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Natural Killer (NK) Cell Activity and Reversal Reaction in Leprosy[1]

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The borderline group of the leprosy spectrum ([18]) contains most of the patients with the disease. Their clinical and immunological status is a highly unstable one. In one prospective study of BT patients, 40% came down with frank reversal reaction (RR) or were highly unstable with variable degrees of inflammation in their lesions ([2]). The reactions were found by Naafs and Wheate ([17]) to occur within the first 6 months of the beginning of dapsone antileprosy chemotherapy. The principal reason for the intense interest in studying reversal reaction is that it is these patients with neuritis who suffer the long-lasting sequelae and deforming stigma that make leprosy a serious disease.

Previous studies have suggested that reversal reactions are accompanied by heightened cell-mediated immunity (CMI) as measured by increased ^{3}H-thymidine incorporation when stimulated with *Mycobacterium leprae* antigens *in vitro* ([3, 5, 11]) and as observed by lymphocyte infiltration in reactive lesions ([20]). A form of CMI postulated to be relevant to defense against viral infections and against tumor development is natural killer (NK) cell activity. NK activity might also be involved in releasing intracellular parasites ([9]) or mycobacteria from infected cells, although the experiments to demonstrate this possibility remain to be done ([1, 26]). Mycobacteria such as the bacille Calmette-Guérin (BCG) are known ([9, 13, 22]) to increase NK cell activity.

We therefore decided to study NK cell reactivity in leprosy patients with and without RR and to compare these data to those of controls.

MATERIALS AND METHODS

Subjects. In our first study, the subjects were newly diagnosed, untreated leprosy patients presenting at the All Africa Leprosy Rehabilitation and Training Centre (ALERT) in Addis Ababa, Ethiopia, during a 6-week period in 1982. They consisted of 9 BT, 8 BL, and 3 LL patients in addition to 6 presenting with acute RR (4 BT and 2 BL). In our second study, 60 patients were drawn from a group completing a 6-month course of multidrug (dapsone, rifampin, and clofazimine) therapy (MDT) and being released from treatment. All patients were lepromin tested during their last month of treatment. Criteria for entrance into the study were that the patient had received antileprosy chemotherapy for at least 3 years, a time span sufficient for RR to have occurred in susceptible individuals, and that the medical history was sufficiently clear to

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allow a definite assignment to the reactional or nonreactional group. This assignment was based entirely on past and present signs of leprosy neuritis, since the degree of skin inflammation is nearly impossible to evaluate in retrospect. RR occurring with skin involvement only is uncommon in Ethiopia [2]. A medical history was taken by one investigator (GB). In both parts of our study, healthy Ethiopian control subjects were staff members from Armauer Hansen Research Institute (AHRI) or the ALERT Hospital or students receiving training there. They represented the same ethnic background as the patients.

Lymphocyte separation. Blood (25 ml) was taken from each individual, defibrinated by gentle rotation with glass beads, diluted 1:2 in RPMI 1640 (Flow Laboratories; Irvine, Scotland, U.K.) and separated on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden; Nyegaard, Oslo, Norway) by the method of Böyum [6]. Briefly, cells were layered on 25 ml of Ficoll and spun at 400 × *g* × 35 min. The mononuclear cells (MNC) at the interface were removed and washed three times. Unfractionated MNC were set aside for lymphocyte stimulation and HLA studies. Seven million cells in 10% autologous serum were placed in plastic Petri dishes for 1 hr at 37°C. The plastic nonadherent fraction (≥95% lymphocytes when cytocentrifuge cell preparations were stained and examined) was gently collected and washed one time. The cells were counted and adjusted to a concentration of 4×10^6/ml in 10% fetal calf serum (FCS)/RPMI. In our first study, effector cells were adjusted to a concentration of 5×10^6/ml.

NK cell assay. K562 cells, an erythroleukemic myeloid cell line that is the conventional human NK target *in vitro,* were labeled with 200 μCi ^{51}Cr for 60 min at 37°C, washed twice in RPMI, resuspended in 10% FCS/RPMI for 45 min to reduce background isotope release, pelleted, and resuspended at a concentration of 8×10^4/ml. The plastic nonadherent cells were incubated with K562 target cells in triplicate, round-bottom, microtiter wells (Titertek; Flow Laboratories) at ratios of 50, 25, 12.5, and 6.25:1 in a final volume of 200 μl. One hundred μl of control target cells were incubated with 100 μl of 10% FCS/RPMI (spontaneous release) or 100 μl of 1% Triton-X 100 (maximum release). The plates were spun at 200 × *g* × 3–4 min and then incubated at 37°C overnight (14–16 hr). It was found that spontaneous release was greater than that in a 4-hr incubation but that the percent specific isotope release was not significantly different in the test cultures. Plates were spun again, and 100 μl of supernatant was harvested from each well and counted in a gamma counter (LKB Wallac: 1270 Rackgamma II; Turku, Finland). In our first study, effectors and targets were incubated in tubes in a final volume of 0.8 ml. Specific ^{51}Cr release was measured as follows:

$$\% \text{ specific } {}^{51}\text{Cr release} = \frac{\text{cpm test} - \text{cpm spon release}}{\text{cpm max release} - \text{cpm spon release}}.$$

Statistics. Specific release calculated at the different effector : target (E:T) ratios was plotted to obtain correlation and regression coefficients. The E:T ratio necessary for 50% target cell lysis (ED-50) was also determined for each patient from the calculation of the slope. Tests for significance between patient and control groups were measured by means of the Wilcoxon rank sum test and by Student's *t* test.

RESULTS

Figure 1 summarizes the results of the first set of experiments assessing NK activity in untreated patients across the leprosy spectrum. High NK activity correlates inversely with the ED-50 value, i.e., a low ED-50 indicates high NK activity. The mean NK activity as measured by the ED-50 was significantly less ($p < 0.05$) in untreated leprosy patients without RR compared to healthy controls. NK activity was also significantly lower in these patients when compared to those patients presenting with acute RR. There was a wide range of values obtained in the group of nine BT patients and also in the BL group. NK activity was also significantly lower ($p < 0.05$) in the three untreated LL patients compared to controls or untreated patients presenting with RR.

Given the results in the first series of NK assessments and that NK activity might be

considered as a stable immunological phenotype ([11, 19]), assays were performed on a large series of BT leprosy patients treated for their disease for at least 3 years and who had just completed 6 months of MDT.

The 60 patients selected for study upon release from MDT included 33 patients with a history of RR neuritis and 27 with no such history. There were 27 healthy Ethiopian control subjects evaluated for NK activity. Low cell numbers or technical errors prevented evaluation in 4 subjects, 3 with a history of RR and 1 control subject.

Comparisons of NK cell activity in patient groups and controls were again made by three measurements: a) E:T ratios, b) the ratio required for 50% target cell lysis (ED-50), and c) the regression coefficient or slope of activity determined from the different E:T ratios.

Significant differences between groups were found by two of the three parameters. Significant differences were found only at the 50:1 E:T ratio between patients and controls (The Table). There were no significant differences between groups for the number of effector cells required to obtain 50% target cell lysis. Patient ED-50 values in both groups were 32 ± 4 and controls were 25 ± 4.

However, highly significant differences were observed between patients and controls when the slopes of NK activity were compared (Fig. 2). Measurement of the slope or regression coefficient permits an assessment of NK activity per cell. The less steep the slope the less activity is decreasing with dilution of effector cells. The slopes of the group of BT patients with a history of RR were significantly less steep ($p < 0.001$) than healthy Ethiopian controls. Patients without a history of RR also had less steep ($p < 0.01$) slopes than did controls. When the two patient groups were combined, the slopes were also less steep than controls ($p < 0.001$). Mean differences between the two patient groups, however, were not statistically significant.

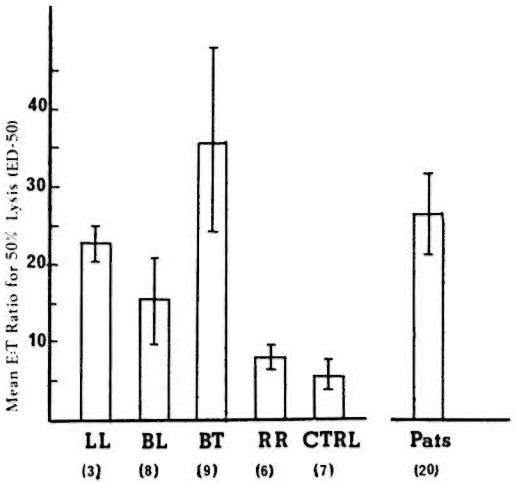

FIG. 1. NK cell activity in untreated leprosy patients. The ED-50 correlates inversely with the degree of NK cell activity. LL = lepromatous leprosy; BL = borderline lepromatous leprosy; BT = borderline tuberculoid leprosy; RR = reversal reaction patients; CTRL = healthy Ethiopian controls; Pats = nonreactional patients combined.

All patients (except for one of the RR group; her NK cell activity was near the median value) were lepromin tested during the last month of MDT. The WHO protocol ([22]) was followed for both the skin test antigen and the measurement of the Mitsuda response. To determine if exposure to *M.*

THE TABLE. *NK cell activity in treated BT leprosy patients and healthy controls.*[a]

Group	No.	E:T ratio[b]			
		50:1	25:1	12.5:1	6.25:1
NR[c]	27	62 ± 17 (5)[d]	50 ± 18 (5)	35 ± 18 (3)	23 ± 14 (3)
RR[e]	30	64 ± 17 (5)[f]	53 ± 20 (5)	38 ± 17 (3)	24 ± 15 (3)
Controls	26	74 ± 15 (5)	56 ± 19 (4)	39 ± 18 (4)	26 ± 15 (3)

[a] Data expressed as mean percent ^{51}Cr release ± S.D. (S.E.M.).
[b] E:T ratio = effector : target cell ratio.
[c] NR = patients without a history of reversal reaction.
[d] Significantly less than controls ($p < 0.001$).
[e] RR = patients with a history of reversal reaction.
[f] Significantly less than controls ($p < 0.025$).

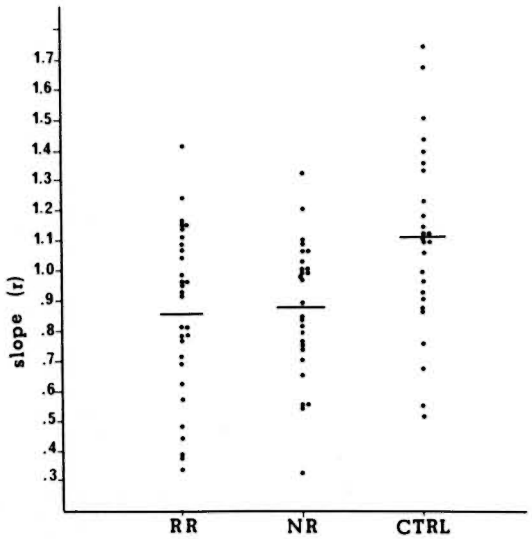

FIG. 2. NK cell activity in treated borderline tuberculoid leprosy patients as measured by regression coefficient (r). A low r value indicates NK activity is decreasing less rapidly with dilution of effector cells. RR = patients with a history of reversal reaction (30); NR = patients with no history of RR (27); CTRL = healthy Ethiopian controls (26).

leprae antigens in the perhaps novel form of the Dharmendra lepromin material was related to subsequent NK activity, responses to lepromin in millimeters were plotted against the various measurements of NK activity *in vitro*. Only one of the parameters of NK activity showed any correlation with lepromin responsiveness *in vivo*. For patients without a history of RR, there was a positive correlation between NK activity per cell and the lepromin reaction.

DISCUSSION

The present studies have assessed the level of NK activity in plastic nonadherent mononuclear cells of leprosy patients. The first part of the studies established that there was a lower NK activity (measured as ED-50) in nonreactional, untreated leprosy patients than in controls. Patients presenting with RR had NK activity at normal levels. Other parameters of CMI have also been found to be lower in untreated leprosy patients, particularly lepromatous patients (refs. in [10]). NK activity has been found to be lower in a study ([7]) of 12 LL patients compared to 11 tuberculoid patients and 11 normal controls. However, these findings were not supported in two other studies ([8, 14]). Treatment status did not appear to affect NK activity in one of these reports ([14]). Among the BT patients in the present study, heterogeneity in levels of NK activity was observed, as had been seen previously in the lymphocyte stimulation tests ([4]).

That there should be higher NK activity in association with RR is not very surprising. Other observers ([20]) have shown that reactions in leprosy are accompanied by reduction or destruction of bacilli. Under these circumstances, release of *M. leprae* antigens from immunologically "privileged" locations such as Schwann cells would be expected. Mycobacteria such as BCG are known to augment NK activity ([9, 13, 23]). *M. leprae* itself is known to have strong adjuvant activity ([18, 24, 25]). The augmentation is associated with interferon (IFN) release. IFN is known to enhance NK activity as well. Monocytes from ENL patients have been shown to suppress NK activity ([14]). Whether enhancement rather than suppression would occur in the presence of monocytes—that may be activated to release alpha-interferon—from RR patients is not known. Reversal reactions are effectively treated with corticosteroids which are able to ablate NK activity ([13]). Whether NK activity is reduced after such treatment in leprosy patients has not been tested. Increased NK activity could thus be a consequence of bacillary destruction and IFN release rather than a cause of RR.

However, according to the literature ([15, 22, 23]) NK activity, either high or low, may in fact have a genetic basis. Examples are low activity in the inbred beige mice ([13]) and in the hereditary Chediak-Higashi syndrome ([12]) in humans. Levels of NK activity have also been shown to be influenced by H-2 type ([20]).

From the results we report here, a predisposition to the development of RR cannot be demonstrated through NK cell activity, nor can it be associated with HLA type (T. Ottenhoff, personal communication). However, BT patients had lower regression coefficients of their NK activity, i.e., less reduction of activity with decreasing E:T ratios, than did normal controls. At the same time, there was no significant difference between NK activity in the two groups as measured by ED-50. This might

indicate that the patients' plastic nonadherent cells are more aggressive at lower (and probably more physiological, i.e., 50 effectors per target may be unlikely *in vivo*) E:T ratios due to *in vivo* activation.

However, differential enhancement of NK activity in response to lymphokines may exist in the two types of patients. Such a difference is suggested by the finding of a correlation of increased NK activity with size of induration in response to lepromin in the nonreactional patients. In addition, cells obtained directly from affected lesions may express activity that is quite different from fresh PBL. Consequently, we are in the process of analyzing the effect of passive addition of lymphokines to the above systems as well as of determining NK cell activity in cells obtained from the local lesions.

SUMMARY

Two studies were conducted to assess natural killer (NK) cell activity in leprosy patients and healthy Ethiopian controls. The first study tested 26 untreated leprosy patients across the spectrum of the disease. It was found that lepromatous leprosy and all untreated, nonreactional patients had lower NK activity than healthy controls. However, patients presenting with reversal reaction (RR) had NK activity within the normal range. Heterogeneity was particularly marked in the NK activity of borderline patients. In the second study, NK cell activity was assessed in treated borderline tuberculoid leprosy (BT) patients. There were 30 patients with a history of RR and 27 BT patients without such a history (NR). All patients had had at least 3 years of dapsone treatment and 6 months of multidrug therapy. There were 26 control subjects. NK activity was higher in controls than in patients only at one effector:target (E:T) ratio tested, but NK cells from the BT patient group appeared to be more "aggressive" in that there was significantly ($p < 0.001$) less reduction of activity with dilution of effector cells. There were no significant differences in NK activity between RR and NR patients. The NK activity of NR patients was positively correlated with the size of induration of the lepromin response. We conclude that higher NK activity in acute RR would appear to be a consequence rather than a cause of reversal reactions.

RESUMEN

Se hicieron 2 estudios para cuantificar la actividad de las células asesinas naturales (NK) en pacientes con lepra y en sus convivientes etiopes sanos. En el primer estudio se incluyeron 26 pacientes con lepra no tratada abarcando todo el espectro de la enfermedad. Se encontró que los pacientes lepromatosos y todos los pacientes no tratados y no reaccionales tuvieron menor actividad NK que los controles sanos. Sin embargo, los pacientes en reacción reversa (RR) tuvieron actividad de NK dentro de limites normales. La heterogeneidad en la actividad de NK fue particularmente marcada en los pacientes con lepra intermedia. En el segundo estudio, la actividad de NK sólo se midió en pacientes con lepra intermedia-tuberculoide (BT) bajo tratamiento. Hubieron 30 pacientes con historia de RR y 27 pacientes sin historia reaccional (NR). Todos los pacientes habían tenido cuando menos 3 años de tratamiento con dapsona y 6 meses con terapia múltiple. Se incluyeron 26 sujetos control. La actividad de NK fue más alta en los controles que en los pacientes sólo a una relación de efectoras:blanco de 1:1, pero las NK de los pacientes BT parecieron ser más agresivas en el sentido que cuando se diluyeron las células efectoras hubo menos reducción de la actividad de NK ($p < 0.001$). No hubieron diferencias significativas en la actividad de NK entre los pacientes RR y NR. La actividad de NK en los pacientes NR correlacionó positivamente con el tamaño de la induración de la leprominoreacción. Se concluye que la mayor actividad de NK en la RR aguda parece ser la consecuencia más que la causa de las reacciones reversas.

RÉSUMÉ

En Ethiopie, chez des malades de la lèpre et chez des sujets sains, on a mené deux études pour évaluer l'activité cellulaire NK ("natural killer cell activity"). La première étude portait sur 26 malades de la lèpre non traités, appartenant aux différents types de la maladie. On a constaté que les malades lépromateux, de même que tous les malades non réactionnels non-traités, présentaient une activité NK plus faible que les témoins sains. Toutefois, les malades qui présentaient une réaction reverse ("reversal reaction") témoignaient d'une activité NK dans les limites normales. L'hétérogénéité était particulièrement marquée en ce qui concerne l'activité NK des malades présentant la forme dimorphe ("borderline"). Dans la deuxième étude, on a évalué l'activité cellulaire NK chez des malades atteints de lèpre tuberculoïde dimorphe (BT) et traités. Ce groupe comprenait 30 malades avec antécédents de réaction réverse, et 27 patients BT chez lesquels de tels antécédents étaient absents. Tous les malades avaient été traités par la dapsone pendant 3 ans au moins, et pendant 6 mois par la polychimiothérapie. On a comparé

les résultats obtenus avec ceux observés chez 26 sujets témoins. Pour un seul ratio effecteur/cible ("effect/target") on a relevé une activité NK plus élevée chez les témoins que chez les malades. Néanmoins, les cellules NK provenant de malades BT se sont révélées plus "agressives" car la réduction de l'activité à la suite de la dilution des lymphocytes effecteurs était significativement moins prononcée ($p < 0.001$). On n'a pas observé de différences significatives dans l'activité NK entre les malades avec anamnèse de réaction réverse et ceux qui ne présentaient pas de tels antécédents. L'activité NK des malades sans antécédent de réaction réverse présentait une corrélation positive avec la dimension de l'induration lors de la réponse à la lépromine. On en conclut que l'augmentation de l'activité NK dans la réaction réverse aiguë paraît être la conséquence plutôt que la cause des réactions réverses.

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