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Suppressive Effect of Circulating Immune Complexes from Leprosy Patients on the Lymphocyte Proliferation Induced by *M. leprae* Antigens in Healthy Responders[1]

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Elevated levels of circulating immune complexes (CICs) have been demonstrated in the sera of leprosy patients, particularly in patients with borderline lepromatous (BL)/lepromatous (LL) types of leprosy and those with erythema nodosum leprosum (ENL) ([1, 4, 28, 29]). Further, CICs from BL/LL and ENL patients have been shown to have efficient complement-activating ability ([19, 28]).

Patients with BL/LL types of leprosy and those suffering ENL already have been shown to have diminished cell-mediated immune (CMI) response against *Mycobacterium leprae* ([26, 27]). The mechanism of the defective CMI response in patients with LL leprosy is still unclear. Studies have, however, shown that the cellular mechanisms may be responsible for this defect. Both macrophages ([21]) and T cells ([9]) from LL patients have been shown to have suppressor activity. Further, Nath, *et al.* ([13]) have shown that some factors from the monocytes of LL patients may induce a suppressive effect.

Godal, *et al.* ([5]) suggested that unresponsiveness in LL patients is due to the lack of circulating T cells reactive to *M. leprae.* Sometimes this *M. leprae*-specific T-cell unresponsiveness can be corrected by the addition of exogenous interleukin-2 (IL-2) ([6, 7]). However, the *M. leprae*-specific response of T cells cannot be restored in all LL patients ([14]). On the contrary, Mohagheghpour, *et al.* ([11]) explained this unresponsiveness as the lack of expression of IL-2 receptors on the lymphocytes of LL patients. Although there have been some studies indicating that blocking antibodies ([15]), lymphocytotoxic antibodies ([23]), and immune complexes (ICs) ([1]) might play some role in immunosuppression in leprosy, the literature is very scanty and needs further evaluation. Whether humoral components (such as CICs) play any significant role in the suppression of *M. leprae*-induced lymphocyte proliferation has, therefore, been studied by us. CICs were isolated from the sera of leprosy patients and their effect on *in vitro* lymphocyte proliferation induced by *M. leprae* antigens (Ags) was studied.

MATERIALS AND METHODS

Cell-free extract (CFE). The cell-free extract (CFE) of armadillo-derived *M. leprae* (kindly supplied by Dr. R. J. W. Rees, National Institute for Medical Research, London, from the IMMLEP Bank) was used as specific antigen for the lymphocyte transformation test (LTT).

Purified protein derivative (PPD). PPD of bovine tuberculin, neutralized and freeze-dried (Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Way Bridge, Surrey, U.K.) was a gift of LEPRA U.K.

Sera for isolation of CICs. Venous blood samples (10 ml each) were collected from the patients in the outpatient department of the Central JALMA Institute for Leprosy, Agra, India. The patients were classified on the clinical criteria of Ridley and Jopling ([18]): 4 tuberculoid (TT)/borderline tuberculoid (BT), 5 BL/LL, and 4 ENL subjects.

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Blood samples from four healthy laboratory volunteers were used as controls.

Isolation of CICs by PEG 6000. CICs were precipitated by 2.5% polyethylene glycol (PEG) 6000 (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) treatment of sera by a previously described method ([28]).

Source of sensitized mononuclear cells. For lymphocyte proliferation, mononuclear cells (MNCs) were isolated from the peripheral blood of healthy personnel working at our institute who have been in constant exposure to leprosy patients for many years and were known to mount a strong CMI response as determined by leukocyte migration inhibition and lymphocyte proliferation to *M. leprae* antigens and by *in vivo* lepromin testing.

Lymphocyte transformation test (LTT). MNCs from healthy responders were isolated by centrifugation on a Ficoll-Hypaque density gradient ([2]) and cultured as described previously ([3]). In brief, 2×10^5 cells were cultured in triplicate in flat-bottom microtiter plates (Nunc, Denmark) in 200 μl of RPMI 1640 containing 10% pooled AB+ serum which was supplemented with 2 mM L-glutamine and antibiotics (100 IU/ml of penicillin and 100 μg/ml streptomycin). The MNCs were stimulated with the optimal antigen doses of 5 μg/well of CFE and 1 μg/well of PPD, and were cultured for 5 days at 37°C in 5% CO_2 in humidified air. The cultures were pulsed with 1.0 μCi of tritiated methyl thymidine (specific activity 2.0 Ci/mmol; ^{3}H-TdR, Radiochemicals, Inc., Amersham, U.K.) for 18 hr before harvest. The cells were harvested by a cell harvester (Ilacon Harvester, U.K.) and ^{3}H-thymidine incorporation of the culture was measured in a liquid scintillation counter (LKB Wallac 1209 Rack Beta, Finland).

The results were expressed as mean counts per minute (cpm) of triplicates. The percent decrease in the mean cpm in the presence of ICs was calculated using the following formula:

$$\text{Percent decrease} = \frac{\Delta\text{cpm with Ag} - \Delta\text{cpm with Ag + CIC}}{\Delta \text{ with Ag}} \times 100$$

Agents used in LTT. CFE 10 μl (5 μg); PPD 10 μl (1 μg); NHS PEG precipitates 10 μl with or without CFE/PPD; TT/BT PEG precipitates 10 μl with or without CFE/PPD; BL/LL PEG precipitates 10 μl with or without CFE/PPD; ENL PEG precipitates 10 μl with or without CFE/PPD were used in the LLT.

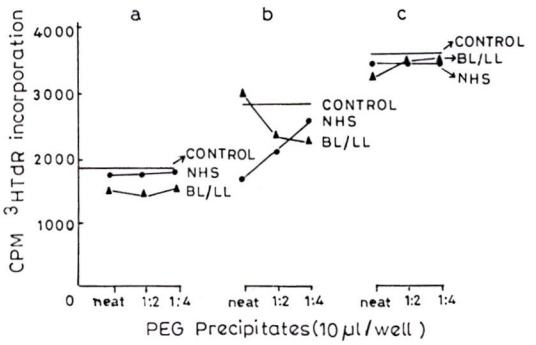

FIG. 1. Dose-dependent effect of PEG precipitates (NHS = ●---●; BL/LL = ▲---▲) on the LLT in three (a, b, and c) healthy responders.

Statistical analysis. Group means (of percent decrease in Δcpm) were compared by Student's *t* test.

RESULTS

Effect of PEG precipitates on the LTT. As can be seen in Figure 1, the PEG precipitates isolated from the sera of NHS and BL/LL groups at various concentrations (neat, 1:2, 1:4) were found to have no significant dose-dependent effect (proliferative or suppressive) on the LTT. In addition to this, 10 μl of (neat) PEG precipitates from TT/BT, BL/LL, and ENL groups were also found to have no significant effect on the LTT. The cpm obtained were similar to those of PEG precipitates from normal controls and RPMI 1640 media alone (Fig. 2).

Effect of PEG precipitates on LTT induced by CFE. PEG precipitates from BL/LL and ENL groups were found to exert a statistically significant suppressive effect on the LTT induced by CFE. PEG precipitates obtained from TT/BT and normal control groups, however, failed to show any such effect. The percent decreases in the mean values of Δcpm obtained were: NHS, 12.32 $\pm$ 9.7; TT/BT, 15.98 $\pm$ 11.51; BL/LL, 46.77 $\pm$ 22.42 and ENL, 65.0 $\pm$ 24.28. The difference in the mean values of percent decrease between NHS vs BL/LL, ENL and TT/BT vs BL/LL, ENL was found to be

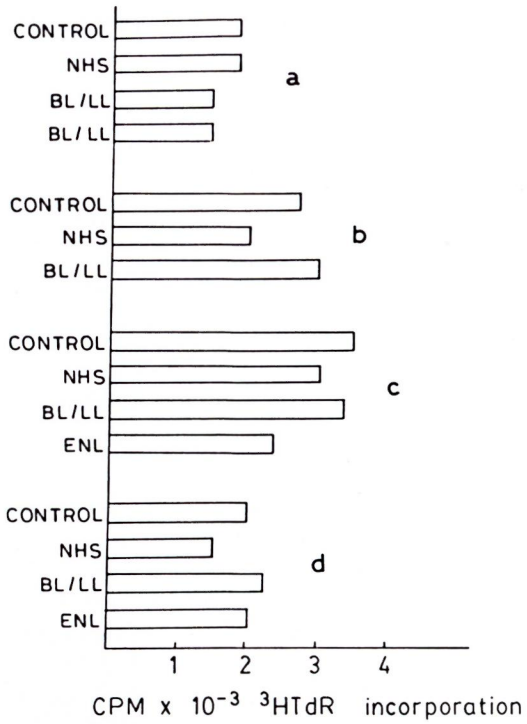

FIG. 2. Effect of PEG precipitates obtained from serum of normal healthy volunteers (NHS), borderline lepromatous (BL)/lepromatous leprosy (LL), and erythema nodosum leprosum (ENL) patients on lymphocyte proliferation of healthy responders (a, b, c, and d); control = RPMI 1640; horizontal bars = mean cpm $\times 10^{-3}$ ^{3}H-TdR uptake of triplicate cultures.

statistically significant ($p < 0.05$); whereas the difference between BL/LL and ENL was not found to be statistically significant ($p > 0.1$) (Table 3).

Effect of PEG precipitates on LTT induced by PPD. PEG precipitates from the ENL group were found to exert a statistically significant suppressive effect on the LTT induced by PPD. PEG precipitates from TT/BT, BL/LL and normal control groups, however, failed to show any such effect. The percent decreases in the mean values of Δcpm were: NHS, 8.46 ± 10.47; TT/BT, 15.68 ± 26.0; BL/LL, 34.94 ± 32.10 and ENL, 49.81 ± 12.48. (NHS vs ENL, $p < 0.01$) (Table 4). However, the PEG precipitates from 2 out of 7 BL/LL patients were found to exert a suppressive effect on the LTT induced by PPD. The percent decreases in their Δcpm were 77.48% and 80.67%, respectively (Table 2).

DISCUSSION

There have been several studies which indicated that in lepromatous leprosy the CMI is depressed specifically to *M. leprae,* but the mechanism of unresponsiveness of lymphocytes against *M. leprae* antigens is far from clear. The failure of T lymphocytes to proliferate in response to *M. leprae in vitro* was, however, interpreted in terms of: a) clonal deletion ([24]); b) selective defect of presensitized T cells in IL-2 secretion ([6, 10]); c) defect in IL-2 receptor expression ([11]); d) specific T-suppressor cells ([9]); and e) specific suppressor macrophages ([21, 22]). In our previous study ([28]), we showed that patients with BL/LL types of leprosy and patients with ENL have elevated levels of mycobacterial IC (MIC) in their circulation. Whether CICs play any suppressive role in leprosy is, however, not known. There have been reports in which ICs have been shown to act as an immunomodulator ([30]). In addition, ICs have been shown to enhance ([12]) as well as to suppress ([25]) immune responses.

In a mouse model in the recent past, Rao, *et al.* ([17]) have shown that pretreatment of H-2b mice with Ag-Ab complexes rendered the mice incapable of mounting an effective immune response to viable allotypic tumor cells. Similarly, our results in an *in vitro* situation showed a significant suppression of *M. leprae*-induced lymphocyte proliferation by PEG precipitates obtained from BL/LL and ENL patients. However, PEG precipitates obtained from TT/BT and normal control groups were found to exert no significant suppressive effect on the lymphocyte proliferation. It is possible that the elevated levels of mycobacterial ICs ([28]) in the BL/LL and ENL patients may have a direct immunosuppressive effect on the LTT induced by CFE. It may also be argued that possibly due to antimycobacterial antibodies in CICs in BL/LL and ENL groups, formed by antibody excess, these antibodies may have bound to *M. leprae* antigens. This might have lowered the concentration of free *M. leprae* antigens available in the culture for the optimal stimulation of the lymphocytes.

The other possible explanation for the suppression of lymphocyte proliferation might be due to the presence of elevated

TABLE 1. *Effect of PEG precipitates isolated from various types of leprosy patients and normal healthy controls on the LTT induced by CFE in healthy responders.*[a]

Healthy responders	Antigen	Total protein concentration in PEG precipitates (μg/ml)	^{3}H-TdR uptake Δcpm	% Decrease in Δcpm (induced by CFE) in presence of PEG precipitates
P.N.S.	CFE	—	6,851	—
	CFE + NHS IC	65	6,254	14.2%
	CFE + BL/LL IC	430	4,447	42.8%
	CFE + ENL IC	580	4,638	34.7%
A.K.G.	CFE	—	38,452	—
	CFE + NHS IC	60	28,608	23.6%
	CFE + TT/BT IC	200	30,321	21.1%
	CFE + BL/LL IC	690	8,231	78.9%
	CFE + ENL IC	700	12,521	68.4%
N.Y.	CFE	—	17,816	—
	CFE + NHS IC	40	17,887	0%
	CFE + TT/BT IC	500	14,901	16.6%
	CFE + BL/LL IC	730	12,703	27.0%
	CFE + ENL IC	800	1,028	94.0%
S.S.	CFE	—	25,956	—
	CFE + NHS IC	40	23,030	11.5%
	CFE + TT/BT IC	430	19,212	26.9%
	CFE + BL/LL IC	730	15,818	38.4%
	CFE + ENL IC	850	9,257	65.3%

[a] Results showing Δcpm induced by CFE and percentage decrease in the Δcpm in the presence of PEG precipitates. NHS = normal human serum; TT/BT = tuberculoid/borderline tuberculoid; BL/LL = borderline lepromatous/lepromatous leprosy; ENL = BL/LL with erythema nodosum leprosum; IC = immune complex.

levels of immunosuppressive molecules, such as phenolic glycolipid-I (PGL-I) and lipoarabinomannan (LAM), in the sera of these patients ([8]). However, in our study PEG precipitates (from the BL/LL and ENL groups) alone were found to have no significant effect on the LTT (Fig. 2), indicating thereby that the concentration of PGL-I and/or LAM (if any) in the complex would be minimal and, hence, may not be optimal for inhibition of the proliferation of the sensitized lymphocytes.

Further, the presence of low or negligible levels of MIC in TT/BT and normal control groups may explain the finding of the insignificant suppressive effect of these PEG precipitates on the lymphocyte proliferation induced by CFE in these patients (Table 3). Similarly, the presence of the insignificant differences in the mean values of MIC levels between BL/LL and ENL groups ([28]) may explain the finding of insignificant differences in the mean values of percent decreases in Δcpm (induced by CFE) between BL/LL and ENL groups (Table 3).

It is interesting to note that PEG precipitates from TT/BT and BL/LL groups were found to have no significant suppressive effect on the LTT induced by PPD. However, the significant suppressive effect of PEG precipitates from the ENL group and the depression noted in two patients with BL/LL leprosy could not be explained from the present study. It is, however, possible that these patients may have elevated levels of MICs that might have induced the suppressive effect.

From these observations, it may be concluded that CICs in leprosy patients have the ability to suppress lymphocyte proliferation induced by *M. leprae* antigens. However, the exact mechanism of suppression for proliferation of lymphocytes is not clear. Further studies are needed at the molecular level to find out the mechanism for

TABLE 2. *Effect of PEG precipitates isolated from various types of leprosy patients and healthy controls on the LTT induced by PPD in healthy responders.*[a]

Healthy responders	Antigen	Total protein concentration in PEG precipitates (μg/ml)	^{3}H-TdR uptake Δcpm	% Decrease in Δcpm (induced by PPD) in presence of PEG precipitates
A.K.G.	PPD	—	58,632	—
	PPD + NHS IC	40	57,764	1.6%
	PPD + TT/BT IC	200	57,554	1.6%
	PPD + BL/LL IC	430	48,726	16.9%
S.A.	PPD	—	3,031	—
	PPD + NHS IC	60	1,100	6.7%
	PPD + BL/LL IC	300	2,677	1.3%
	PPD + ENL IC	721	1,763	43.3%
H.R.D.	PPD	—	1,449	—
	PPD + NHS IC	40	2,329	0%
	PPD + BL/LL IC	770	321	77.8%
	PPD + BL/LL IC	793	280	80.7%
	PPD + ENL IC	735	543	64.2%
K.K.K.	PPD	—	3,961	—
	PPD + NHS IC	270	3,005	25%
	PPD + TT/BT IC	340	3,016	25%
	PPD + TT/BT IC	150	3,861	2.5%
	PPD + TT/BT IC	431	1,425	65%
	PPD + BL/LL IC	500	2,465	37.5%
P.N.S.	PPD	—	3,988	—
	PPD + NHS IC	40	3,295	17.5%
	PPD + TT/BT IC	120	4,191	0%
	PPD + BL/LL IC	495	3,320	17.5%
U.D.	PPD	—	3,113	—
	PPD + NHS IC	40	3,012	0%
	PPD + TT/BT IC	150	3,259	0%
	PPD + BL/LL IC	478	2,760	12.9%
	PPD + ENL IC	540	1,846	41.9%

[a] Results showing Δcpm induced by PPD and percentage decrease in the Δcpm in the presence of PEG precipitates. NHS = normal human serum; TT/BT = tuberculoid/borderline tuberculoid; BL/LL = borderline lepromatous/lepromatous leprosy; ENL = BL/LL with erythema nodosum leprosum; IC = immune complex.

suppression of the LTT responses by CICs in leprosy patients.

SUMMARY

The effect of circulating immune complexes, isolated in the form of polyethylene glycol (PEG) precipitates from leprosy patients, on lymphocyte proliferation was studied. The results obtained showed that PEG precipitates obtained from the borderline lepromatous/lepromatous (BL/LL) types of leprosy patients and those undergoing erythema nodosum leprosum (ENL) had significant suppressive effects on the lymphocyte proliferation induced by *Mycobacterium leprae* antigens in healthy responders. The percent decreases in the mean values of Δcpm in the presence of PEG precipitates from the BL/LL and ENL groups were found to be 46.8 ± 22.4 and 65.0 ± 24.3, respectively. However, no significant suppressive effects (except for ENL PEG precipitates) of these PEG precipitates were observed on the lymphocyte proliferation induced by tuberculin (PPD). Further, PEG precipitates alone (in the absence of *M. leprae* antigen) from the BL/LL and ENL groups were found to have no effect on the lymphocyte proliferation.

RESUMEN

Se estudió el efecto de los complejos inmunes circulantes aislados por precipitación con polietilén glicol (PEG) de los sueros de pacientes con lepra, sobre la

TABLE 3. *Values of percent decrease in Δcpm (induced by CFE) in the presence of PEG precipitates from various types of leprosy patients and control groups in healthy responders.*

Group[a]	No.	% Decrease in Δcpm (induced by CFE) in presence of ICs	
		Mean ± S.D.	Range
NHS	4	12.3 ± 9.7	0%–23.6%
TT/BT	4	16.0 ± 11.5	0%–26.9%
BL/LL	4	46.8 ± 22.4	27.0%–78.9%
ENL	4	65.0 ± 24.3	34.7%–94%
Statistical analysis			
NHS vs TT/BT		NS[b]	
NHS vs BL/LL		$p < 0.05$	
NHS vs ENL		$p < 0.05$	
TT/BT vs BL/LL		$p < 0.05$	
TT/BT vs ENL		$p < 0.05$	
BL/LL vs ENL		NS	

[a] NHS = normal human serum; TT/BT = tuberculoid/borderline tuberculoid leprosy; BL/LL = borderline lepromatous/lepromatous leprosy; ENL = BL/LL with erythema nodosum leprosum.
[b] NS = Not significant.

TABLE 4. *Values of percent decrease in Δcpm (induced by PPD) in the presence of PEG precipitates isolated from various types of leprosy patients and control groups in healthy responders.*

Group[a]	No.	% Decrease in Δcpm (induced by PPD) in presence of ICs	
		Mean ± S.D.	Range
NHS	6	8.5 ± 10.5	0%–17.5%
TT/BT	6	15.7 ± 26.0	0%–65%
BL/LL	7	34.9 ± 32.1	1.3%–80.7%
ENL	3	49.8 ± 12.5	43.3%–64.2%
Statistical analysis			
NHS vs BL/LL		NS[b]	
NHS vs ENL		$p < 0.01$	
TT/BT vs BL/LL		NS	
TT/BT vs ENL		NS	
BL/LL vs ENL		NS	

[a] NHS = normal human serum; TT/BT = tuberculoid/borderline tuberculoid leprosy; BL/LL = borderline lepromatous/lepromatous leprosy; ENL = BL/LL with erythema nodosum leprosum.
[b] NS = Not significant.

proliferación de linfocitos de individuos respondedores sanos. Los resultados obtenidos mostraron que los precipitados PEG obtenidos de pacientes con lepra lepromatosa/lepromatosa subpolar (LL/BL) y de aquellos pacientes con eritema nodoso leproso (ENL), tuvieron significantes efectos supresivos de la proliferación de linfocitos inducida por los antígenos del *Mycobacterium leprae.* Los porcentajes de disminución en los valores medios de las CPM en presencia de precipitados PEG de los grupos BL/LL y ENL, fueron 46.8 ± 22 y 65.0 ± 24.3, respectivamente. Sin embargo, los precipitados PEG de los pacientes LL/BL no tuvieron efectos supresivos significantes sobre la proliferación de linfocitos inducida por la tuberculina (PPD). Los precipitados PEG sólos de los grupos BL/LL y ENL, en ausencia de antígenos del *M. leprae,* no tuvieron ningún efecto sobre la proliferación de linfocitos.

RÉSUMÉ

On a étudié l'effet, sur la prolifération lymphocytaire, des complexes immuns circulants, isolés de patients lépreux sous la forme de précipités de polyéthylène glycol (PEG). Les résultats ont montré que les précipités de PEG obtenus à partir de patients atteints de lèpre borderline lépromateuse ou lépromateuse (BL/LL) et ceux présentant un érythème noueux lépreux (ENL) avaient des effets suppresseurs significatifs sur la prolifération lymphocytaire induite par des antigènes de *Mycobacterium leprae* chez des personnes en bonne santé. La diminution des valeurs moyennes de Δcpm en présence de précipités de PEG provenant des groupes de patients BL/LL et ENL était respectivement de 46.8 ± 22.4% et de 65.0 ± 24.3%. Cependant, aucun effet suppresseur significatif (à l'exception des précipités de PEG provenant des patients ENL) de ces précipités de PEG n'était observé vis-à-vis de la prolifération lymphocytaire induite par la tuberculine (PPD). De plus, on n'a pas trouvé d'effet des précipités de PEG seuls (en l'absence d'antigène de *M. leprae*) provenant des patients BL/LL et ENL sur la prolifération lymphocytaire.

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