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ConA-induced Suppressor Cells in Lepromatous Leprosy Patients During and After Erythema Nodosum Leprosum[1]

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Mycobacterium leprae produces in man different clinical and immunological alterations. According to the classification of Ridley and Jopling ([25]), two polar forms of leprosy have been described: one of them, known as tuberculoid leprosy (TT), shows high resistance to *M. leprae*; while the other, with lower resistance to the agent, is a highly disseminated bacilliferous form known as lepromatous leprosy (LL). The immunological aspects of LL have been widely studied in the last decades in order to elucidate the mechanisms involved in the etiopathogenesis of the disease. Recent investigations have shown that cell-mediated immunity (CMI) in LL patients is diminished. Low responses to both specific ([11,12,20]) and nonspecific antigens have been observed ([6,9]). Furthermore, an absence of reactivity to cutaneous sensitizing agents has also been found ([10]). The alterations could be due either to intrinsic defects of the disease that may or may not be linked to genetic factors and/or to a failure in immunoregulatory mechanisms ([8]).

Functional alterations in immunoregulatory cells in different human pathologic conditions have already been described. In autoimmune diseases ([4,29]), sarcoidosis, schistosomiasis ([7]), and some immunodeficiencies, elevation, diminution, or even absence of suppressor functions have been observed. Suppressor activity can be induced and measured *in vitro* by cultivating lymphocytes in the presence of mitogens such as concanavalin A (ConA), antigens, or allogeneic cells. ConA can be used as an inducer of T suppressor cells and as a nonspecific mitogen. Recent works show that LL patients have a failure in their suppressor functions, while those with TT have a normal suppressor activity ([22]).

Erythema nodosum leprosum (ENL) occurs in patients at or close to the lepromatous end of the leprosy spectrum. ENL is found in highly bacilliferous patients, especially when they are put on antileprosy treatment. This clinical feature is characterized by the presence of subcutaneous nodules, frequently associated with fever and, on some occasions, with neuritis, iridocyclitis, orchitis, proteinuria, and lymphadenopathies. Histologically, it is very similar to the Arthus reaction with perivascular infiltrations of neutrophils and immunoglobulin and complement deposits in the lesions ([5,27]). The presence of immune complexes has been reported to be important in the pathogenesis of ENL ([19,26]), and authors such as Bjorvatn, *et al.* ([3]) believe that extravascular complexes might be involved. On the other hand, Bullock and Fasal ([6]) have reported that ENL patients show less alterations in their lymphocyte blastogenic responses to mitogens than do patients in the quiescent form of the disease. During ENL, there is a sudden liberation of bacilli from macrophages ([13,35]), and high antibody titers and complement values have been observed.

On the basis of these observations, we decided to study suppressor T cell functions, using ConA as the inducer, in the patients with LL during ENL and in some of the patients after the ENL episode.

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MATERIALS AND METHODS

Patients

A total of 16 patients with lepromatous leprosy (LL) classified according to Ridley and Jopling were studied during an episode of ENL. Six were women and ten were men, ranging in age from 27–60 years. All the patients were under specific treatment for a period of time longer than 28 months with dapsone (diaminodiphenylsulfone) (DDS). Some of the cases also received aspirin. Thalidomide was added in patients with ENL. Nine of these patients returned 20–30 days after stopping treatment with thalidomide and a second blood sample was obtained. The length of time between the first and second blood samples was 30–40 days. Simultaneously, 12 healthy controls (6 men and 6 women between 15–50 years of age) were studied.

Isolation of mononuclear cells

Peripheral blood of healthy controls and patients was defibrinated with glass beads for 10 min. Mononuclear cells (MNC) were isolated by Ficoll-Hypaque discontinuous density gradient centrifugation according to Thronsby and Bratlie ([31]). Cells at the interphase were harvested, washed, and resuspended in RPMI-1640 (Gibco Biocult, Gibco Laboratories, Grand Island, New York, U.S.A.) supplemented with 10% normal AB serum, 1% amphotericin B, and 50 μg/ml of gentamycin (RPMI-AB).

Suppressor cell assay

Activation of MNC with ConA (first culture) ([28]). MNC were cultured at 2×10^6 cells/ml in RPMI-AB: a) with 10 μg/ml of ConA (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) (ConA-activated MNC), and b) without the addition of ConA (control cells) for 24 hr. Subsequently, ConA-activated and control cells were washed three times and treated with mitomycin C (Sigma Chemical Company) 50 μg/ml for 30 min to block further DNA synthesis. They were washed three times with RPMI-1640 and resuspended in RPMI-AB at 1×10^6 cells/ml.

Assay for suppressor activity (second culture). Each preparation from the first culture (ConA-stimulated cells and control cells) was then cultured with fresh autologous MNC obtained from a second blood sample of the same donor or patient drawn one day later. In this second culture, 2.5×10^5 fresh responding cells and 2.5×10^5 ConA-prestimulated cells or control cells were cultured with or without two different mitogens: ConA (20 μg/ml) and PHA-P (50 μl of 1/100 stock solution, Difco Laboratories, Detroit, Michigan, U.S.A.) for 72 hr in plastic tubes (10 mm × 7.5 mm, Falcon Plastics, Oxnard, California, U.S.A.) at 37°C. The final volume of the incubation mixture was 0.5 ml.

Thymidine incorporation was measured after a terminal 3 hr pulse with 1 μCi/ml of ^{3}H-thymidine (20 Ci/mM, New England Nuclear Corp., Boston, Massachusetts, U.S.A.). The counts per minute (cpm) of trichloracetic precipitable material dissolved in Soluene-100 (Packard Instrument Co., Inc., Rockville, Maryland, U.S.A.) were measured in a Packard Tricard scintillation counter. Each reaction was carried out in triplicate.

Data are expressed as mean cpm ± standard error of the mean (SEM); Δcpm was calculated by subtracting the cpm of the unstimulated cultures from the cpm of the stimulated cultures with PHA or ConA. The percentage of suppression was calculated according to the following formula:

$$\% \text{ suppression} = \left(1 - \frac{\Delta\text{cmp}^{CA}}{\Delta\text{cpm}^{NA}}\right) \times 100$$

where Δcpm^{CA} is the Δcpm after the addition of ConA-activated cells to the second assay culture, and Δcpm^{NA} is the Δcpm after the addition of nonactivated or control cells to the second assay culture.

Proliferative response to PHA

The proliferative responses to PHA were measured in the same samples. MNC were isolated as described above, and cultured at 5×10^5 cells/0.5 ml in RPMI-AB. Cultures were stimulated with 50 μl of a 1/100 dilution of PHA-P (Difco Laboratories); control cells were incubated without PHA. Cultures were maintained at 30°C for 74 hr, and ^{3}H-thymidine incorporation was measured as described for the ConA-induced suppressor activity.

The PHA stimulation indexes (SI) were calculated as:

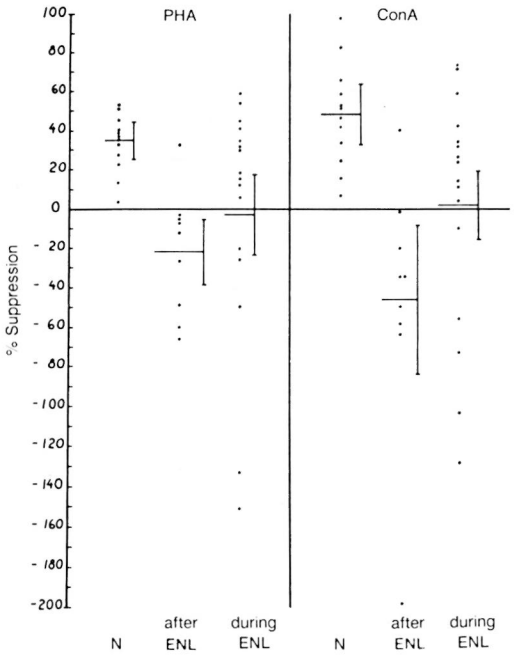

FIG. 1. Suppression of PHA and ConA-induced mitogenesis by ConA-activated lymphocytes.

The percentage of suppression calculated, as described in the Materials and Methods section, was measured in 9 patients with lepromatous leprosy after the ENL episode (after ENL), 16 patients at the time of the ENL episode (during ENL), and compared with 12 healthy controls (N). Statistical differences (Wilcoxon-Mann-Whitney test) were found between N vs after ENL vs during ENL ($p < 0.01$).

$$SI = \frac{\text{cpm stimulated culture}}{\text{cpm control culture}}$$

Statistical methods

The Wilcoxon-Mann-Whitney rank test and the Student *t* test were used for statistical analysis.

RESULTS

The results of measuring blast transformation by PHA are shown in The Table. The normal SI value is 75 ± 20 ($\bar{x}$ ± SEM, N = 20) with the technique used in our laboratory. In the 16 LL patients the SI in response to PHA was 63.5 ± 15.8 ($\bar{x}$ ± SEM). This difference was not statistically significant. SI values did not change significantly in the nine patients that could be studied during and after the ENL episode.

The *in vitro* response of normal control

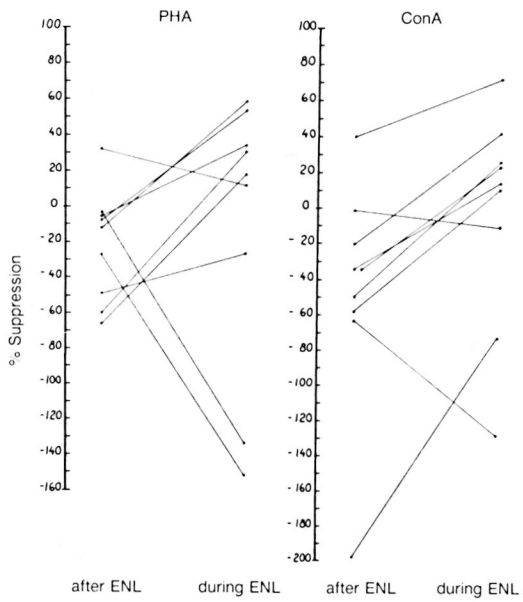

FIG. 2. Suppressor activity of lymphocytes from nine patients during and after the ENL episode. Statistical differences were only found in the ConA proliferative responses ($p < 0.01$).

lymphocytes to stimulation with PHA and ConA was suppressed by autologous ConA-pretreated lymphocytes. As shown in Figure 1, inhibition for both mitogens was observed in the control cases. The range of

THE TABLE. *Stimulation indexes in PHA blast transformation in LL patients with ENL.*

Patient no.	Stimulation indexes
1	106.7
2	48.9
3	76.8
4	18.5
5	43.3
6	31.2
7	65.6
8	56.8
9	59.6
10	64.9
11	30.0
12	98.7
13	100.0
14	135.0
15	37.5
16	44.0
$\bar{x}$ ± SEM	63.5 ± 15.8
Normal $\bar{x}$ ± SEM (N = 20)	75 ± 20

inhibition of the PHA mitogenic response was between 3%–52% ($\bar{x} \pm SEM = 34.41 \pm 4.54$, N = 12). Considerable inhibition also occurred in ConA-induced mitogenesis, with values between 16%–52% ($\bar{x} \pm SEM = 48.4 \pm 7.67$, N = 12).

The ConA-induced suppressor response of T cells was markedly less than normal in LL patients during the ENL episode. The mean suppressor value was $-2.18 \pm 8.09\%$ ($\bar{x} \pm SEM$, N = 16) for the PHA challenge and $1.25 \pm 8.87\%$ for ConA proliferative stimulus. These reductions are highly significant ($p < 0.01$) (Fig. 1). There was even less suppressor capacity in the nine LL patients after the ENL episode. The responses in both the ConA and PHA mitogenesis challenges gave negative values for suppression, $-46.4 \pm 18.92\%$ and $-22 \pm 8.09\%$, respectively (Fig. 1).

Nine patients were studied both during the ENL episode and after its remission (Fig. 2). A separate analysis of these cases demonstrated that the suppressor activity in the LL patients during the ENL episode was higher than that in the same patients after the ENL episode. These results were significant ($p < 0.01$) for suppression of the proliferative response to ConA (Fig. 2).

DISCUSSION

Leprosy is a disease characterized by an abnormal immune response. The clinical form resulting from an infection with *M. leprae* depends on the integrity of the specific cellular immunity of the host. A failure in the immune response to the bacterium leads to the development of the lepromatous form of the disease. On the other hand, when the immune response is preserved, high resistance is conferred and the tuberculoid form appears.

Recent findings [13] suggest that patients develop LL as a result of a specific defect in the T lymphocyte population. According to some authors, this defect would be genetically controlled; this hypothesis is based on the fact that certain genes of the major histocompatibility complex (HLA) appear with greater frequency in TT than in LL patients [8,14]. Perhaps a certain clone of T cells with receptors for *M. leprae* can be actively suppressed. On the other hand, the balance of suppressor mechanisms might be impaired [15]. Therefore, the study of the CMI of the host would be the first step to take in exploring these possibilities.

Some authors [6,16] have reported a low PHA response in LL patients. However, the response was restored under specific treatment [18,21]. Although several authors [16,24,33] have reported that PHA response was normal during the ENL episode, we have observed that only 60% of the patients (9/16) had normal PHA stimulation indexes (The Table). We did not find statistical differences between normal controls and patients. Our results are in agreement with previous reports demonstrating a normal PHA proliferative response in LL patients during the ENL episode [1,16].

Augmented cellular immune responses and higher levels of sensitization to DNCB [23] were observed in ENL patients when compared to LL patients during the quiescent phase. Evidence of activation of the immune system during reactional episodes is also offered by the increase in specific antibody titers [27], production of immune complexes, and elevation of complement levels [19].

It is known that antibody production is altered in leprosy patients; it is possible that specific antibodies which would act directly against *M. leprae* could be correlated with the antigenic challenge [10,15,32]. Up to the present time, no protective action of these antibodies has been demonstrated; in contrast, they might even be deleterious for the patient, as occurs during ENL [10]. Clinical, histopathological, and immunological investigations indicate that immune complexes are of great importance in the pathogenesis of ENL. Immune complexes could induce a defensive cellular inflammatory reaction but, in some cases, painful inflammatory lesions can take place and lead to generalized impairment. On the other hand, excessive immune reactivity could be of benefit to the patients, leading to activation of immunoregulation mechanisms.

We have shown that ConA-induced suppressor activity was less in LL patients during the ENL episode than in normal individuals (Fig. 1). Nine patients could be studied during and after ENL, and in eight of these, the suppressor activity was even less after the ENL episode (Figs. 1 and 2).

Nath, *et al.* [22] observed low suppressor activity in untreated LL patients. In their

series, and in contrast with our data, suppressor activity during the ENL episode was similar to that of normal controls. Bach, *et al.* ([2]) demonstrated that ConA-induced suppression of mixed leukocyte culture proliferation was decreased after ENL. These authors postulate that the T helper/suppressor (H/S) cell ratio is altered in these patients. Increased T helper activity would be responsible for the high responses to PHA in LL patients during the ENL and immediately after the episode. On the other hand, decreased numbers of circulating T suppressor cells would account for the low functional values of ConA-induced suppressor activity observed in these patients.

Our results show a defect in the induction of suppression by ConA in LL patients after the ENL episode and in this regard they differ from those of Mehra, *et al.* ([17]). These authors showed that specific antigens (Dharmendra lepromin) induced suppression of mitogenic responses in LL or borderline patients but not in TT or normal individuals. However, the two experimental systems are quite different, and it is possible that suppressor responses are enhanced by a specific antigenic stimulus in patients in the absence of a reactional episode (ENL).

The ratios of H/S T cells in LL patients with recent ENL episodes have been studied with monoclonal antibodies by Wallach, *et al.* ([34]). They reported that the H/S ratio was increased through a relative diminution of the suppressor/cytotoxic T cell subset. These results could be correlated to the decreased T suppressor function observed by us in lepromatous patients during and after the ENL episode.

Reduced levels of suppressor activity in LL patients reveal a defect in central mechanisms of control of the immune response. With the present data it is not possible to determine if the regulatory imbalance that produces the sharp decrease in suppressor cell activity in LL patients during and after ENL has a relevant pathogenic role in the production of the ENL or is a consequence of its development.

SUMMARY

ConA-induced suppressor activity in patients with lepromatous leprosy (LL) was studied. Patients were studied during and after erythema nodosum leprosum (ENL) reactions. The study included 16 patients with ENL, nine of whom returned once the ENL episode was over. Patients were compared to 12 normal controls. Suppressor activity was evaluated *in vitro* by cultivating peripheral blood lymphocytes (PBL) with an inducer of T suppressor cells, concanavalin A (ConA), and with two different mitogens, phytohemagglutinin (PHA) and ConA, in order to measure the inhibition of the proliferative responses in all cases. In contrast, in LL patients during ENL the ConA-induced suppressor response was markedly reduced. The reduction in suppressor responses was even more marked in the LL patients after the ENL episode. Reduced levels of suppressor activity in LL patients reveal a defect in central mechanisms of control in the immune response.

RESUMEN

Se estudió la actividad supresora inducida por ConA en pacientes con lepra lepromatosa (LL). Los pacientes se estudiaron durante y después de episodios reaccionales tipo eritema nodoso leproso (ENL). El estudio incluyó a 16 pacientes con ENL, nueve de los cuales se reestudiaron después de que el episodio reaccional hubo terminado. Los pacientes se compararon con 12 controles sanos. La actividad supresora se evaluó *in vitro,* cultivando linfocitos de sangre periférica en presencia de concanavalina A, un inductor de células T-supresoras, y con otros dos mitógenos diferentes, fitohemaglutinina (PHA) y ConA, para medir la inhibición de las respuestas proliferativas. Los pacientes LL en reacción leprosa al momento del estudio mostraron una respuesta supresora inducida por ConA, muy reducida. La reducción en la respuesta supresora fue aún más marcada en los pacientes LL después del episodio reaccional, ENL.

Los reducidos niveles de la actividad supresora en los pacientes LL, revelan un defecto en los mecanismos centrales de control de la respuesta inmune.

RÉSUMÉ

On a étudié l'activité de suppression induite par la Concanavaline-A chez des malades atteints de lèpre lépromateuse (LL). Ces patients ont été étudiés pendant et après des réactions d'érythème noueux lépreux (ENL). Cette étude a porté sur 16 malades atteints d'ENL, dont neuf sont revenus après que l'épisode réactionnel ait été terminé. Ces malades ont été comparés à 12 témoins normaux. L'activité de suppression a été évaluée *in vitro,* par la culture de lymphocytes de sang périphérique, avec un inducteur des cellules T suppressives, avec la Concanavaline A (ConA), et avec deux mitogènes différents, la phytohémagglutinine et la Concanavaline A, afin de mesurer l'inhibition des ré-

ponses prolifératives chez tous ces malades. A l'opposé, chez les malades LL au cours de l'épisode d'érythème noueux lépreux, la réponse suppressive induite par la Concanavaline A était notablement réduite. La diminution des réponses suppressives était encore plus marquée chez les malades LL après un épisode d'ENL. Une réduction des taux de l'activité suppressive chez les malades LL révèle un défaut dans les mécanismes centraux de contrôle de la réponse immunitaire.

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