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Restoration of Proliferative Response to *M. leprae* Antigens in Lepromatous T Cells Against Candidate Antileprosy Vaccines[1]

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Leprosy is a chronic disease with a well-defined clinical, histopathological and immunological spectrum. Cell-mediated immunity (CMI) plays the most important role in protection against leprosy ([5]). The causative agent, *Mycobacterium leprae,* induces a strong and long-lasting CMI response in healthy humans ([6–8, 26]) and tuberculoid patients with self-limiting disease ([5]), but lepromatous leprosy patients with disseminated disease are anergic to *M. leprae* antigens in CMI assays ([5]). More recent studies demonstrate that the nonresponsiveness of lepromatous T cells to *M. leprae* antigens is not absolute. *In vivo* nonresponsiveness of T cells to *M. leprae* antigens has been overcome in a large proportion of lepromatous patients by immunization with candidate antileprosy vaccines, e.g., *M. bovis* BCG ([16, 32]), *Mycobacterium w* ([2, 41, 44, 45]), and a mixture of *M. bovis* BCG + killed *M. leprae* ([3, 4, 38]). The *in vitro* nonresponsiveness of T cells to *M. leprae* can be abrogated in about 60% of lepromatous patients by providing exogenous interleukin-2 (IL-2) to T-cell cultures stimulated with *M. leprae* ([11–13]). *M. leprae*-reactive T cells can be demonstrated in lepromatous patients during erythema nodosum leprosum (ENL) ([18]) and *M. leprae*-specific T-cell clones have been generated from long-term-treated lepromatous patients ([9]). All of these studies suggest that *M. leprae*-reactive T cells in the lepromatous patients do exist but probably at a low frequency.

Lepromatous T cells respond to the antigens of cultivable mycobacteria which have been selected as candidate antileprosy vaccines ([5, 11, 30]). The selected mycobacteria have antigens that crossreact with *M. leprae* in T-cell functions, i.e., delayed-type hypersensitivity (DTH) skin response, antigen-induced proliferation, and IL-2 production ([19, 24, 29]). Since the basic defect in lepromatous T cells lies in their inability to produce IL-2 and other regulatory cytokines in response to *M. leprae* ([11, 33]), one of the ways by which immunizations with candidate antileprosy vaccines might be elevating the CMI response to *M. leprae* is by producing IL-2 and other regulatory cytokines which enrich and expand pre-existing *M. leprae*-responsive T cells in lepromatous patients. Once *M. leprae*-reactive T cells are enriched and expanded, they may subsequently respond to *M. leprae* antigens upon retesting. The present study was undertaken to determine if the above proposition can be verified *in vitro.*

M. leprae-reactive T cells in the peripheral blood mononuclear cells (PBMC) of lepromatous patients were enriched by establishing T-cell lines against the antigens of candidate antileprosy vaccines. When tested for reactivity to *M. leprae,* T-cell lines from some lepromatous patients responded to *M. leprae* antigens in proliferative assays. The best restoration of *M. leprae* response was observed among T-cell lines established against *M. bovis* BCG + *M. leprae.*

MATERIALS AND METHODS

Antigens. Killed preparations of *M. leprae* were kindly supplied by Dr. R.J.W. Rees through the WHO/IMMLEP Bank. *M. bovis* BCG was obtained from Serum Institute, Copenhagen, Denmark. Killed *Mycobacterium w* was a kind gift from Pro-

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fessor G. P. Talwar, National Institute of Immunology, New Delhi, India. These mycobacteria were used at concentrations optimal for T-cell proliferation, i.e., *M. leprae* at 5×10^7 bacilli/ml, *M. bovis* BCG at 10 μg/ml (wet weight), and *Mycobacterium w* at 5×10^6 bacilli/ml.

Escherichia coli lysates containing 65 kilodalton (kDa), 36 kDa, 28 kDa, 18 kDa, 12 kDa ([43]) and 13B3 ([27]) recombinant antigens of *M. leprae* and 65 kDa, 19 kDa and 14 kDa recombinant antigens of *M. tuberculosis* ([42]) were prepared according to the protocols described earlier ([20, 28, 34]). *E. coli* lysates lacking recombinant antigens were used as the control. The lysates at a protein concentration of 50 μg/ml were used for T-cell proliferation assays ([20, 28, 34]).

Patients and T-cell lines. Heparinized blood was obtained from leprosy patients attending the outdoor clinic of the All Africa Leprosy Research and Training Centre, Addis Ababa, Ethiopia. The patients were classified according to the Ridley and Jopling scale ([39]). PBMC were separated from the heparinized blood by flotation on Ficoll/Hypaque gradients (Lymphoprep). T-cell lines were established from the PBMC of leprosy patients as described earlier ([25]). In brief, 2×10^6 PBMC/ml complete medium (RPMI-1640 + 15% heat inactivated AB serum + 1% pencillin-streptomycin) were stimulated with optimal concentrations of *M. leprae, M. bovis* BCG + *M. leprae,* and *Mycobacterium w* in the wells of 24-well costar plates (Costar, Cambridge, Massachusetts, U.S.A.). The plates were incubated for 6 days at 37°C in an atmosphere of 5% CO_2 and 95% air. Thereafter, to expand the antigen-reactive T cells, recombinant IL-2 was added to the cultures at 100 U/ml twice a week. After 3–4 weeks, the T-cell lines were tested for antigen responsiveness in proliferation assays.

Proliferation assays. Assays of antigen-induced proliferation of PBMC were performed by using 1×10^5 cells/well in complete medium in 96-well, flat-bottom microtiter plates (Costar). In proliferation assays of T-cell lines, 2×10^4 T cells were added together with antigen presenting cells from 5×10^4 irradiated autologous PBMC ([23]). Experimental cultures were stimulated with antigens in triplicate. The control cultures did not have antigen. To test the proliferative potential of the T-cell lines, 2×10^4 cells were cultured in the presence of 10 U/ml of IL-2 alone. To assess the response against recombinant mycobacterial antigens, the experimental cultures were stimulated with *E. coli* lysates containing recombinant antigens. The cultures with *E. coli* lysates lacking recombinant antigens were taken as controls. The total culture volume was kept at 200 μl. The plates were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. One μCi ^{3}H-thymidine was added to each well of the PBMC cultures on day 6 and to the cultures with T-cell lines on day 3. The plates were further incubated for 4 hr at 37°C. Cultures were harvested and the radioactivity incorporated was determined by standard methods ([21]). Mean counts per minute (cpm) ± standard deviation (S.D.) of the triplicate values have been used to express the results. The cells were considered responding to a given antigen when the cpm in antigen-stimulated cultures was > 500 and the Stimulation Index (SI) was > 2. Such values are underlined in the tables. The SI is defined as: SI = cpm in cultures with antigen/cpm in cultures without antigen.

RESULTS

In this study, we investigated 10 lepromatous (2 LL and 8 BL) leprosy patients whose PBMC did not show a detectable response to *M. leprae* antigens in proliferative assays (Table 1). Two tuberculoid (BT) leprosy patients responding to *M. leprae* antigens were also included for comparison purposes (Table 1). The nonresponsiveness of PBMC from the lepromatous patients was specific to *M. leprae* antigens because the same PBMC responded to *M. bovis* BCG, *M. bovis* BCG + *M. leprae* and *Mycobacterium w* (Table 1).

T-cell lines were established after stimulation of the patients' PBMC with *M. leprae, M. bovis* BCG, *M. bovis* BCG + *M. leprae,* and *Mycobacterium w.* All of the tested T-cell lines established against different antigens responded to recombinant IL-2 (Tables 2–5), suggesting that the T cells were capable of responding to an appropriate stimulus.

Only 1 of the 10 *M. leprae*-induced T-cell lines established from lepromatous patients responded to *M. leprae* on restimulation;

TABLE 1. *Proliferative response of leprosy patients' PBMC to mycobacterial antigens.*

Patients No.	Type	Proliferation[a] in response to No antigen	M. leprae		M. bovis BCG		M. bovis BCG + M. leprae		Mycobacterium w	
1	LL	273±50	355±63	(1.3)	17331±1521	(63.5)	7952±1200	(29.1)	8065±1355	(29.5)
2	LL	663±154	877±187	(1.3)	20728±2356	(31.3)	13798±2400	(20.8)	1616±225	(2.4)
3	BL	253±31	352± 72	(1.3)	1315±908	(5.2)	1244±158	(4.9)	535±495	(2.1)
4	BL	1559±320	2342±421	(1.5)	12066±1677	(7.7)	10782±1155	(6.9)	8228±1478	(5.2)
5	BL	750±221	514±128	(0.7)	33192±2355	(44.2)	40195±4723	(53.6)	31622±4872	(42.1)
6	BL	20302±2148	14470±1527	(0.7)	51450±9211	(2.5)	34936±6412	(1.8)	16779±3704	(0.8)
7	BL	953±179	1483±327	(1.5)	46020±3496	(48.3)	35666±4772	(37.4)	5632±2758	(5.9)
8	BL	1091±70	1264±192	(1.2)	87114±5204	(79.8)	66975±7625	(61.4)	19516±5575	(17.8)
9	BL	389±127	284±159	(0.7)	5610±2423	(14.4)	3370±1643	(8.7)	413±83	(1.0)
10	BL	796±242	888±543	(1.1)	25020±1365	(31.4)	17103±4817	(21.5)	3004±2406	(3.7)
Positive/Tested			0/10		10/10		10/10		9/10	
11	BT	295±92	870 ± 91	(2.9)	9857±473	(30.4)	ND[b]		14736±4530	(49.9)
12	BT	1032±572	32069±4200	(31.1)	61320±4294	(59.4)	39400±3620	(38.2)	33920±10698	(32.8)
Positive/Tested			2/2		2/2		1/1		2/2	

[a] Proliferation results in response to each antigen are presented as cpm ± S.D. (SI). SI = cpm in antigen-stimulated culture/cpm in culture without antigen; cpm values with significant proliferation, as defined in Materials and Methods, are underlined.

[b] ND = Not done.

whereas both of the T-cell lines from tuberculoid patients responded to *M. leprae* and other mycobacterial antigens (Table 2). Most of the *M. leprae*-induced T-cell lines from the lepromatous patients did not respond to *M. bovis* BCG, *M. bovis* BCG + *M. leprae*, and *Mycobacterium w*, suggesting that the T cells which might have been nonspecifically activated and expanded by IL-2 did not contribute much to the T-cell response driven by the specific antigens.

Among *M. bovis* BCG-induced T-cell lines, 3 of the 10 T-cell lines from lepromatous patients responded to *M. leprae*; whereas all of them responded to *M. bovis* BCG and *M. bovis* BCG + *M. leprae*. Six of

TABLE 2. *Proliferative response of* M. leprae-*induced T-cell lines to mycobacterial antigens.*

Patients No.	Type	Proliferation[a] in response to No antigen	M. leprae		M. bovis BCG		M. Bovis BCG+ M. leprae		Mycobacterium w		IL-2	
1	LL	387±60	425±121	(1.1)	516±78	(1.3)	501±187	(1.3)	344±82	(0.9)	ND[b]	
2	LL	357±70	365±98	(1.0)	439±111	(1.2)	347±69	(1.0)	93±15	(0.3)	ND	
3	BL	297±40	191±13	(0.6)	414±28	(1.4)	215±70	(0.7)	228±13	(0.8)	ND	
4	BL	488±45	723±157	(1.5)	296±33	(0.6)	239±35	(0.5)	297±45	(0.6)	2635±231	(5.3)
5	BL	350±38	735±112	(2.1)	696±78	(2.0)	189±68	(0.5)	243±55	(0.7)	805±129	(2.3)
6	BL	624±99	198±86	(0.3)	170±44	(0.3)	148±45	(0.2)	149±36	(0.2)	2710±880	(4.3)
7	BL	905±211	497±88	(0.5)	1018±499	(1.1)	640±166	(0.7)	845±92	(0.9)	ND	
8	BL	410±105	299±43	(0.7)	229±27	(0.6)	154±81	(0.4)	94±4	(0.2)	ND	
9	BL	426±65	560±126	(1.3)	2141±323	(5.0)	1209±351	(2.8)	582±37	(1.3)	ND	
10	BL	675±221	1111±153	(1.6)	3076±730	(4.5)	2680±541	(4.0)	1412±229	(2.1)	ND	
Positive/Tested			1/10		2/10		2/10		1/10		3/3	
11	BT	647±131	2615±179	(4.0)	1841±613	(2.8)	1436±174	(2.2)	1122± 211	(1.7)	2058± 574	(3.1)
12	BT	452±136	1798±403	(3.9)	1638±223	(3.6)	1733±614	(3.8)	ND		2226±445	(4.9)

[a] Proliferation results in response to each antigen are presented as cpm ± S.D. (SI). SI = cpm in antigen-stimulated culture/cpm in culture without antigen; cpm values with significant proliferation, as defined in Materials and Methods, are underlined.

[b] ND = Not done.

TABLE 3. *Proliferative response of* M. bovis-*BCG-induced T-cell lines to mycobacterial antigens.*

Patients No.	Type	Proliferation[a] in response to No antigen	M. leprae	M. bovis BCG	M. bovis BCG + M. leprae	Mycobacterium w	IL-2
1	LL	289±13	332±33 (1.1)	30676±4893 (106.0)	24300±3354 (84.1)	9700±2143 (33.5)	ND[b]
2	LL	167±28	254±57 (0.9)	8809±2121 (33.0)	7050±1568 (26.4)	163±22 (1.0)	ND
3	BL	401±21	252±63 (0.6)	ND	3190±415 (8.0)	300±29 (0.7)	ND
4	BL	341±41	344±33 (1.0)	1515±187 (4.4)	1258±229 (3.7)	363±57 (1.1)	3853±358 (13.3)
5	BL	282±28	881±75 (3.1)	3091±351 (11.0)	2025±349 (7.2)	1087±185 (3.8)	4455±756 (15.7)
6	BL	165±13	495±29 (3.0)	5363±2793 (32.5)	3876±661 (23.5)	322±140 (1.9)	7540± 1940 (45.6)
7	BL	396±201	220±33 (0.5)	11030±1093 (27.8)	9350±345 (23.6)	802±310(2.0)	ND
8	BL	238±19	640±41 (2.7)	6207±564 (26.1)	5036±445 (21.1)	2107±66 (8.8)	ND
9	BL	647±115	549±260 (0.8)	68735±2215 (102.0)	35256±3550 (52.3)	3881±886 (6.0)	ND
10	BL	780±308	3476±1224(4.5)	13116±3414 (16.8)	10791±184 (13.8)	4558±672 (5.8)	ND
Positive/Tested			3/10	9/9	10/10	6/10	3/3
11	BT	240±35	768±318 (3.2)	903±154 (3.8)	6569±636 (27.4)	1316±229 (5.4)	2856±295 (11.9)
12	BT	218±48	ND	ND	2528±180 (11.6)	2554±46 (11.7)	3039±478 (13.9)

[a] Proliferation results in response to each antigen are presented as cpm ± S.D. (SI). SI = cpm in antigen-stimulated culture/cpm in culture without antigen; cpm values with significant proliferation, as defined in Materials and Methods, are underlined.

[b] ND = Not done.

these T-cell lines responded by *Mycobacterium w* antigens (Table 3).

Six of the 10 T-cell lines established against *M. bovis* BCG + *M. leprae* from lepromatous patients responded to *M. leprae* on restimulation (Table 4). All of the 10 T-cell lines responded to *M. bovis* BCG and *M. bovis* BCG + *M. leprae* and seven of them responded to *Mycobacterium w* (Table 4).

Two of the 10 *Mycobacterium w*-induced T-cell lines established from lepromatous patients responded to *M. leprae* antigens. Most of these T-cell lines responded to *M. bovis* BCG, *M. bovis* BCG + *M. leprae,* and *Mycobacterium w* antigens (Table 5).

M. leprae-induced T-cell lines were also tested for reactivity to different recombinant antigens of *M. leprae* and *M. tubercu-*

TABLE 4. *Proliferative response of* M. bovis-*BCG* + M. leprae-*induced T-cell lines to mycobacterial antigens.*

Patients No.	Type	Proliferation[a] in response to No antigen	M. leprae	M. bovis BCG	M. bovis BCG + M. leprae	Mycobacterium w	IL-2
1	LL	358±79	626±129 (1.7)	33088±5623 (92.4)	28169±4700 (78.7)	9731±1656 (27.1	ND[b]
2	LL	154±53	192±41 (1.2)	4230±2298 (27.5)	3564±1833 (23.1)	152±21 (1.0)	ND
3	BL	283±257	1418±276 (5.0)	1000±114 (3.5)	584±69 (2.1)	646±61 (2.2)	ND
4	BL	228±54	519±101 (2.3)	1681±419 (7.1)	1512±278 (6.6)	366±59 (1.6)	3921±676 (17.1)
5	BL	115±18	1043±329 (9.1)	2111±518 (18.3)	1329±288 (11.6)	633±188 (5.5)	2231±622 (19.4)
6	BL	181±12	747±239 (4.1)	2921±173 (16.1)	2714±276 (15.0)	293±48 (1.6)	4940±1821 (27.2)
7	BL	310±130	301±116 (1.0)	9233±394 (29.8)	8000±961 (25.8)	1178±314 (3.8)	ND
8	BL	175±3	422±185 (2.4)	15893±510 (90.8)	15357±1194 (87.8)	5573±368 (31.8)	ND
9	BL	367±83	794±174 (2.2)	52760±3775 (143.8)	40123±1488 (109.3)	4618±1072 (12.5)	ND
10	BL	971±245	2594±556 (2.7)	12842±802 (13.2)	8337±1041 (8.6)	4459±924 (4.5)	ND
Positive/Tested			6/10	10/10	10/10	7/10	3/3
11	BT	432±121	945±15 (2.2)	11499±1085 (26.6)	8566±722 (19.8)	1422±260 (2.6)	2639±61 (4.9)
12	BT	214±166	1392±457 (6.5)	2421±101 (11.3)	2240±101 (10.5)	1886±97 (8.8)	3072±610 (14.3)

[a] Proliferation results in response to each antigen are presented as cpm ± S.D. (SI). SI = cpm in antigen-stimulated culture/cpm in culture without antigen; cpm values with significant proliferation, as defined in Materials and Methods, are underlined.

[b] ND = Not done.

TABLE 5. *Proliferative response of* Mycobacterium w-*induced T-cell lines to mycobacterial antigens.*

Patients No.	Type	Proliferation[a] in response to No antigen	M. leprae		M. bovis BCG		M. bovis BCG + M. leprae		Mycobacterium w		IL-2	
1	LL	686±293	368±148	(0.5)	10121±3758	(14.7)	6352±2119	(9.2)	13930±4586	(20.3)	ND[b]	
2	LL	167±31	300±67	(1.7)	4847±679	(29.0)	1131±358	(6.7)	ND		ND	
3	BL	597±52	367±62	(0.6)	182±52	(0.3)	143±8	(0.2)	ND		ND	
4	BL	362±77	695±159	(1.9)	633±211	(1.7)	385±78	(1.0)	1846±459	(5.0)	6335±2118	(17.5)
5	BL	277±58	725±121	(2.6)	1169±389	(4.2)	626±257	(2.2)	ND		1218±415	(4.3)
6	BL	1025±354	968±232	(0.9)	2867±341	(2.7)	2195±260	(2.1)	2729±124	(2.6)	22749±1266	(21.9)
7	BL	670±105	309±180	(0.4)	5840±366	(8.7)	4380±982	(6.5)	3866±464	(5.7)	ND	
8	BL	698±158	1466±252	(2.1)	9629±451	(13.7)	9484±960	(13.5)	11468±533	(16.4)	ND	
9	BL	134±22	320±146	(2.3)	24791±236	(185)	1865±1132	(13.9)	13455±2035	(100.4)	ND	
10	BL	395±90	682±152	(1.7)	3089±690	(7.8)	3109±952	(7.8)	3286±1231	(8.3)	ND	
Positive/Tested			2/10		8/10		8/10		7/7		3/3	
11	BT	315±79	317±69	(1.0)	1300±393	(4.1)	549±281	(1.7)	3249±365	(10.3)	5021±1267	(15.9)
12	BT	571±68	816±155	(1.4)	2562±340	(4.4)	2374±128	(4.1)	1997±59	(3.5)	2680±606	(4.6)

[a] Proliferation results in response to each antigen are presented as cpm ± S.D. (SI). SI = cpm in antigen-stimulated culture/cpm in culture without antigen; cpm values with significant proliferation, as defined in Materials and Methods, are underlined.

[b] ND = Not done.

losis to determine if T cells capable of responding to defined antigens of mycobacteria existed in lepromatous patients. The 65 kDa, 36 kDa, 28 kDa and 12 kDa recombinant *M. leprae* antigens stimulated 3, 2, 1, and 2 T-cell lines, respectively (Table 6). None of the *M. leprae*-induced T-cell lines responded to the 18 kDa and 13B3 recombinant antigens of *M. leprae* or to the 65 kDa, 19 kDa and 14 kDa recombinant antigens of *M. tuberculosis* (Table 6).

DISCUSSION

Our earlier studies have shown that proliferative nonresponsiveness of lepromatous T cells to *M. leprae* antigens is a manifestation of the inability of the T cells to produce IL-2 in response to *M. leprae* ([11]). If IL-2 is provided exogenously, *M. leprae*-specific proliferation can be induced in lepromatous T cells ([11–13]). The *in vivo* relevance of these *in vitro* findings has been

TABLE 6. *Proliferative response of* M. leprae-*induced T-cell lines to the recombinant antigens of* M. tuberculosis *and* M. leprae.

Patient no.	Proliferation[a] in response to Control[b] E. coli lysate	E. coli lysates containing recombinant antigens of M. tuberculosis 65 kDa	19 kDa	14 kDa	M. leprae 65 kDa	36 kDa	28 kDa	18 kDa	12 kDa	13B3
4	179±76 (0.9)	159±22 (0.6)	118±114 (0.9)	169±9 (3.5)	634±112 (3.2)	566±165 (0.9)	166±38 (2.3)	419±94 (2.6)	476±74 (1.5)	277±65
5	205±26	265±94 (1.3)	308±140 (1.5)	237±73 (1.1)	282±38 (1.4)	997±252 (4.9)	180±41 (0.9)	265±166 (1.3)	671±233 (3.0)	257±36 (0.9)
7	195±23	269±135 (1.4)	169±22 (0.9)	420±66 (2.1)	805±331 (4.1)	479±132 (2.4)	531±83 (2.7)	438±290 (2.2)	453±354 (2.3)	432±268 (1.6)
10	267±77	215±66 (0.8)	314±128 (1.2)	309±30 (1.1)	1346±229 (5.0)	535±6 (2.0)	215±50 (0.8)	303±118 (1.1)	937±40 (3.5)	352±165 (1.6)
Positive/Tested		0/10	0/10	0/10	3/10	2/10	1/10	0/10	2/10	0/10

[a] The proliferative responses are presented only for those lepromatous T-cell lines which responded to one or more recombinant antigens. Proliferation results in response to each antigen are presented as cpm ± S.D. (SI). SI = cpm in antigen-stimulated culture/cpm in culture without antigen; cpm values with significant proliferation, as defined in Materials and Methods, are underlined.

[b] Control *E. coli* lysate was prepared from *E. coli* cells infected with wild-type λgt11 phage.

demonstrated in clinical trials with IL-2 ([14, 15]). Injections of low doses of IL-2 into the cutaneous lesions of lepromatous patients have shown the generation of an effective CMI response, recapitulating an antigen-driven event and leading to striking local reductions in *M. leprae* ([15]). In other studies, immunizations of lepromatous leprosy patients with candidate antileprosy vaccines based on crossreactive cultivable mycobacteria either alone, e.g., *M. bovis* BCG ([16, 32]) and *Mycobacterium w* ([2, 41, 44, 45]), or along with *M. leprae,* i.e., *M. bovis* BCG + killed *M. leprae* ([3, 4, 38]) have shown upgrading of bacterial and immunological status similar to what has been reported by injecting IL-2. It is possible that immunizations with the mycobacteria of candidate antileprosy vaccines, which have antigens that crossreact with *M. leprae* in T-cell functions ([19, 24, 29]), may restore the response to *M. leprae* by enriching pre-existing *M. leprae*-responsive T cells. Since T cells from lepromatous patients are anergic to *M. leprae* antigens but respond to the antigens of candidate antileprosy vaccines ([5, 11]; Table 1), IL-2 produced in response to the antigens of candidate antileprosy vaccines may enrich the T cells responsive to *M. leprae* antigens.

The results of this study suggest that the enrichment of pre-existing *M. leprae*-responsive T cells by activation with candidate antileprosy vaccines may contribute to the restoration of the *M. leprae* response in some lepromatous patients. Among the T-cell lines established against *M. bovis* BCG and *Mycobacterium w,* two and three T-cell lines, respectively, responded to *M. leprae*; whereas 6 of the 10 T-cell lines established against *M. bovis* BCG + *M. leprae* responded to *M. leprae.* These results could be explained on the basis that *M. bovis* BCG and *Mycobacterium w* can enrich only those *M. leprae*-reactive T cells which recognize crossreactive antigens; whereas the activation of PBMC with *M. bovis* BCG + *M. leprae* can also enrich the T cells responsive to *M. leprae*-specific antigens. Consistent with our findings, this should lead to an increased possibility of the restoration of the *M. leprae* response among T-cell lines established against *M. bovis* BCG + *M. leprae* as compared to the T-cell lines established against *M. bovis* BCG and *Mycobacterium w.*

Although, 3/10, 6/10 and 2/10 T-cell lines from lepromatous patients, established against *M. bovis* BCG, *M. bovis* BCG + *M. leprae,* and *Mycobacterium w,* respectively, showed positive response to *M. leprae,* the responses in general were low (SI range 2.1 to 9.1), especially when compared with *M. bovis* BCG responses (SI range 2.7 to 106). These results are comparable with what has been reported by others after immunization of lepromatous patients with *M. bovis* BCG + killed *M. leprae* ([38]) and *Mycobacterium w* ([41]). When tested for *M. leprae*-induced proliferative response of PBMC, none of the lepromatous patients showed significant proliferation (SI > 2) prior to immunizations ([38, 41]). Following immunizations and improvements in clinical, bacteriological, histopathological and immunological (conversion to lepromin positivity) status, PBMC from 60%–70% of the patients showed significant proliferation (SI > 2) in response to *M. leprae* ([38, 41]). However, the extent of the positive response to *M. leprae* was considerably lower [SI ranges 2 to 10 and 2 to 8.4 after immunizations with *M. bovis* BCG + *M. leprae* ([38]) and *Mycobacterium w* ([41]), respectively] compared to the proliferation in response to *M. bovis* BCG [SI range 2 to 50 ([38])] and purified protein derivative [SI range 3.0 to 38.3 ([41])].

The addition of IL-2 to PBMC cultures stimulated with *M. leprae* can restore *M. leprae*-specific responsiveness in T cells from 60% of lepromatous patients ([11–13]), but in this study only 1 of the 10 *M. leprae*-activated and IL-2-expanded T-cell lines responded to *M. leprae* (Table 1). The discrepancy between the results of the present and earlier studies may be explained on the basis of the difference in the time of adding IL-2 to the cultures. In the earlier studies, IL-2 was added along with *M. leprae* antigens on day 0; in the present study, IL-2 was added on day 6 of antigen stimulation. IL-2-induced proliferation of T cells requires expression and up-regulation of high-affinity IL-2 receptors. Specific antigen as well as IL-2 is required for the expression and up-regulation of high-affinity IL-2 receptors on T cells ([22]). In earlier experiments where *M. leprae* and IL-2 were provided simultaneously to the T-cell cultures, antigen-induced expression of high-affinity IL-2 receptors on T cells would

have been up-regulated by IL-2 and the T cells would have been triggered to proliferate by the interaction of IL-2 with high-affinity IL-2 receptors. In the present experiments, the addition of IL-2 to the cultures was delayed for 6 days and, therefore, the receptors expressed in response to *M. leprae* antigens would have mostly disappeared by the time exogenous IL-2 was added to the cultures. The experiments with T-cell lines established against *M. bovis* BCG + *M. leprae* will be comparable to the activation of PBMC in cultures by the simultaneous addition of *M. leprae* and IL-2. In these experiments, IL-2 produced in response to *M. bovis* BCG during the early phases of cell activation will up-regulate the high-affinity IL-2 receptors induced on T cells in response to *M. leprae* and drive *M. leprae*-specific T cells to proliferate. This will enrich *M. leprae*-specific T cells in addition to the enrichment of T cells responsive to crossreactive antigens. The results of this study support the above view since the *M. leprae* response was restored in T cells from 60% of the lepromatous patients by either establishing T-cell lines against *M. bovis* BCG + *M. leprae* or by the stimulation of PBMC with *M. leprae* + IL-2 ([11–13]).

As compared to only 1 of the 10 *M. leprae*-induced T-cell lines, established from lepromatous patients, responding to whole *M. leprae,* four of these T-cell lines responded to different recombinant antigens of *M. leprae.* The presence of antigens/epitopes activating suppressor and helper T cells have been demonstrated in *M. leprae* ([1, 37]). The observed nonresponsiveness of lepromatous T cells to whole *M. leprae* or to total sonicates may result from the activation of suppressor cells that suppress the response of helper T cells ([1, 37]). In experimental models of nonresponsiveness, it has been shown that the suppression mediated by complex antigens having suppressor as well as helper epitopes could be overcome by using amputated antigens having only helper epitopes ([17, 40]). Our results suggest that a similar mechanism may also be operating in some lepromatous leprosy patients (Table 6). The restoration of the proliferative response to isolated antigens of *M. leprae* prepared by one- and two-dimensional gel electrophoresis has been reported, but the exact nature of the stimulating antigens was not identified ([10, 36]). By using recombinant antigens of *M. leprae,* we have for the first time shown that 65 kDa, 36 kDa, 28 kDa and 12 kDa recombinant proteins possess helper T-cell epitopes capable of overcoming the possible effect of suppressor T cells in lepromatous leprosy. Since full length protein antigens may still have both helper and suppressor T-cell epitopes ([31]), the identification of epitopes recognized by helper T cells from lepromatous patients may be useful in designing subunit vaccine(s) against leprosy.

An interesting observation was the reactivity of T-cell lines to the *M. leprae* 65 kDa recombinant antigen but not to the *M. tuberculosis* 65 kDa recombinant antigen. Similar observations were made by Ottenhoff, *et al.* who have reported that lepromatous T cells responded to 61 kDa–68 kDa antigenic fractions of *M. leprae* prepared by one-dimensional gel electrophoresis but not to the 65 kDa recombinant antigen of *M. bovis* BCG ([36]). Ottenhoff, *et al.* did not test the *M. leprae* 65 kDa recombinant antigen but, on the basis of high amino acid sequence homology (>90%) between *M. leprae* and *M. bovis* BCG 65 kDa recombinant antigens, they suggested that the 65 kDa recombinant mycobacterial antigen was not involved in the proliferative response of lepromatous T cells. However, the existence of species-specific T-cell epitopes on the 65 kDa mycobacterial proteins has been demonstrated by testing *M. leprae* and *M. tuberculosis* 65 kDa recombinant antigen-specific T-cell clones obtained from killed *M. leprae*-vaccinated healthy subjects and tuberculosis patients, respectively ([26, 35]). The results of this study suggest that the inability of lepromatous T cells to respond to the *M. bovis* BCG 65 kDa recombinant antigen ([37]) could have been due to the *M. leprae* specificity of the T-cell epitopes present on the *M. leprae* 65 kDa recombinant antigen.

SUMMARY

Several studies conducted in the last decade suggest that *Mycobacterium leprae*-reactive T cells exist in lepromatous patients, but their number may be too few to yield a detectable response in cell-mediated immunity (CMI) assays. Immunizations

with candidate antileprosy vaccines and stimulation of T cells with *M. leprae* + interleukin-2 restore the *M. leprae*-induced CMI response in lepromatous leprosy patients. These immunizations and stimulation may enrich the pre-existing *M. leprae*-responsive T cells in lepromatous patients and, thereby, induce a detectable CMI response to *M. leprae* antigens upon repeat testing. To verify this proposition, we carried out a study in a group of 10 lepromatous leprosy patients. Peripheral blood mononuclear cells (PBMC) obtained from these patients were anergic to *M. leprae* antigens in proliferative assays, but they responded to the antigens of candidate antileprosy vaccines, i.e., *M. bovis* BCG, *M. bovis* BCG + *M. leprae,* and *Mycobacterium w.* The enrichment of *M. leprae*-responsive T cells was performed by establishing T-cell lines from the PBMC after *in vitro* stimulation with *M. leprae, M. bovis* BCG, *M. bovis* BCG + *M. leprae,* and *Mycobacterium w.* When tested for their proliferative responses, 1/10, 3/10, 6/10 and 2/10 T-cell lines established against *M. leprae, M. bovis* BCG, *M. bovis* BCG + *M. leprae,* and *Mycobacterium w,* respectively, responded to *M. leprae.* These results suggest that enrichment of pre-existing *M. leprae*-responsive T cells may contribute to the restoration of the T-cell response to *M. leprae* in some lepromatous patients. Four of the 10 *M. leprae*-induced T-cell lines proliferated in response to the 65 kDa, 36 kDa, 28 kDa, and 12 kDa recombinant antigens of *M. leprae,* suggesting that the nonresponsiveness of T cells in some lepromatous patients may be overcome by using recombinant antigens of *M. leprae.*

RESUMEN

En la última década se han realizado varios estudios que sugieren que en los pacientes con lepra lepromatosa existen células reactivas con *Mycobacterium leprae* pero que su número es tan pequeño que es dificil detectarlas en los ensayos de immunidad celular. La inmunización con vacunas potenciales contra la lepra y la estimulación de las células T con *M. leprae* + interleucina 2, restauran la respuesta de inmunidad celular en los pacientes lepromatosos. Las inmunizaciones y la estimulación podrían enriquecer la población preexistente de células reactivas con *M. leprae* facilitando así la detección de las respuestas celulares hacia los antígenos del microorganismo. Para verificar esta suposición se hizo un estudio en un grupo de 10 pacientes con lepra lepromatosa. Las células mononucleares de sangre periférica (PBMC) de estos pacientes fueron anérgicas a los antígenos de *M. leprae* en los ensayos de proliferación pero respondieron a los antígenos de las vacunas potenciales contra la lepra (*M. bovis* BCG, BCG + *M. leprae,* y *Mycobacterium w*). El enriquecimiento de las células T reactivas con *M. leprae* se logró estableciendo líneas de células T a partir de las PBMC estimuladas *in vitro* con *M. leprae, M. bovis* BCG, *M. bovis* BCG + *M. leprae,* y *Mycobacterium w,* En los ensayos de proliferación, 1 de10, 3 de10, 6 de10 y 2 de10 líneas de células T establecidas contra *M. leprae, M. bovis* BCG, *M. bovis* BCG + *M. leprae,* y *Mycobacterium w,* respectivamente, respondieron a *M. leprae.* Estos resultados sugieren que el enriquecimiento de las células T reactivas con *M. leprae* preexistentes contribuye a la restauración de la respuesta de células T hacia el microorganismo en algunos de los pacientes lepromatosos. Cuatro de las 6 líneas de células T inducidas con *M. leprae* proliferaron en respuesta a los antígenos recombinantes de 65 kD, 36 kD, 28 kD, y 12 kD de *M. leprae,* sugiriendo que la falta de respuesta de las células T en algunos pacientes con lepra se puede revertir utilizando antígenos recombinantes de *M. leprae.*

RÉSUMÉ

Différentes études réalisées au cours de la denière décennie suggèrent que des cellules T réactives à *Mycobacterium leprae* existent chez les malades lépromateux, mais leur nombre pourrait être trop faible pour provoquer une réponse détectable par les tests de l'immunité à médiation cellulaire (IMC). Des immunisations avec des candidats vaccins anti-lèpre et la stimulation des cullules T par *M. leprae* + interleukine-2 restaurent la réponce d'IMC induite par *M. leprae* chez les lépreux lépromateux. Ces immunisations et la stimulation peuvent enrichir des cellules T réactives à *M. leprae* pré-existantes chez des malades lépromateux, et, par là, provoquer ine réponse IMC détectable vis-à-vis des antigènes de *M. leprae* lors de tests à répétition. Pour vérifier cette hypothèse, nous avons réalisé une étude dans un groupe de dix malades lépromateux. Les cellules mononucléaires du sang périphérique (CMSP) obtenues de ces patients étaient anergiques aux antigènes de *M. leprae* dans les tests de prolifération, mais répondaient aux antigènes de candidats vaccins anti-lèpre BCG de *M. bovis,* BCG de *M. bovis* + *M. leprae,* et *Mycobacterium w.* L'enrichissement des cellules T réactives à *M. leprae* a été réalisé en établissant des lignées de cellules T à partir de CMSP après stimulation in vitro par du *M. leprae,* BCG de *M. bovis,* BCG de *M. bovis* + *M. leprae,* et *Mycobacterium w.* Quand on les a testées pour leurs réponses prolifératives, respectivement 1/10, 3/10, 6/10 et 2/10 des lignées de cellules T établies contre *M. leprae,* BCG de

M. bovis, BCG de *M. bovis* + *M. leprae*, et *Mycobacterium w* ont répondu à *M. leprae*. Ces résultats suggèrent que l'enrichissement de cellules T pré-existantes réactives à *M. leprae* pourraient contribuer à la restauration de la réponse des cellules T vis-à-vis de *M. leprae* chez certains malades lépromateux. Quatre des 10 lignées de cellules T induites par *M. leprae* ont proliféré en réponse aux antigènes recombinants de 65 kDa, 36 kDa, 28 kDa et 12 kDa; ceci suggère que la non-réactivité des cellules T chez certains malades lépromateux pourrait être vaincue par l'utilisation d'antigènes recombinants de *M. leprae*.

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