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Study of HLA-DR Expression on Skin Lesions of Leprosy Before and During Multiple Drug Therapy[1]

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Leprosy is a chronic inflammatory disease caused by *Mycobacterium leprae*. The human response to this pathogen exhibits intriguing aspects which are until now not well understood ([16]). The disease is characterized by a broad spectrum of clinical forms depending on the patient immune status. T-helper 1 (Th1) cells are associated with tuberculoid leprosy patients, who have strong cell-mediated immune response, while Th2 cells are expressed in lepromatous leprosy patients who have strong humoral response with a lack of T-cell response ([8]). However, patients often suffer from immunologically mediated reactions either spontaneously or during treatment. Two major reactions are recognized, the first is the reversal reaction in which skin becomes red and swollen with tenderness of peripheral nerves, which may lead to additional disability. Such reaction seemed to be due to increased CMI response following increased release of antigen after starting treatment of either tuberculoid or lepromatous leprosy. The second reaction is erythema nodosum leprosum in which painful inflamed nodules occur in the skin of lepromatous leprosy patients which seemed to be immune complex mediated reaction ([4]).

Fortunately, multiple drug therapy (MDT) has revolutionized the treatment of leprosy where many patients are now cured within six months to two years depending on their bacterial load ([7]).

There is an increasing interest in the immunomodulatory role of MDT because its beneficial effect may be accompanied by important changes in the immune cell profile which have a great role in overcoming such infection. In some studies, it was suggested that the CMI was reactivated in most patients under MDT, which is not restricted to those who developed immunologically mediated adverse reactions during the therapeutic course, such as reversal reaction or erythema nodosum leprosum ([8]).

In the present study, we tried to investigate the immunomodulatory effect of MDT on the skin lesions of patients with leprosy by studying the HLA-DR display before and a few weeks after starting MDT using immunofluorescent staining. In addition, new cases who did not receive any treatment for 2–4 weeks were included for comparison. Patients who developed reversal reaction during MDT were studied for the effect of prednisolone administration on the HLA-DR expression in their granuloma.

PATIENTS AND METHODS

This study included 35 patients with leprosy from the Outpatient Clinic of the Department of Dermatology and Venereology, Tanta University Hospital. There were 24 males and 11 females with an age range of 18 years to 50 years (mean = 35.5 years). They included 30 newly-diagnosed patients and five patients who developed reversal reaction during MDT.

Skin punch biopsy specimens (4 mm) were taken before and at least once at 2–4 weeks after starting MDT in 20 cases who were newly-diagnosed clinically and who were confirmed in the first biopsy by histopathology. Two biopsies, 2–4 weeks apart, were also taken from each of 10 newly-diagnosed patients who did not yet receive any treatment for comparison (control group). In addition, two or more biop-

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TABLE 1. *Classification of the studied patients based on Ridley and Jopling scale according to bacterial load.*

Patients	Paucibacillary (PBL)			Multibacillary (MBL)		Total
	TT[a]	BT[b]	BB[c]	BL[d]	LL[e]	
Newly diagnosed	5	7	3	7	8	30
Reversal reaction	0	2	1	1	1	5
Total	5	9	4	8	9	35

[a] Tuberculoid.
[b] Borderline tuberculoid.
[c] Borderline.
[d] Borderline lepromatous.
[e] Lepromatous.

sies were taken before and during corticosteroid therapy of reversal reaction in the five patients who were clinically diagnosed as being in reversal reaction. Slit-skin smear was done for all patients to determine the bacteriological index in order to classify the patients into paucibacillary leprosy (PBL) and multibacillary leprosy (MBL). In all cases, biopsies were taken from the edge of the same skin lesion to ensure reproducibility of histology ([5]). Biopsies were fixed in 10% formol saline then routinely processed. Paraffin-embedded sections were cut at 5 μm, stained with hematoxylin and eosin (to confirm the histologic diagnosis) and with immunofluorescent staining in order to detect the HLA-DR display.

Procedure of indirect immunofluorescence. Sections of biopsies from the same patient both before and after MDT were stained on parallel, on the same day. This was performed according to manufacturer's instructions as follows: The paraffin sections of skin biopsies were dewaxed (by xylol for 1–2 hrs) and rehydrated (by alcohol 100%–70%–50% and 30%), then dried overnight at 37°C. The sections were rinsed in Tris saline buffer, pH 7.8, and then put in a solution of Tris saline buffer containing 0.1% calcium chloride (w/v) and 0.05% trypsin for 40 minutes. After rinsing in Tris saline buffer, the sections were left in this buffer overnight at 4°C. The sections were then incubated at room temperature (RT) for 30 minutes with monoclonal antibodies specific for HLA-DR (BioGenex, Abu Dhabi, United Arab Emirates). After rinsing with PBS, the sections were incubated at RT for 30 minutes with antimouse IgG fluorescein conjugate (Ortho Diagnostics, Rochester, New York, U.S.A.). Lastly the sections were rinsed with PBS and examined with a fluorescent microscope in a darkened room using UVR light of 350–400 μm wavelength. An excitation filter was used to produce a wavelength capable of causing fluorescent activation and a barrier filter was also used to removed the interfering waves of light. An isotype control was included, i.e., sections stained only with antimouse IgG fluorescein conjugate.

Positively stained material (HLA-DR) had bright yellowish-green fluorescence, while the negatively stained one appeared dull green in color. The degree of HLA-DR expression was determined by the degree of brightness in the yellowish-green fluorescence, which was scored as faint (+), moderate (++), and strong (+++) expression.

RESULTS

This study included 30 cases of leprosy who were newly diagnosed and 5 cases who were clinically diagnosed as being in reversal reaction during MDT. Table 1 shows the classification of the patients based on the Ridley and Jopling scale according to their bacterial load which was detected after making slit-skin smears.

The newly diagnosed patients included 12 with PBL and 18 with MBL, while the 5 patients with reversal reaction included 2 with PBL and 3 with MBL. Indirect immunofluorescence showed an increased expression of HLA-DR in the second skin biopsy of 7 out of 8 PBL patients (87.5%) and 10 out of 12 MBL patients (83.3%) within 2–4 weeks after starting MDT, for a total number of 17 out of 20 (85%) (Table 2). On the other hand, none of the 10

TABLE 2. *The changes in HLA-DR expression in the second bipsies from 20 newly diagnosed cases 2–4 weeks after starting multidrug therapy (MDT).*

HLA-DR expression after MDT	PBL	BL	Total
Increased	7 (87.5%)	10 (83.3%)	17 (85%)
Decreased	0 (0%)	0 (0%)	0 (0%)
No change	1 (12.5%)	2 (16.7%)	3 (15%)
Total	8 (100%)	12 (100%)	20 (100%)
	Z = 3.21 p = <0.01	Z = 3.10 p = <0.01	Z = 4.38 p = <0.001

newly-diagnosed patients, who did not receive any treatment for 2–4 weeks, had increased expression in the second biopsies compared to the first ones (no change in the expression). A significant difference was found between the number of new cases who had increased expression of HLA-DR in the second biopsies, in those who had received MDT, and those who did not receive MDT as shown in Table 3. However, no significant difference was found between the percentage of cases which had increased HLA-DR expression after MDT in either PBL or as compared to MBL cases ($p > 0.05$) (Table 4).

The Figure shows the HLA-DR expression in the granuloma of a newly-diagnosed patient before (A) and 3 weeks after starting MDT (B). Weak expression of HLA-DR was noticed in The Fig. (A) as indicated by the weak brightness of the immunofluorescent-stained material while visual increase in the degree of brightness was noticed in The Fig. (B), indicating increased expression of HLA-DR after MDT (in the second biopsy).

The five patients who developed reversal reaction during MDT had strong HLA-DR expression at presentation (first biopsy) which decreased in the subsequent biopsies (2–6 weeks after starting prednisolone therapy), as indicated by the weaker, brightness of the fluorescence in the second biopsy specimens when compared to the first ones.

DISCUSSION

Leprosy is a chronic infectious disease characterized by a broad spectrum of clinical forms depending on the patient's immune response ([11]). Recently, cytokines are thought to play an immunoregulatory role in both the immunopathogenesis and the protection of the host. Recombinant cytokines for immunotherapy have been used for controlling mycobacterial infections, including leprosy. This has stimulated an increasing interest in the immunomodulatory role of MDT as its beneficial effect may be accompanied by important changes in the immune cell profile which have a great role in overcoming such infection ([8]).

To examine the influence of MDT on the immune status of leprosy skin lesions, we studied the expression of class HLA (HLA-DR) on these lesions in 20 newly-diagnosed patients before and 2–4 weeks after starting MDT, by using immunofluorescent staining. There was increased expression after MDT in 7 out of 8 patients with PBL (87.5%) and in 10 out of 12 patients with MBL (83.3%), with a total number of 17 out of 20 patients (85%). These are considered to be significant findings in compari-

TABLE 3. *Comparison between the number of new cases who had increased expression of HLA-DR in the second biopsies with and without multidrug therapy (MDT)*[a].

Newly diagnosed patients	Total number	Patients with increased expression of HLA-DR in the second bipsies
Patients who received MDT for 2–4 weeks	20	17 (85%)
Patients without treatment for 2–4 weeks	10	0 (0%)

[a] $p < 0.001$.

TABLE 4. *Comparison between the number of cases which had increased HLA-DR display after multidrug therapy (MDT) in both paucibacillary (PBL) and multibacillary (MBL)*[a].

	Total number	Cases with increased expression after MDT
PBL	8	7 (87.5%)
MBL	12	10 (83.3%)

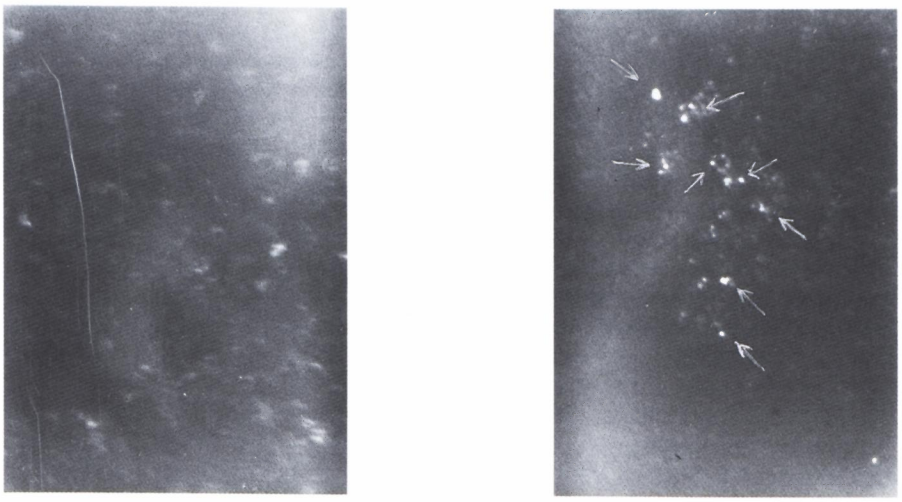
[a] $p > 0.05$.

son to the results obtained from the 10 new cases who did not receive any treatment for 2–4 weeks (during the period between the first and second biopsies), in whom no changes were noticed in HLA-DR expression. This is strong evidence of an increase in macrophage/epithelioid cell activation with more enhanced CMI response in such lesions. This process may involved CD4-T lymphocytes of Th1 type, NK cells, alpha delta cells or a combination of them all ([19]).

In some studies, increased HLA-DR expression was noticed following the injection of IFN-γ into lesions ([9]), or exposure of lepromatous macrophages in culture to IFN-γ ([6]). Therefore, our results might reflect the increased local production of IFN-γ by lymphocytes within the granuloma after starting MDT. This is possibly due to the stimulation of lymphocytes by the large quantities of mycobacterial antigens released from killed bacilli ([10]). Similarly, Moubasher, *et al.* ([11]) mentioned that the change in the antigenic stimulation of the immune system might have an effect on cytokine production and HLA-DR expression.

In the present study, the five cases of leprosy that developed reversal reaction during MDT had strong HLA-DR expression in the first biopsy specimens, which declined, subsequently, after prednisolone therapy. This indicates an exaggerated CMI response in such patients which is widely believed to be the cause of this reaction ([4]). The excessive stimulation of lymphocytes by antigen released during treatment leads to more influx of lymphocytes with increased macrophage activation and giant cell formation ([14]). So it is evident that reversal reaction is cell-mediated, whereas erythema nodosum leprosum is essentially an immune complex diseases ([11]).

Our results go well with those of Cree, *et al.* ([3]) who studied HLA-DR display in the granuloma of leprosy before and during MDT using immunohistochemical technique. They stated that the increased ex-



THE FIGURE. HLA-DR expression in the granuloma of a newly diagnosed patient before and during multidrug therapy (MDT). **A**: Before MDT; **B**: 3 weeks after MDT. Increased expression of HLA-DR is more noticeable in **B** than in **A** as indicated by the visual increase in the brightness of the immunofluorescent stained material as shown by the arrows.

pression of HLA-DR within a short time after starting MDT may be marker for the tendency to develop reversal reaction ([2, 15]). They also suggested that activation of cell-mediated immunity in leprosy lesions occurs in most patients during MDT and is not restricted to those with clinical evidence of reversal reaction.

It has been noticed that treatment of leprosy leads to an increase in both cytokine expression within the lesion ([1, 17]) and serum soluble IL2 receptor concentrations ([18]), providing additional evidence for activation of CMI response during MDT. However, the difference between patients who develop reversal reaction during treatment and those who do not is likely to be quantitative rather than qualitative with more exaggerated CMI response in the former, although this requires further investigation ([13]). So, it was suggested that reversal reaction represents a clinically identifiable degree of an immunological process which occurs (but with different degrees) in all patients treated for leprosy ([3]). Therefore, the ability to follow immunological changes within the granuloma quantitatively should be considered as a tool for use in the trials of modified MDT regimes in the future ([13]).

SUMMARY

Leprosy is a dynamic disease in which cell mediated immunity (CMI) plays an important role in host defense and control of the clinical spectrum. This study was carried out to detect immune activation in the granuloma of leprosy during multiple drug therapy (MDT) by studying the expression of human leukocytic antigen-DR (HLA-DR) in the granuloma before and during therapy. Skin punch biopsies were taken before and at least once 2–4 weeks after starting MDT in 20 newly diagnosed patients. Two biopsies, 2–4 weeks apart, were also taken from 10 new patients who did not yet receive any treatment, for comparison. Furthermore, biopsies were taken before and during corticosteroid therapy in five patients who developed reversal reaction during MDT. The biopsy specimens were studied for the expression of HLA-DR using the immunofluorescent staining which was found to be visibly increased in 17 out of 20 new cases (85%) within 2–4 weeks after starting MDT, while no change in the expression was noticed in those who did not receive any treatment ($p < 0.001$). This might reflect the increased production of interferon gamma (IFNγ) specially from granuloma lymphocytes after being stimulated with the excessive release of mycobacterial antigen from killed bacilli during therapy. The five patients who developed reversal reaction during MDT had strong HLA-DR expression in the first biopsies which declined subsequently 2–6 weeks after starting prednisolone therapy. Our results suggest that CMI was activated in skin lesions of leprosy during MDT. Such activation was not only restricted to those who developed reversal reaction across the therapeutic course, which indicates that the difference between patients who developed such reaction and those who did not, was likely to be quantitative rather than qualitative, with a more exaggerated CMI response in the former. Furthermore, it seems that the beneficial effect of MDT is accompanied by important changes in the immune cell profile which have a great role in overcoming such infection.

RESUMEN

La lepra es una enfermedad dinámica en la que la inmunidad mediada por células (IMC) juega un papel importante en la defensa del huésped y en el control del espectro clinico de la enfermedad. El presente estudio se realizó con el fin de detectar la activación inmunológica en el granuloma de la lepra durante la poliquimioterapia (PQT) en función de la expresión de los antígenos leucocitarios HLA-DR en el granuloma, antes y durante la PQT. Se incluyeron 20 pacientes con lepra recién diagnosticada, de los cuales se tomaron biopsias de piel con un "sacabocado", antes y después de 2 a 4 semanas de iniciar el tratamiento. Para comparación, también se tomaron dos biopsias, a intervalos de 2 a 4 semanas, de 10 pacientes similares que todavía no habían recibido ningún tratamiento. Además se tomaron biopsias antes y durante el tratamiento con corticoesteroides de 5 pacientes que desarrollaron una reacción reversa por efecto de la PQT. Las biopsias se estudiaron para buscar la expresión de antígenos HLA-DR usando una tinción inmunofluorescente. La expresión de antígenos HLA-DR se encontró visiblemente aumentada en 17 de los 20 casos nuevos de lepra (85%) entre las primeras 2 a 4 semanas de tratamiento con PQT mientras que no se observó ningún cambio en aquellos pacientes que no recibieron tratamiento ($p < 0.001$). Esto podría reflejar una producción incrementada de interferón gamma (IFNγ) por los linfocitos del granuloma estimulados por la excesiva liberación de antigenos micobacterianos a partir de los bacilos muertos durante la terapia. Los 5 pacientes que desar-

rollaron reacción reversa durante la PQT mostraron una marcada expresión de antígenos HLA-DR en las primeras biopsias pero su expresión declinó 2 a 6 semanas después de iniciar el tratamiento con prednisolona. Nuestros resultados sugieren que la IMC se activó durante el tratamiento con PQT. Sin embargo, tal activación no estuvo solo restringida a los pacientes que desarrollaron la reacción reversa durante la PQT, lo que indica que la diferencia entre los pacientes que desarrollaron la reacción y aquellos que no la desarrollaron fue probablemente cuantitativa más que cualitativa, con una IMC más exagerada en los primeros. Además, et efecto benéfico de la PQT se acompaña de cambios importantes el et perfil de las células inmunitarias que participan en et control de la infección.

RÉSUMÉ

La lèpre est une maladie évolutive où l'immunité à médiation cellulaire (IMC) joue un rôle important dans la défense de l'hôte et dans le caractère spectral des manifestations cliniques. Cette étude fut mise en œuvre dans le but de détecter les signes d'une activation immunitaire au sein des granulomes de la lèpre pendant une polychimiothérapie (PCT). Cette activation fut étudiée en évaluant le niveau d'expression des antigènes leucocytaires humains de type DR (HLA-DR) présent dans les granulomes avant et après la prise en charge thérapeutique. Des biopsies de peau furent prélevées à partir de lésions de 20 patients récemment diagnostiqués avant et au moins 2 à 4 semaines après la mise en œuvre de la PCT. Pour comparaison, 10 nouveaux patients hanséniens furent aussi biopsiés à 2–4 semaines d'intervalle avant la mise en œuvre de tout traitement. De plus, des biopsies furent obtenues à partir de 5 patients qui développèrent des réactions reverses pendant la PCT, avant et après la mise en œuvre de la corticothérapie. L'expression de HLA-DR au sein des biopsie fut étudiée par immunofluorescence. Elle était visiblement augmentée chez 17 parmi 20 nouveaux cas (85%) dans les 2 à 4 semaines suivant le début de la PCT, tandis qu'aucun changement de niveaux d'expression de HLA-DR ne furent détectés chez les patients sans traitement ($p < 0.001$). Cette augmentation pourrait être la conséquence d'une production augmentée d'interféron gamma (IFNγ), en particulier par les lymphocytes présents dans le granulome, après avoir été stimulés par un libération accrue d'antigènes mycobactériens provenant de bacilles tués par le traitement. Les 5 patients qui ont développés une réaction reverse durant la PCT ont montré une forte expression HLA-DR au sein des premières biopsies. Cette expression fut ensuite plus faible 2 à 6 semaines après le début du traitement par la prednisolone. Nos résultats semblent donc indiquer que l'IMC est activée au sein des lésions cutanées des patients hanséniens pendant la PCT. Une telle activation n'était pas seulement restreinte aux patients montrant une réaction reverse pendant la mise en œuvre du traitement, ce qui indique que la différence entre les individus qui développent et ceux qui ne développent pas une telle réaction est plus probablement quantitative que qualitative, avec notamment une IMC exagérée chez les premiers. De plus, il semble que les effets bénéfiques de la PCT soient accompagnés par des modifications importantes du profil des cellules immunitaires, qui ont un rôle éminent pour juguler une infection de ce type.

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