum pool developed with the anti-human IgG antibody conjugated to either alkaline phosphatase (direct) or biotin followed by avidin conjugated to alkaline phosphatase (indirect). Both systems gave acceptable signals when tested with antigen at a concentration of 0.1 μ g/ml. Higher signals were observed with the avidin-biotin system than with the direct alkaline phosphatase system. The avidin-biotin system was then used to compare the antibody reactivity of the three 18-kDa antigen preparations.

Figure 2 shows the reactivity with the positive pool as well as two individual sera with high and low activity to the 18-kDa antigen. All three antigens were tested under identical conditions in one single assay. The background responses to the β gal control

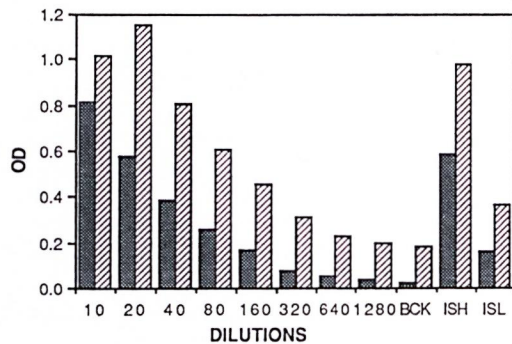


FIG. 1. Comparison of ELISAs for recombinant *M. leprae* 18K Asn. ■ = assay using alkaline phosphatase conjugate; ▨ = results of the avidin-biotin conjugate system. Double dilution of a high-titered serum pool starting at 1/10 dilution was tested in plates coated with 0.1 µg/ml of antigen. BCK = background responses of blocked wells with no antigen and tested with 1/10 dilution of the positive serum pool; ISH and ISL = individual sera (tested at 1/10 dilution) with high and low antibody activity to 18K Asn.

interfered with evaluation of the antibody activity to 18K βgal. 18K Asn showed higher positivity with the pool than did 18K JDW at all dilutions of the positive pool. However, both 18-kDa preparations (18K Asn and 18K JDW) showed similar reactivity with the individual patient sera. The higher reactivity of 18K Asn with the pool may have been due to a bias in the selection of sera for the pool which were chosen on the basis of their high reactivity with the 18K Asn. To assess the differences in reactivity of the three preparations further, individual sera from leprosy patients were also tested.

IgG antibody to the 18-kDa antigens in leprosy sera. Sera were tested in both the direct alkaline phosphatase and the avidin-biotin system. In general, more consistent results were obtained with the direct alkaline phosphatase system. The avidin-biotin system gave not only variable backgrounds with endemic-control sera but, in addition, the values obtained in this assay system were found to be less consistent with the sera of leprosy patients. Therefore, results are only shown for the direct alkaline phosphatase system. Figure 3 shows correlation between the antibody activity to 18K Asn and the other two preparations. Antibody reactivity with 18K Asn was consistently higher than with 18K JDW or 18K βgal. The cut off for

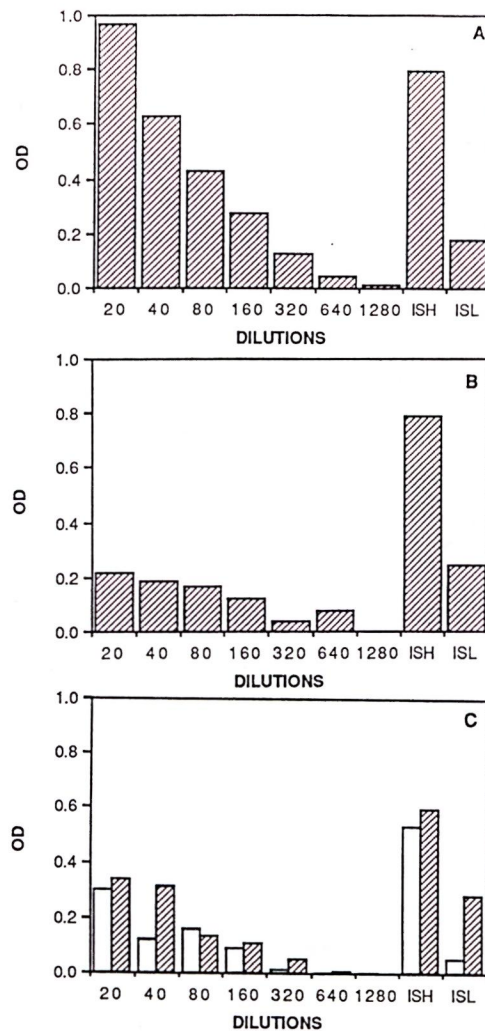


FIG. 2. Comparison of three recombinant *M. leprae* 18-kDa preparations by ELISA using the avidin-biotin conjugate system. Dilutions of a high-titered serum pool and two individual sera (ISH and ISL) were tested against the 18-kDa antigen as described in the legend for Fig. 1. A = results with 18K Asn; B = 18K JDW; C = 18K-βgal (□) or βgal (▨).

normal endemic sera (mean + 2 S.D.) is shown as vertical or horizontal lines which were used to determine the seropositivity. Only 9.2% (N = 6) were positive to both 18K Asn and 18K βgal while 25% (N = 16) were positive to 18K Asn alone in the same set (Fig. 3A). A slightly higher percentage was positive to both 18K Asn and 18K JDW (18%) and to 18K Asn alone (26%) (Fig. 3B). The major discrepancy was in sera with lower activity. Sera highly positive to 18K Asn

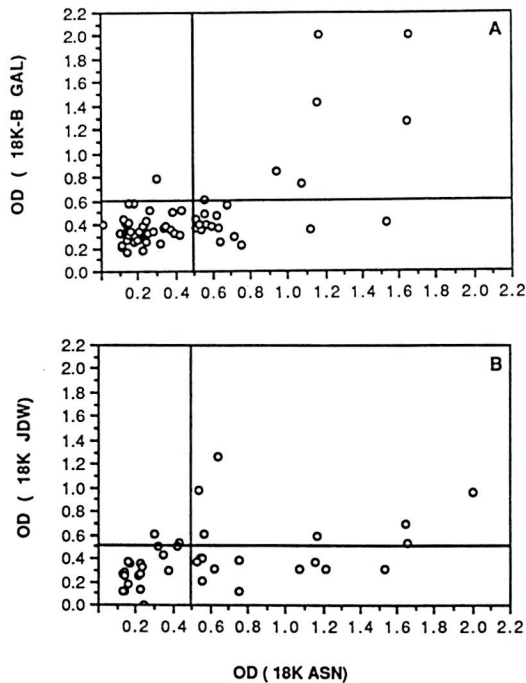


FIG. 3. Comparison of 18K Asn antibody reactivity in leprosy sera to *M. leprae* 18K β gal and 18K JDW. A: N = 65; B: N = 39; horizontal and vertical lines indicate a cut-off value (endemic control mean \pm 2 S.D.) for seropositivity.

(> 0.8 OD) generally show positivity to the other two preparations. The exceptions were two sera with the 18K β gal preparation where the high reactivity was due to antibodies to β gal (data not shown).

Because of the higher immunological activity of 18K Asn, this preparation was further used to determine the seropositivity across the leprosy disease spectrum (Table 1). Patients with lepromatous leprosy showed the highest antibody responses to 18K Asn. It was interesting to note that seropositivity to 18K Asn was restricted even within the lepromatous leprosy group (41.6%) which usually showed close to 100% positivity to *M. leprae* sonicate in both the IgM and IgG isotypes.

Lymphocyte proliferation to the 18-kDa antigens. These antigens were next compared for their ability to induce lymphocyte proliferation. Lymphocytes from peripheral blood obtained from leprosy patients, healthy contacts of leprosy patients, or healthy endemic controls with no known contact with leprosy patients were tested

TABLE 1. Seropositivity to 18K Asn.

Donor ^a sera	No.	% Positive ^b
LL	24	41.6
BL	22	13.6
BT	40	10.0
TT	10	0
EC	27	7.4

^a LL, BL, BT and TT = untreated patients across the leprosy spectrum as diagnosed by histology; EC = healthy control living in the same area.

^b Positivity is defined as values above mean + 2 S.D. of the endemic controls.

against *M. leprae* soluble sonicate (MLSON), 18K Asn and 18K JDW. 18K β gal was not included in these experiments because of the previously reported high T-cell reactivity to β gal itself⁽¹⁵⁾. T-cell reactivity to these three antigens in individual donors is shown in Table 2. Donors with positive lymphocyte proliferation to MLSON are shown. Significant correlation was observed with MLSON for both 18K Asn ($p < 0.048$) and 18K JDW ($p < 0.005$). The strongest responses to the 18-kDa antigens were observed in the donors showing the highest responses to *M. leprae*. Four out of seven

TABLE 2. Lymphocyte proliferation to recombinant *M. leprae* 18-kDa preparations.

Donor ^b	Stimulation index ^a		
	18K Asn ^c	18 K JDW ^c	MLSON ^c
LS 936	24.1 ^d	68.9	50.0
LS 570	3.6	2.0	29.0
LS 938	19.2	15.5	26.2
LS 726	1.8	5.3	15.3
LS 935	6.3	10.8	10.7
LS 571	3.1	0.99	8.3
LS 926	8.8	10.1	6.8
LC 317	31.1	93.8	168.0
LC 316	17.8	52.7	77.9
NS 010	32.8	33.9	18.5
NS 006	1.5	7.6	14.8
NS 008	5.2	1.0	7.4
NS 004	3.8	1.2	2.3

^a Stimulation index is expressed as a ratio of mean values of triplicate cultures with antigen divided by the mean values of triplicate cultures without antigen.

^b Leprosy patients are designated as LS, leprosy contacts as LC, endemic controls as NS. Each individual donor is identified by a number.

^c All antigens were tested at a final concentration of 10 μ g/ml.

^d A stimulation index \geq 2.0 was considered significant.

leprosy patients showed similar lymphocyte proliferative responses. One leprosy patient and the two contacts of leprosy patients responded more strongly to the full-length molecule, while some donors failed to respond to one or the other 18-kDa antigen.

DISCUSSION

The gene for the *M. leprae* 18-kDa protein was one of the first leprosy genes cloned in λ gt11¹⁹. Several laboratories have been using 18-kDa preparations obtained by different purification protocols which may result in differential preservation of antigenic epitopes. We have, therefore, compared IgG antibody responses to three 18-kDa preparations obtained using different protocols (two 18-kDa antigens containing amino acids 38–148 and the full-length protein) in leprosy. Our observation that the IgG responses to the asparagine 18-kDa antigen was higher than to the other two 18-kDa antigens implies that the major human B-cell epitopes are located within the carboxy-terminal two-thirds of the molecule. It is perhaps surprising that the 18K Asn gave higher antibody responses than the full-length molecule; the asparagine leader sequence which protects the recombinant antigen from degradation during its expression in *E. coli*¹⁴ may provide the protein with increased solubility as well as additional charges giving better binding to the plastic surfaces during ELISAs. It was difficult to assess antibody binding to the purified asparagine leader sequence since small peptides tend to wash off the plastic more easily, resulting in inconsistent binding. Despite this technical problem, extensive testing with the leader peptide failed to demonstrate significant antibody recognition to this peptide (data not shown), indicating that the antibody responses were directed to the 18-kDa portion of the molecule. This is further supported by the observation that comparable antibody responses were obtained with other 18-kDa preparations lacking the asparagine leader sequence.

The purified, full-length 18-kDa protein and the synthetic peptides covering its sequence have been used previously to define B-cell epitopes in mice (BALB/c) immunized with the synthetic peptides. An IgG response to six peptides was observed, five of which were also recognized by B10.BR

mice⁴). Helper T-cell responses were inferred by the responses in the IgG isotype to four peptides (1–20, 16–35, 31–50 and 76–95) in B10.BR mice. Interestingly, mouse T-cell proliferative responses mapped to 116–120⁷ which is adjacent to the L5 epitope located in the region of 109–115⁵, indicating that T-helper cells providing help for IgG isotype switching may be distinct from those inducing proliferative responses. Such a dichotomy of T-cell proliferation and IgG antibody responses has been shown for murine immune responses to human basement collagen¹⁶ which was shown to be under Ir gene control. Our results with lepromatous leprosy patients who are high responders for IgG antibody to *M. leprae* 18K Asn but show little or no proliferative responses³ would indicate a similar dichotomy of T-cell help for antibody and proliferative responses. Furthermore, the restricted antibody response to the 18-kDa protein, even within the lepromatous leprosy patients, may indicate some genetic restriction which also has been suggested by studies in mice; high, intermediate and low responder strains have been identified in terms of their IgG responses to the 18-kDa proteins which show linkage to genes in the H2 Complex⁴).

Epitopes recognized by human T cells during proliferative responses may be distinct from those recognized by mouse T cells primed using immunization protocols. Human T-cell responses to the 18-kDa protein were first reported using a crude lysate containing the 18-kDa antigen as a β gal fusion protein with T-cell clones derived from volunteers immunized with killed *M. leprae* vaccine¹¹. Five out of 10 human T-cell clones, selected to respond to *M. leprae* but not to PPD or *M. bovis* BCG, recognized the 18-kDa antigen, indicating that this protein contained a leprosy-specific, dominant, T-cell epitope. However, these clones did respond to *M. scrofulaceum*¹¹ and, in one case, to *Mycobacterium w*¹⁰. The T-cell epitope recognized by human T-cell clones was shown to be located in the region of 38–50, and was recognized in a DR4 Dw4-restricted fashion by the four T-cell clones tested¹³.

We have been looking at T-cell recognition in leprosy patients and their contacts who may respond differently during an ac-

tive or subclinical infection than donors immunized with *M. leprae* vaccine. We have previously shown that the 18K Asn (38–148) with a short asparagine leader sequence, but without the β -galactosidase, was strongly recognized by long-term healthy leprosy contacts⁽³⁾. Recombinant antigens derived from *E. coli* may also be contaminated with different amounts of endotoxin and *E. coli*-derived proteins which may interfere with the evaluation of the immune response to the antigen of interest, particularly at the T-cell level. We believe that our immune responses are directed to the 18-kDa proteins since T-cell proliferation to the 18-kDa proteins correlated well with proliferation to the *M. leprae* soluble sonicate. In addition, all three proteins showed comparable T-cell responses in the majority of the donors, and some have shown higher T-cell reactivity to 18K JDW which contains little or no endotoxin (H. M. Dockrell and S. K. Young, unpublished observations).

Most donors who gave lymphocyte proliferation to the 18K Asn antigen also responded to the full-length 18-kDa protein. It was interesting to note that 5 out of 13 donors responded more strongly to the full-length molecule, indicating that the region between 1–38 amino acids may contain an additional T-cell epitope. The only human T-cell epitope reported so far is located within the region of 38–50. Our results further indicate that the 18-kDa antigen may also contain an epitope crossreactive with *M. bovis* BCG, as shown by the responses in BCG-vaccinated donors in the U.K.⁽³⁾ and as shown in this study by the responses to the 18-kDa proteins in healthy endemic controls who are likely to have been vaccinated with BCG. Further epitope mapping should identify if one of the T-cell epitopes is species-specific for *M. leprae*.

SUMMARY

Three different, purified, *Escherichia coli*-derived, recombinant preparations of the *Mycobacterium leprae* 18K protein were compared for their immunological recognition in leprosy. The preparations tested were 18K fusion proteins containing 70% (amino acids 38–148) of the full 18K protein fused to either a short leader sequence containing six asparagine residues or to

β -galactosidase, and the full length 18K protein. All three recombinant antigens were recognized by IgG antibodies which were restricted mostly to lepromatous leprosy patients. The 18K antigen with the asparagine leader sequence showed better reactivity with IgG antibodies compared with the other two 18K preparations. In lymphocyte proliferation assays, the truncated 18K and the full-length 18K showed equivalent responses in the same donors with strongest recognition in donors who were also strongly responsive to the *M. leprae* soluble sonicate. These results indicate that the major human B- and T-cell epitopes are located within the segment 38–148, although some individuals may recognize additional epitopes at the NH₂-terminal end.

RESUMEN

Tres preparaciones recombinantes de la *Mycobacterium leprae* de la proteína de 18 kDa purificadas a partir de *Escherichia coli*, se compararon en cuanto a su reconocimiento inmunológico por los pacientes con lepra. Las preparaciones probadas fueron proteínas de fusión de 18 kDa conteniendo 70% (aminoácidos 38–148) del total de la proteína de 18 kDa completa, fusionadas a una corta secuencia líder conteniendo 6 residuos de asparagina o a la beta-galactosidasa, y la proteína de 18 kDa completa. Los 3 antígenos recombinantes fueron reconocidos por anticuerpos IgG restringidos casi exclusivamente a los pacientes lepromatosos. El antígeno de 18 kDa con la secuencia líder de asparagina mostró mejor reactividad con los anticuerpos IgG que los otros dos antígenos recombinantes. En los ensayos de proliferación de linfocitos, los antígenos truncados y el antígeno completo de 18 kDa, indujeron respuestas equivalentes en los mismos donadores. Las respuestas más intensas se observaron en aquellos donadores que también dieron respuestas intensas con sonificados solubles del *Mycobacterium leprae*. Estos resultados indican que los epítipes más importantes reconocidos por los linfocitos T y B, están localizados dentro del segmento 38–148, aunque algunos individuos pueden reconocer epítipes adicionales en el extremo NH₂-terminal.

RÉSUMÉ

Trois préparations différentes de recombinants de la protéine 18K de *Mycobacterium leprae* purifiées obtenues à partir d'*Escherichia coli* ont été comparées quant à leur pouvoir de reconnaissance immunologique de la lèpre. Les préparations testées étaient des protéines de fusion 18K contenant 70% (acides aminés 38–148) de la protéine 18K complète attachées soit à une courte séquence contenant 6 résidus asparagine ou au β -galactosidase, et la protéine 18K complète. Les trois antigènes recombinants ont été reconnus par des

anticorps IgG essentiellement spécifiques à des patients lépromateux. L'antigène 18K avec la séquence asparagine montrait une meilleure réactivité avec les anticorps IgG comparé aux deux autres préparations 18K. Dans les tests de prolifération lymphocytaire, les préparations tronquées et complètes 18K montraient des réponses équivalentes chez les mêmes donneurs avec la reconnaissance la plus forte chez les donneurs qui répondaient également fortement aux sonicates solubles de *M. leprae*. Ces résultats indiquent que les épitopes principaux des cellules humaines B et T sont localisés dans le segment 38–148, bien que certains individus puissent reconnaître des épitopes additionnels au niveau de l'extrémité NH₂.

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