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A Peptidoglycan Protein Complex Purified from *M. leprae* Cell Walls Contains Most or All Immunodominant *M. leprae* T-Cell Antigens[1]

Tuna Mutis, Wim C. A. van Schooten,
and René R. P. de Vries[2]

Leprosy presents a continuous spectrum of clinical manifestations that closely parallel the T-cell-mediated immunity which is developed by the host against *Mycobacterium leprae* ([1, 14, 16]). The location and the nature of many antigens that are recognized by either helper- or suppressor-T cells, however, remain to be established. Recently five *M. leprae* proteins have been identified by monoclonal antibodies ([6, 21]), three of which could stimulate a relatively small number of T-cell clones (TC clones) derived from leprosy patients ([12, 13, 15]). However, many *M. leprae*-reactive T cells apparently do not recognize these proteins.

Several reports have indicated that the cell-wall skeletons of various mycobacteria induce cellular immune responses such as delayed-type hypersensitivity reactions in the skin ([11]). However, the antigenic properties of the cell wall of *M. leprae* have not been widely studied, although it is known that its peptidoglycan unit differs from those of other mycobacteria ([4, 5]) and there is evidence for the existence of cell-wall-associated proteins ([7]) which might carry some T-cell epitopes.

Recently, Melancon-Kaplan, *et al.* ([10]) showed that purified cell walls stimulated proliferation of T cells from tuberculoid leprosy patients, and suggested that all or most of this activity was contained in a complex of peptidoglycan and proteins. We have systematically explored the possibility that this peptidoglycan-protein complex (PPC) purified from *M. leprae* cell walls contains important antigens involved in T-cell-mediated immunity against *M. leprae*. To this purpose, we performed standard lymphoproliferation assays and presented this complex to different TC clones from a tuberculoid (TT) leprosy patient and T-cell lines (TC lines) obtained from patients with different types of leprosy as well as from healthy individuals, including leprosy contacts. Our results indicate that PPC contains all or more of the antigens that are recognized by *M. leprae*-reactive proliferative T cells.

MATERIALS AND METHODS

TC clones. Peripheral blood mononuclear cells (PBMC) of a TT patient were isolated by Ficoll-Isopaque density centrifugation and restimulated with Dharmendra lepromin (1 μg/ml; Dr. R. C. Good, Centers for Disease Control, Atlanta, Georgia, U.S.A.) in Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, New York, U.S.A.) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml) (both Flow Laboratories, Scotland) and 10% heat-inactivated human serum (complete medium). The cultures were incubated for 5 days in 24-well tissue culture trays (Falcon 3047; Becton, Dickinson & Co., Oxnard, California, U.S.A.) at 37°C in a fully humidified CO_2-air mixture. T-cell blasts were then enriched by Percoll density centrifugation, diluted to 5 blasts/ml in a feeder cell mixture consisting of 50 Gy irradiated autologous Epstein-Barr-transformed B cells (10^5 cells/ml), 30 Gy irradiated PBMC of 3–4 random donors (10^6 cells/ml), and Dharmendra lepromin (1 μg/ml), plated in 96-well flat-bottom microtiter plates (Falcon 3072; Becton, Dickinson) as 0.5 blast/well and incubated as described above. Growing cultures were transferred into 24-well tissue culture trays and restimulated with a feeder mixture supplemented with Leuko Agglutinin A (Pharmacia, Uppsala, Sweden). Three to five days later 10% interleukin-2 (IL-2) (Lympocault-T; Biotest, Federal Re-

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[2] T. Mutis, M.D.; W. C. A. van Schooten, M.Sc.; R. R. P. de Vries, Ph.D., M.D., Department of Immunohematology and Bloodbank, University Hospital, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
Reprint requests to Dr. de Vries.

TABLE 1. *Proliferative response of 12 T-cell clones from a tuberculoid leprosy patient to peptidoglycan protein complex (PPC).*[a]

Clones Specificity[c]	1G5 A	2B3 A	2F9 B	3B4 C	3E8 D	4C11 C	1E4 A	1F3 A	1F9[b] D	2E4[b] D	2F10[b] D	3E10[b] D
	^{3}H-Thymidine incorporation (cpm × 10^{-3})											
Medium	0.3	0.8	0.2	0.9	0.6	0.6	0.8	0.5	0.5	0.2	0.2	0.3
PHA (1:200 dil.)	42.2	80.2	97.6	29.6	32.4	78.4	44.9	57.2	54.4	38.4	39.8	26.8
M. leprae (1 μg/ml)	41.9	52.4	54.7	23.7	21.0	8.9	45.2	11.6	83.1	44.2	13.2	5.8
IL-2 (1:10 dil.)	20.3	20.6	45.8	2.8	3.4	6.6	17.8	20.1	16.6	12.8	15.9	4.0
PPC (0.1 μg/ml)	7.2	29.1	34.8	1.8	2.6	12.0	9.3	19.2	10.5	20.7	NT[d]	5.7
PPC (1.0 μg/ml)	37.5	89.7	49.3	18.5	20.8	8.1	43.1	19.4	18.5	49.4	29.9	13.6
PPC (10.0 μg/ml)	60.7	120.6	118.2	28.6	33.9	8.8	46.2	40.0	20.5	63.3	NT[d]	36.2

[a] Results are expressed as the mean of triplicate cultures. The S.E.M. (standard error of the mean) never exceeded 15%. Positive cultures are defined as exceeding the mean background value (medium value) by at least 3 × S.E.M. and are underlined.

[b] Reactive with recombinant mycobacterial 65-kDa protein ([20]).

[c] A = *M. leprae* specific; B = crossreactive with *M. vaccae* and *M. lepraemurium*; C = partly crossreactive; D = completely crossreactive ([15]).

[d] NT = not tested.

public of Germany) was added to expand the clones. All clones used in this study had the phenotype CD3+, CD4+, CD8− and were restricted via DR2 and DR3 molecules. Four of them were previously described as "*M. leprae*-specific" while the others were crossreactive with two or more mycobacteria ([15]). Four of these TC clones were also known to react with the recombinant mycobacterial 65-kDa protein ([20]).

TC lines. PBMC of leprosy patients, healthy individuals, or leprosy contacts were restimulated with either Dharmendra lepromin (1 μg/ml), PPD (10 μg/ml; Statens Serum Institute, Denmark), or tetanus toxoid (1.5 Lf/ml; National Institute of Public Health, The Netherlands) as described above. On day 6, 10% IL-2 was added to expand the lines. After 7–10 days the cells were frozen at −196°C in 1 ml ampules (Nunc, Denmark) containing 1–5 × 10^6 cells, 70% RPMI 1640 (Gibco), 20% pooled human serum, and 10% dimethylsulfoxide.

Antigens. Armadillo-derived *M. leprae* antigen was kindly provided by Dr. R. J. W. Rees, London, England. The peptidoglycan-protein complex (PPC) was purified from the cell walls of armadillo-derived *M. leprae* as described by Melancon-Kaplan, *et al.* ([10]), and was a kind gift of Dr. P. J. Brennan, Colorado State University, Fort Collins, Colorado, U.S.A.

Proliferative assays. In complete medium in the presence of 0.2 ml antigen, 1 × 10^4 TC clones or TC lines and 5 × 10^4 40 Gy irradiated autologous or allogeneic PBMC as antigen-presenting cells (APC) were cultured together. The antigens tested were PPC (0.01–10 μg/ml) and, in some cases, PPD. PHA (1:200 dilution; Welcome Diagnostics, England), IL-2 (1:10 dilution; Biotest, Federal Republic of Germany), soluble *M. leprae* (1 μg/ml), tetanus toxoid (1.5 Lf/ml), and plain IMDM were used as controls. The cultures were set up in triplicate and incubated in conditions as described above for 88 hr. Sixteen hours before termination 1 μCi of [^{3}H]-thymidine (Radiochemical Centre, England) was added to each culture. The samples were harvested on glass-fiber filters using a semi-automatic sample harvester. [^{3}H]-Thymidine incorporation was assessed by liquid scintillation counting.

RESULTS

To study the T-cell reactivity induced by peptidoglycan-protein complex (PPC), we first selected 12 *M. leprae*-reactive T-cell clones (TC clones) of a TT leprosy patient. The antigens that are recognized by eight of these TC clones were not known, while the other four were known to be reactive with the recombinant mycobacterial 65-kDa protein. In proliferation assays, PPC was presented at different concentrations to these TC clones. Over a concentration range of 0.1–10.0 μg/ml PPC, all of these TC clones

TABLE 2. *Proliferative response of T-cell clones to peptidoglycan-protein complex is specific and HLA-DR restricted.*[a]

	T-cell clone								T-cell line	
HLA restriction	2F9 DR2		1G5 DR2		2E4 DR3		1E4 DR3		Tetanus toxoid[b] DR4	
Allogeneic APC	VIJF	HAR	VIJF	HAR	VIJF	HAR	VIJF	HAR	VIJF	BSM
DR of APC	2	3	2	3	2	3	2	3	2	4
	^{3}H-Thymidine incorporation (cpm × 10^{-3})									
Medium	1.0	0.2	0.6	0.4	0.4	0.3	0.3	0.2	1.2	0.2
PHA (1:200 dil.)	81.2	79.2	82.3	75.1	60.0	52.0	60.2	57.0	40.9	29.4
M. leprae (1 μg/ml)	18.0	0.5	19.8	0.8	0.6	13.8	1.1	12.5	0.5	0.3
IL-2 (1:10 dil.)	40.0	40.4	47.0	44.1	15.1	14.1	20.5	20.4	14.2	19.6
Tetanus tox. (1.5 Lf/ml)	NT[c]	NT	NT	NT	NT	NT	NT	NT	0.8	31.2
PPC (1.0 μg/ml)	55.8	0.5	39.7	0.4	0.4	31.7	0.8	37.3	1.2	0.3

[a] Results are expressed as the mean of triplicate cultures. The S.E.M. never exceeded 15%. Positive cultures for PPC are underlined and defined as exceeding the mean background by at least 3 × S.E.M.
[b] Generated by stimulation with tetanus toxoid.
[c] NT = not tested.

showed a significant proliferative response (Table 1). The responses were comparable to that seen with whole *M. leprae* stimulation. However, when PPC was presented to some of these clones by antigen-presenting cells (APC) which were not HLA-DR matched, we did not observe any proliferation (Table 2). Furthermore, a tetanus-toxoid-reactive T-cell line (TC line) of a healthy individual used in similar assays was not stimulated by PPC (Table 2).

Further experiments were performed by using three TC lines from randomly selected healthy individuals generated by PPD stimulation and one TC line from a leprosy contact generated by *M. leprae* stimulation. The data presented in Table 3 show that all of these TC lines recognize PPC. Finally, PPC was presented to TC lines derived from different types of leprosy patients. According to the classification of Ridley and Jopling ([16]), 1 patient was diagnosed as borderline tuberculoid (BT), 1 as borderline lepromatous (BL), 1 had midborderline (BB) leprosy, and 2 were polar lepromatous (LL) leprosy patients. The lines used were generated by either PPD or *M. leprae* stimulation. The proliferative responses of these lines to PPC are shown in Table 3. We observed that the lines from BL, BT and BB patients were stimulated by whole *M. leprae* as well as by PPC. However, the lines from the LL patients which were generated by PPD failed to demonstrate any proliferative response to either *M. leprae* or PPC. This indicates that at least the part(s) of the 65 kDa protein that contain the epitopes for these TLC are still present in PPC.

Recently, two papers were published in which the antigen reactivity of T-cell lines and clones raised with PPC was analyzed ([8, 9]). The protein nature of the immunodominant cell-wall-associated antigens recognized by T cells was established, and these antigens were further defined using an immunoblot technique ([9]). The 65-kDa heat-shock protein appeared to be present in cell wall preparations ([8]) and reactivity to a 65-kDa immunoblot fraction was observed ([9]). However, the data indicated that thus far unknown low molecular weight (7 kDa and 16 kDa) proteins might be the most immunogenic constituents of *M. leprae* cell walls ([9]).

DISCUSSION

In this study we have defined the T-cell antigenic characteristics of the peptidoglycan-protein complex (PPC) which was purified from the cell wall of *M. leprae*. There were mainly two reasons why we were interested in performing this study. First, the peptidoglycan of *M. leprae* differs from that of other mycobacteria in its chemical composition: glycine rather than L-alanine is found in the cross-linking tetrapeptide ([4, 5]). This specific structure might play a role in *M. leprae*-specific immunosuppression observed in LL patients. The second reason was the presence of large amounts of protein

TABLE 3. *Response of* M. leprae- *or PPD-reactive T-cell lines from healthy individuals, one healthy leprosy contact, and leprosy patients to peptidoglycan-protein complex (PPC).*[a]

	Healthy individuals				Leprosy patients[b]				
T-cell line Clinical state	QBL Healthy	CAA Healthy	DAA Healthy	N15 Lepr. contact	GRA BL	GRO BT	BOT BB	DUT LL	AHR LL
T-cell line is generated by	PPD	PPD	PPD	*M. leprae*	*M. leprae*	*M. leprae*	*M. leprae*	PPD	PPD
	^{3}H-Thymidine incorporation (cpm × 10^{-3})								
Medium	1.7	0.9	0.5	0.7	0.1	0.8	1.0	0.3	0.3
PHA (1:200 dil.)	60.2	70.2	90.1	144.2	45.6	52.1	21.8	87.0	23.0
M. leprae (1 μg/ml)	2.7	16.1	1.9	1.5	1.1	12.1	1.8	0.4	0.4
IL-2 (1:10 dil.)	30.8	23.1	47.0	36.7	1.1	2.8	4.0	41.8	14.4
PPC (1 μg/ml)	2.0	15.7	2.5	1.3	1.5	6.1	3.1	0.3	0.2
PPC (10 μg/ml)	14.7	39.0	7.9	3.4	2.8	12.1	3.8	0.8	0.4

[a] Results are expressed as the mean of triplicate cultures. The S.E.M. never exceeded 15%. Positive cultures are defined as exceeding the mean background by at least 3 × S.E.M. and are underlined for PPC.

[b] BL = borderline lepromatous; BT = borderline tuberculoid; BB = borderline; LL = lepromatous leprosy (Ridley-Jopling classification, see text).

(60.6%) in this complex. It is generally believed that the antigens that are recognized by T cells are proteins. Until now, five *M. leprae* proteins have been identified by monoclonal antibodies ([6, 21]) but only three of them could stimulate a relatively small number of *M. leprae*-reactive TC clones derived from leprosy patients ([12, 13, 15]). The antigens that are recognized by many TC clones remain unknown. Thus, some other antigenic molecules, probably proteins, carrying important epitopes must be present in *M. leprae*. The identification or at least localization of these structures is essential to understand the factor(s) playing role(s) in either protective immunity against, or immunopathology induced by, *M. leprae*.

To explore the possibility that PPC might contain important T-cell epitopes, we performed *in vitro* lymphoproliferation assays in which PPC was presented to 12 carefully selected *M. leprae* TC clones of a TT leprosy patient and either *M. leprae*- or PPD-reactive TC lines from healthy individuals and leprosy patients. We observed that all TC clones and TC lines from healthy individuals and *M. leprae*-reactive TC lines from BL, BT and BB patients were stimulated by PPC, while PPD-reactive TC lines from two LL patients did not show any proliferative response to this complex.

Since a large variety of peptidoglycan preparations, including peptidoglycans from some mycobacteria, are known to act as mitogens ([17, 19]), we checked whether the strong T-cell stimulatory effect of *M. leprae* PPC observed by us might be due to a mitogenic effect on T cells. To this purpose, PPC was presented to some of the TC clones mentioned above by APCs which were not carrying relevant HLA class II molecules, and to a tetanus-toxoid-reactive TC line from a healthy individual. However, none of the T-cell APC-combinations which were not reactive with *M. leprae* were stimulated by PPC. Thus, our first conclusion is that PPC does not have any mitogenic effect on T cells *in vitro*. Therefore, we also conclude that this *M. leprae* cell wall PPC contains most if not all of the immunodominant T-cell epitopes of *M. leprae*, since this complex, in association with HLA class II molecules, stimulated all *M. leprae*-reactive TC clones used in this study. Some of these epitopes are *M. leprae*-specific, because four of the TC clones used in this study were previously defined to react only with *M. leprae*. The *M. leprae* cell wall PPC also contains cross-reactive epitopes because it stimulates TC lines restimulated *in vitro* with *M. tuberculosis*. These epitopes include both the known ones, such as the 65-kDa protein epitopes, and those which are as yet undefined. Some authors have described the 65-kDa protein of *M. leprae* as "cell wall associated" ([7]), while others have proposed a periplasmic location and have shown the release of it into culture supernatants of *M. bovis* ([3]). In our study, 65-kDa protein-reactive TC clones were strongly stimulated

by the *M. leprae* cell wall PPC, comparable with that seen by whole *M. leprae*. This indicates that at least the part(s) of the 65-kDa protein that contain the epitopes for these TC clones are still present in PPC.

SUMMARY

The outcome of an infection with *Mycobacterium leprae* is correlated with the T-cell-mediated immune response developed against this pathogenic agent. The identification of *M. leprae* antigens that are recognized by T cells is therefore of great importance. In this paper we present the results of *in vitro* lymphoproliferation assays in which T-cell reactivity was measured against a peptidoglycan-protein complex (PPC) which was purified from the cell wall of *M. leprae*. Twelve *M. leprae*-reactive T-cell clones with different antigen specificities from a tuberculoid (TT) leprosy patient showed proliferative responses, but only when PPC was presented by HLA-DR-matched antigen-presenting cells (APCs). Four of these clones were known to react with the recombinant mycobacterial 65-kDa protein. A tetanus-toxoid-reactive T-cell line from a healthy control was not stimulated by this complex, supporting the idea that the stimulation by PPC was antigen specific. Both PPD-reactive and *M. leprae*-reactive T-cell lines from healthy individuals were stimulated by PPC. However, when this complex was presented to PPD-reactive T-cell lines derived from two lepromatous (LL) leprosy patients, we did not observe any proliferative responses. From these results we conclude that PPC contains most or all of the antigens which stimulate *M. leprae*-reactive T cells in association with relevant HLA class II molecules, including the 65-kDa protein or at least some immunogenic parts of it.

RESUMEN

La evolución de la infección por el *Mycobacterium leprae* está relacionada con el desarrollo de una respuesta inmune específica mediada por células T. Por lo tanto, la identificación de los antígenos del *M. leprae* que son reconocidos por las células T resulta de gran importancia. En este trabajo se presentan los resultados de ensayos de linfoproliferación *in vitro* en los cuales se mide la reactividad de las células T contra un complejo de peptidoglicana-proteína (PPC) purificado a partir de la pared celular del *M. leprae*. Doce clonas de células T reactivas contra *M. leprae* (TLC), con diferentes especificidades antigénicas y derivadas de un paciente con lepra tuberculoide (TT), mostraron respuestas proliferativas sólo cuando el PPC fue presentado por células presentadoras de antígeno (APCs) portadoras del mismo HLA-DR. Se sabía que 4 de estas clonas reaccionaban con la proteína micobacteriana recombinante de 65 kDa. Una línea de células T reactivas al toxoide tetánico (TCL) derivada de un control sano no fue estimulada por este complejo. Esto apoyó la idea de que la estimulación por el PPC fue antígeno-específica. Tanto la TCL reactiva al PPD como la TCL reactiva al *M. leprae* (ambas derivadas de individuos sanos) fueron estimuladas por el PPC. Sin embargo, cuando este complejo fue presentado a las TCLs reactivas al PPD derivadas de 2 pacientes lepromatosos (LL) no se observó ninguna respuesta proliferativa. De estos resultados concluímos que el PPC contiene la mayoría (o todos) los antígenos (incluyendo a la proteína 65 kDa o al menos algunas de sus partes inmunogénicas) que estimulan las células T reactivas al *M. leprae* en asociación con las moléculas HLA clase II relevantes.

RÉSUMÉ

L'évolution ultime d'une infection par *Mycobacterium leprae* est étroitement associée à la réponse immunitaire que développent les cellules-T de cet agent pathogène. L'identification des antigènes de *M. leprae* reconnus par les cellules-T est dès lors d'une grande importance. On présente ici les résultats d'épreuves *in vitro* sur la prolifération des lymphocytes, pour lesquelles la réactivité des cellules-T a été mesurée en utilisant un complexe-protéine peptidoglycan-protéine (PPC) purifié à partir de la membrane cellulaire de *M. leprae*. Douze clones de cellules réagissant à *M. leprae* (TLC), mais ayant des spécificités antigéniques différentes, qui avaient été développés à partir de cellules obtenues d'un malade atteint de lèpre tuberculoïde (TT), ont témoigné de réponses prolifératives, mais ceci uniquement lorsque le complexe PPC était présenté par des cellules APC assorties pour les antigènes tissulaires HLA-DR. Pour quatre de ces clones, on savait qu'ils réagissaient avec la protéine mycobactérienne recombinante 65-kDa. Ce complexe protéine ne stimulait pas l'anatoxine tétanique (TCL) obtenue chez un témoin en bonne santé. Ceci renforce l'hypothèse qui suppose que la stimulation par le PPC possède une spécificité d'antigène. Le PPC stimulait tant les cellules-T réagissant au PPD, que celles qui réagissaient à *M. leprae*, lorsque celles-ci étaient obtenues à partir d'individus en bonne santé. Néanmoins, lorsque ce complexe a été présenté aux lignées de cellules obtenues à partir de deux malades lépromateux (LL), aucune réponse proliférative n'a été observée. Ces résultats permettent de conclure que le complexe PPC-protéine contient la plupart ou même tous les antigènes qui stimulent les cellules-T qui réagissent à *M. leprae* en association avec les molécules HLA de classe II, pour autant qu'elles comprennent la protéine 65-kDa ou tout au moins certaines de ses composantes immunogéniques.

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