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Characterization of the 36 K antigen of *Mycobacterium leprae*

P R KLATSER, R A HARTSKEERL, W C A VAN SCHOOTEN, A H J KOLK, M M VAN RENS & M YL DE WIT
N.H. Swellengrebel Laboratory of Tropical Hygiene, Royal Tropical Institute, Meibergdreef 39, 1105 AZ Amsterdam, The Netherlands

Introduction

Relatively little is known about the complex and diverse immune response, involving both cellular and humoral immune reactions in leprosy patients. With the availability of large amounts of *Mycobacterium leprae* from infected armadillos (1) and the introduction of the hybridoma and recombinant DNA technologies in leprosy research, it has now become feasible to isolate well-defined antigens from the leprosy bacillus.

Isolated antigens of *M. leprae* should help to identify the antigenic determinants involved in the humoral- and cell mediated immunity against *M. leprae* (2). Furthermore, well-defined antigens might be crucial for vaccine development and have already been shown to be useful for diagnostic purposes (3,4,5).

We have previously reported the identification of specific *M. leprae* antigenic determinants which were recognized by leprosy patients' sera (6). To several of these antigens monoclonal antibodies (MCAb) have been prepared and their specificities have recently been characterized in a WHO/IMMLEP-organized workshop (7).

Our *M. leprae* specific MCAb F47-9 (4,7) recognizes a 36K protein, one of the antigens previously identified using patients'sera (6). We have employed this MCAb for the development of an ELISA competition test for serological investigation of patients with leprosy (4). Furthermore, the 36K antigen has been shown to play a role in the cellular immune-response of leprosy patients. Several T-cell clones from tuberculoid leprosy patients recognized common as well as specific epitopes on this antigen (8,9). Continuation of the study of the antigen at the molecular level should further elucidate its role in evoking an immune response against *M. leprae*.Since genes for the major protein antigens of *M. leprae* recognized by mouse MCAbs have been isolated (7,10), it has become feasible to isolate large amounts of protein comprising the antigenic determinant recognized by our MCAb F47-9.

Isolation of the 36K antigen from *M. leprae*

The 36K antigen was isolated from a 10,000 g supernatant of sonicated *M. leprae* by various chromatographic techniques: affinity-, ion-, exchange- and gelpermeation chromatography. MCAb F47-9 bound to Sepharose 4B was used for the affinity purification. This procedure gave the highest purification in one step. Eluates were screened in ELISA with MCAb F47-9 for the presence of the 36K antigen. Fractions showing a positive reaction with F47-9 were pooled and applied to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). After coomassie brilliant blue (CBB) and periodic acid Shiff (PAS) staining of the gel, a diffuse band at position 36K was seen. However, in the SDS-PAGE immuno

peroxidase assay (SGIP) (6) using MCAb F47-9 a sharp band at position 36K was revealed indicating that the 36K antigen was present as a distinct protein. The diffuse band appearing after CBB and PAS staining could be attributed to the fact that the purified fractions always contained lipoarabinomannan (LAM), recently purified and characterized by Hunter and coworkers (11). This suggests that the 36K antigen and LAM are associated with each other. However both components could be separated when *M. leprae* was first delipidated, refluxed in 50 % ethanol, followed by phenol biphasic partition of the extracted material (11). Analysis of both phases in ELISA and SGIP with MCAb F47-9 and anti-LAM MCAb revealed that the 36K antigen was only present in the phenol phase while LAM was exclusively present in the water phase.

Isolation of a beta-galactosidase-36K fusion protein

Recombinant DNA clone Y3180, isolated from the *M. leprae* library in lambda gt 11 (10) with MCAb F47-9, codes for a fusion protein consisting of the C-terminal part of the 36K protein and beta-galactosidase (R.A.H., Manuscript in preparation). The *E. coli* strain Y1089 (12) was infected with Y3180 and the resulting lysogen was induced as described by Huynh et al. (13). Proteins produced by the lysogen were analysed by SDS-PAGE and «Western › (14) with the MCAb probes F47-9 and anti-beta-galactosidase (Promega Biotec). The fusion protein showed to have an apparent molecular weight of 116K. Since the beta-galactosidase portion of the fusion accounts for 114K daltons (13,15), the 36K protein portion is about 2K. Consistent with this, the *M. leprae* DNA of the fused gene appeared to encode a peptide of 18 amino acids with a total molecular weight of 1.9K daltons. Thus, the *M. leprae* specific epitope recognized by MCAb F47-9 is located within the C-terminal 18 amino acids of the 36K antigen.

The beta-galactosidase-36K fusion protein was isolated from a crude lysate of the lysogen Y1089 (Y3180) by immuno affinity chromatography using either MCAb F47-9 bound to Sepharose 4B or a commercially available anti-beta-galactosidase column (Promega Biotec). Eluates were initially screened in ELISA with MCAb F47-9 or anti-beta-galactosidase for the presence of the fusion product. Fractions containing the fusion product were further analysed by SDS-PAGE and SGIP. Whereas large amounts of the fusion protein were obtained when the anti-beta-galactosidase column was used, the isolation of the protein was ineffective when the F47-9 column was used. This suggests that MCAb F47-9 cannot bind the native fusion protein. Apparently the *M. leprae* specific epitope is hidden in the native fusion product. Consistent with this in the ELISA the native fusion protein reacted strongly with anti--beta-galactosidase and weakly with F47-9, whereas the denatured protein in the Western blot and SGIP assays reacted strongly with both antibodies. The purity of the fusion protein after immuno affinity chromatography as estimated by SDS-PAGE was about 90 %.

Recognition of purified mycobacterial antigens by human T-cell clones

T clones from a non treated tuberculoid leprosy patient were obtained from peripheral blood lymphocytes (PBL) stimulated in vitro with *M. leprae*. After culturing for 5-10 days the T lymphoblasts were cloned by limiting dilution. Clones were screened for antigen specificity after 14 days in a proliferation assay measuring the [^{3}H]-thymidine incorporation. Irradiated autologous PBLs or autologous Epstein Barr virus transformed B-cells were used as antigen presenting cells.

In this way sixteen T_H clones from this tuberculoid leprosy patient were isolated. The specificity of the clones was established with twelve different mycobacterial sonicates from *M. leprae, M. lepraemurium, M. tuberculosis, M. africanum, M. bovis, M. avium, M. scrofulaceum, Armadillo Derived Mycobacteria, M. fortuitum, M. kansasii, M. vaccae* and *M.*

Table 1. Recognition of purified mycobacterial antigens by T-cell clones from a leprosy patient

T-cell clone	[^{3}H]thymidine incorporation (cpm x 10^{-3})						
		antigen					
	medium	sonicate *M. leprae*	36K *M. leprae*	64K *M. leprae*	64K *M. tub.*	64K rec. DNA[2]	36K rec. DNA[3]
Specificity A.[1]							
Br2	1.2	30.8	0.8	1.3	1.5	1.0	0.1
Br6	0.2	20.7	0.6	0.2	0.3	0.3	0.1
Specificity B.							
Br3-3	0.2	31.3	10.4	13.2	0.5	0.5	6.2
Br2-8	2.1	25.6	2.5	3.6	2.1	2.2	0.2
Br4-1	1.2	20.4	1.2	1.3	1.2	1.2	0.1
Specificity C.							
Br2-1	0.1	18.8	19.6	0.2	0.1	0.4	0.1
Br2-5	0.3	3.4	2.8	0.2	0.3	0.2	0.2
Br4-2	1.8	19.3	1.2	1.9	1.2	nt	0.1
Specificity D.							
Br2-10	0.1	29.8	0.4	15.3	17.1	11.2	0.1
Br2-17	0.4	9.9	0.3	8.6	6.9	5.6	0.3
Br3-5	0.4	10.1	11.8	11.2	5.7	6.5	0.2
Br3-10	0.2	7.8	7.2	3.8	4.1	2.5	0.1

[1] specificity (A): T-cell clones recognizing determinants present only in *M. leprae;* specificity (B): T-cell clones reactive with determinants present in *M. leprae* and two or three other mycobacterial strains: *M. lepraemurium, M. vaccae* and *M. scrofulaceum;* specificity (C): T-cell clones reactive with many mycobacteria; specificity (D): T-cell clones responding to cross-reactive determinants present in all mycobacterial strains tested. Four representative clones are shown.

[2] antigen isolated from recombinant *M. bovis* BCG DNA-clone (16).

[3] beta-galactosidase 36K fusion protein (10).

nonchromogenicum. Four different patterns of reactivity were found: 2 (12 %) T-cell clones were reactive with *M. leprae* only (designated specificity A): 3 (19 %) T-cell clones were reactive with *M. leprae* and two or three other mycobacterial strains *(M. lepraemurium, M. vaccae* and *M. scrofulaceum* : specificity B): 3 (19 %) T-cell clones were reactive with many mycobacteria (8/12) (specificity C) and ((50 %) T-cell clones were reactive with all mycobacteria tested (12/12) (specificity D).

The clones were tested with five purified antigens: the 36K and the 64K proteins from *M. leprae,* the 64K protein from *M. tuberculosis,* the 64K protein from a recombinant *M. bovis* BCG DNA clone in *E. coli* (16) and the beta-galactosidase-36K fusion protein from Y3180 (Table 1). The T-cell clones with specificity A from this tuberculoid leprosy patient do not

react with either the 36K protein or the 64K protein. We have not yet defined the antigens recognized by these clones. Many of the clones with specificity B, C and D react with an epitope present on the 36K or 64K proteins. Three cross-reactive T-cell clones (Br3-3, Br3-5 and Br3-10) recognize a shared epitope on the 36K and 64K proteins. The possibility that the 36K antigen is contaminated with the 64K antigen is excluded since the dose response curves for both antigens were the same for the clones Br3-5 and Br3-10. Furthermore the clones Br3-5 and Br3-10 also recognize the 64K protein from a recombinant *M. bovis* BCG DNA-clone. Alternatively, both preparations could have a common contaminant. Proliferation due to contamination with LAM can be excluded since non of the T-cell clones reacted with purified LAM. 45 % of the clones reacted with the 36K and 64K antigens, which suggests that these proteins are immuno dominant.

The *M. leprae* specific T-cell clones from this patient did neither react with the 36K protein, nor with the 64K protein. T-cell clones from another tuberculoid patient (8) recognized a *M. leprae* specific epitope on the 36K protein. This epitope is unique for the 36K antigen since it is not present on other proteins so far tested.

T-cell clones with specificity B, C and D recognized unique (Br2-1; Br2-10) as well as shared epitopes on the 36K and 64K proteins (Br3-5; Br3-3; Br3-10). One clone, Br3-3, recognized an epitope present on the *M. leprae* 36K and 64K proteins as well as on the beta-galactosidase-36K fusion protein. It has to be investigated whether the isolated *M. leprae* 36K and 64K antigens and the beta-galactosidase-36K fusion protein are capable to induce a delayed hypersensitivity reaction in immunized animals, in leprosy patients and in contacts of leprosy patients. The 36K protein is the first isolated protein that has been shown to evoke a humoral and cellular immune response in leprosy patients. It will be fascinating to compare the T-cell repertoire of different leprosy patients exhibiting the whole spectrum of the disease and of healthy contacts. These studies could result in a set of antigens useful for a specific skin test or a vaccine.

The results of the purification of the 36K protein from armadillo-derived *M. leprae* illustrate the difficulties in antigen purification due to the scarcity and complexity of the starting material. These problems can be overcome by using recombinant DNA techniques to obtain *M. leprae* proteins.

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