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Antibodies to Mycobacterial Arabinomannan in Leprosy: Correlation with Reactional States and Variation During Treatment[1]

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Sera from patients with leprosy contain specific antibodies directed at several distinct mycobacterial antigens ([1, 2, 7, 8, 9, 15, 17]). These antibodies are often present in high concentration although they have no documented role in host immunity to *Mycobacterium leprae.* The intensity of this humoral response varies over the clinical spectrum of leprosy, in general being greatest at the lepromatous pole and weakest in polar tuberculoid disease ([2, 8, 15, 21]). However, the correlation between antibody level and bacillary load, and the pattern of change in antibody level during therapy in individual patients, are poorly characterized. Bjorvatn, *et al.* ([5]) tested serial specimens from 16 lepromatous patients by crossed immunoelectrophoresis using sonicated *M. leprae* as antigen. Over the study period of 4–12 months they found virtually no change in the number or intensity of precipitin bands formed. The occurrence of erythema nodosum leprosum (ENL) reactions in four of the 16 patients had no discernable effect on the band patterns. In another study which used a radioimmunoassay for the detection of antibody to antigen 7 of *M. leprae,* Melsom, *et al.* ([12]) found only a slight tendency for antibody activity to decline over the first year of dapsone treatment in a group of 15 patients with lepromatous leprosy. Finally, in a recent study, Melsom, *et al.* ([11]) using a solid phase radioimmunoassay demonstrated gradual declines in IgG and IgA antibody activity against antigen 7 and a smaller but significant decline in specific IgM activity during the initial 2–4 years of therapy in lepromatous patients. Four of the seven patients with ENL reactions were noted to have had transient increases in antibody activity, but these increases were not quantified in the paper.

We have developed an enzyme-linked immunosorbent assay (ELISA) utilizing a purified, chemically characterized mycobacterial antigen, arabinomannan (AM), purified from *M. smegmatis.* This complex cell wall associated polysaccharide is common to all mycobacterial species and is a

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strong immunogen ([4, 14]). The antibody levels to this characterized antigen were quantitated in pretreatment serum specimens obtained from nine patients with leprosy and compared with an estimate of bacillary load based on the results of six-site scrapings. Subsequent changes in antibody level over the initial 12–31 months of therapy were then measured in serial serum specimens collected from these patients.

MATERIALS AND METHODS

Enzyme-linked immunosorbent assay. Arabinomannan (AM) was extracted from *M. smegmatis,* purified, and characterized biochemically and immunochemically as previously described ([13]). An ELISA was performed using a modification of the technique reported by Buchanan ([6]). The AM was suspended in 0.067 M phosphate buffer, pH 5.0, at a concentration of 20 μg of carbohydrate per ml. One hundred μl of this solution was placed in each well of a 96-well, flat-bottom polystyrene microtiter plate (Dynatech, Alexandria, Virginia, U.S.A.) and incubated at 37°C for 3 hr, then stored at 4°C. On the day of the test the AM was removed by aspiration, 100 μl of phosphate buffered saline with 5% bovine serum albumin (BSA) was added to each well, and the plate was incubated at 37°C for 90 min. Following a wash with phosphate buffered saline with 1% BSA (PBS-BSA), the study sera were added to the wells. Each serum specimen was diluted 1:100 in PBS-BSA and assayed in triplicate, except for Patients 8 and 9 whose serum was diluted 1:200. Each plate also had internal controls consisting of: a) serial dilutions of pooled human leprosy sera, b) standard dilutions of pooled normal human sera, and c) PBS-BSA alone. The diluted sera were incubated on the plates for 60 min at room temperature. Following further washing, peroxidase-conjugated IgG fraction goat anti-human IgG (Fc fragment, gamma chain specific) (Cappel Labs, Cochranville, Pennsylvania, U.S.A.) was added at a dilution of 1:500 in PBS-BSA. Plates were incubated for 60 min at room temperature. After washing, H_2O_2/o-phenylenediamine was added as a substrate/chromogen, and the plates were incubated in the dark at 37°C for approximately 30 min. The reaction was stopped with 8 N H_2SO_4 and the OD_{492} was read using a Titertek Multiskan (Flow Labs, Helsinki, Finland). The arithmetic mean of the three values for each serum was calculated and used in all subsequent analyses. Through the linear range of optical densities measured by the ELISA (approximately 0.4–1.7), a decline of 25% of the OD_{492} roughly corresponded to a two-fold dilution of the serum.

Patients. All of the leprosy patients followed at the Seattle Public Health Hospital who met the following criteria were included in this study: a) banked sera were available from the date of initiation of therapy (except in Patient 9 whose first serum was drawn two weeks after the start of therapy); b) stored sera were available over at least the first full year of therapy (range 55–136 weeks, mean 78 weeks); and c) there were at least four sera available for testing. All patients were followed by one of the investigators (JH) over the entire period of the study. There were seven males and two females in the study population; age range was 20–52 years. All clinical diagnoses were confirmed with skin biopsies from affected areas for histologic classification. Scrapings from six separate skin sites (earlobes and the extensor surfaces of the knees and elbows) were performed at the time of diagnosis using the Carville technique as reported by Kirchheimer ([10]). Based on the standard Ridley-Jopling criteria ([18]), there were 3 borderline lepromatous (BL), 3 borderline tuberculoid (BT), and 3 borderline (BB) cases among the 9 patients. All reactional episodes (except Patient 8) were confirmed by biopsy—Patients 4, 5, and 6 had reversal reactions and Patients 8 and 9 had erythema nodosum leprosum (ENL). The frequency of clinic visits and sera collections was based on each patient's clinical course. No patient had a history of active or inactive tuberculosis or atypical mycobacterial illness.

Serum specimens. Serum was obtained at most clinic visits. Samples were preserved with the addition of 0.1% sodium azide or sterilized by passage through a 200 nm filter (Millipore, Bedford, Massachusetts, U.S.A.), then aliquoted and stored at −70°C. All sera were tested on the same day.

Therapeutic regimens. Dapsone and rifampin therapy was initiated in all patients

at the time of diagnosis (except Patient 9, who received dapsone alone for three months prior to the addition of rifampin to the regimen). In most instances, the dosage was 100 mg per day of dapsone and 600 mg per day of rifampin. One patient (number 5) had rifampin discontinued after six months because of persistently elevated liver function tests. All reactional episodes were treated with corticosteroids, usually prednisone 40 mg per day tapered as rapidly as the clinical course allowed. Patient 9 eventually required thalidomide for control of ENL; this was begun during week 99 of therapy.

RESULTS

Correlation of ELISA results and six-site scrapings. Data from the six-site scrapings and simultaneous antibody levels to AM are presented in The Table. There is a trend towards higher levels of antibody in BL cases than in BT, but as noted by other investigators ([15, 21]) there are both low and high responders within each clinical and histologic class. The technique of examining scrapings from six clinically uninvolved skin sites for quantitation of bacillary load may provide a more accurate assessment of antigenic load than simple classification based on clinical criteria and the histopathology of leprous lesions ([10]). Correlation of antibody levels to AM measured by ELISA with the results of the six-site scrapings (either the bacterial index of involved sites or the bacterial index totaled for all six sites) is better than the comparable correlation with clinical class of disease. The correlation coefficient of the absolute OD_{492} to the totaled bacterial index is 0.75 ($p = 0.03$) by the Spearman test.

Variation in antibody levels with time in patients with uncomplicated leprosy. Four of the patients studied (1, 2, 3, and 7) were free of reversal reactions or ENL during the initial 13–28 months of therapy. Their antibody levels over time are shown in Figure 1. Patient 3 discontinued his dapsone and rifampin therapy between weeks 18 and 26 with resultant clinical relapse after an initially excellent clinical response. This may account for his atypical rise in antibody level in the week 26 and subsequent sera. The remaining three patients all had declining or stable amounts of specific antibody after the initial two months of therapy.

Influence of ENL on antibody levels. Two of the patients (8 and 9) experienced ENL reactions during their initial 1–2 years of therapy. Both of these patients had extremely high levels of antibody to AM throughout the study period, requiring additional dilution of their sera prior to assay. Serial measurement of antibody levels showed gradual declines with no consistent effect of the ENL reactions on the titers (Fig. 1). Patient 9 did have a small increase in antibody level at the onset of ENL but, relative to high baseline levels, it amounted to only an 8% increase and is of no signifi-

THE TABLE. *Pretreatment arabinomannan ELISA and six-site skin smear results from patients with leprosy.*

Patient no. (age/sex)	Type of leprosy	Reactional state	Pretreatment OD	Six-site smears	
				No. sites positive	Total BI[a]
1 (41/M)	Borderline tuberculoid	None	0.116	0	0
2 (45/M)	Borderline tuberculoid	None	0.182	1	2
3 (36/M)	Borderline	None	0.304	0	0
4 (43/M)	Borderline lepromatous	RR[b]	0.718	1	1
5 (20/F)	Borderline tuberculoid	RR	0.857	1	5
6 (26/M)	Borderline	RR	1.066	0	0
7 (52/M)	Borderline	None	1.549	3	8
8 (42/F)	Borderline lepromatous	ENL[c]	1.704[d]	5	22
9 (33/M)	Borderline lepromatous	ENL	1.807[d]	6	20

[a] Total bacterial index (BI) of six sites; each scored 0–6+. Maximum total = 36.
[b] RR = reversal reaction.
[c] ENL = erythema nodosum leprosum.
[d] Serum diluted 1:200 because of high titer.

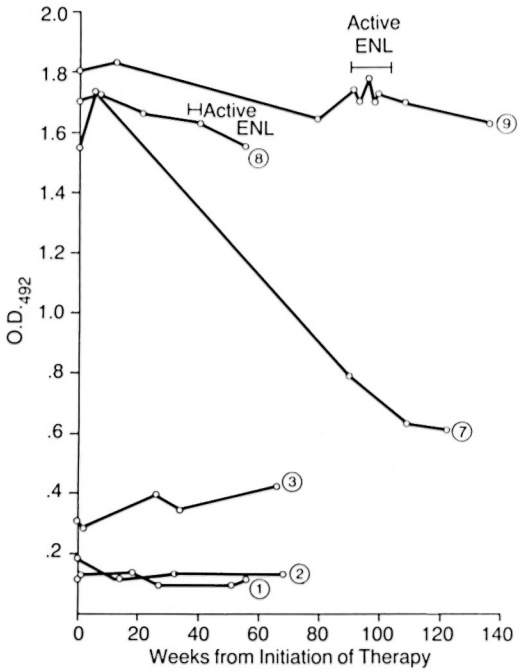

FIG. 1. Serial arabinomannan antibody levels in patients with uncomplicated treatment courses (Patients 1, 2, 3 and 7) or with ENL reactions (Patients 8 and 9). Periods of active ENL are delineated by brackets.

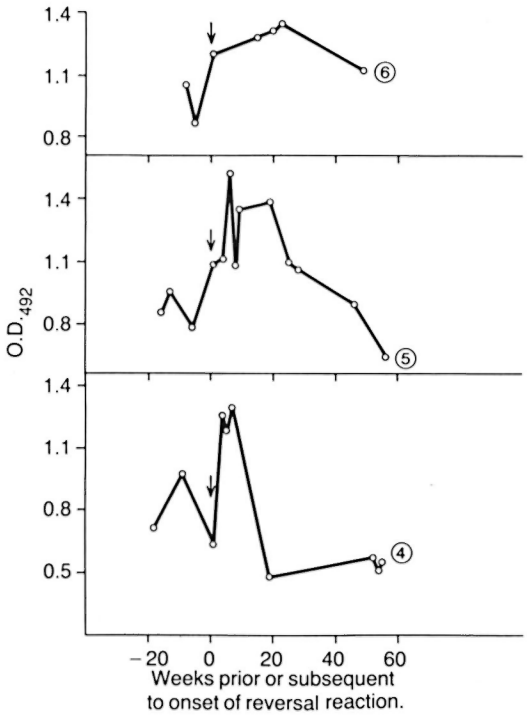

FIG. 2. Serial arabinomannan antibody levels in the three patients with reversal reactions. Arrows mark the onset of the reactional state. Note: The OD scale (ordinate axis) is discontinuous, but covers the same range of values for each patient.

cance. There was no evidence to suggest that antibody variation in patients undergoing ENL reactions differed significantly from that of patients with uncomplicated clinical courses.

Variations in antibody levels during reversal reactions. Figure 2 presents the pattern of variation in antibody levels during reversal reactions as measured in the AM ELISA. The graphs are standardized for the three patients (4, 5 and 6) by defining the date of clinical onset of the reversal reaction as "day 0". Corticosteroids were begun on all patients within one week of this date and, in most cases, there was rapid and steady improvement in symptoms. During the 4–6 weeks preceding the onset of the reversal reactions, the antibody levels in all had declined to a value below the pretreatment level. Coincident with or shortly after the development of the clinical symptoms of reversal reaction, all patients had abrupt increases in the quantity of specific antibody to AM which persisted for several weeks, eventually peaking between six and 23 weeks after the reactional state began. In all cases the reversal reaction was clinically resolving at the time of peak antibody levels. Thereafter, the antibody levels fell steadily in all three patients, and reached levels below the pretreatment value in two of them.

DISCUSSION

The correlation of the antibody level with the bacterial index of the six-site smears implies that the level of antibody may serve as a rough measure of the amount of AM accessible to the host immune system. This relationship appears to hold true across the clinical spectrum of leprosy in the population studied. The documented increase in seropositivity as one moves along the spectrum towards polar lepromatous disease has led to speculation that antibody levels reflect antigen load, but none of the previous studies attempted to relate antibody titer to an objective measure of bacillary load.

The finding that antibody titers to AM from *M. smegmatis* remain constant or decline at a slow rate is in agreement with the two studies in the literature that tested serial sera on the same patients ([5, 11, 12]) and the three studies that reported only mean antibody levels in patient groups during treatment ([3, 16, 21]). This persistence of antibody despite adequate therapy and excellent clinical response is likely due to the continued presence in the host of mycobacterial antigens. Mouse foot pad inoculation studies ([10]) have confirmed the validity of the bacterial index (BI) and morphological index (MI), and it is now firmly established that nonviable mycobacterial "skeletons" can persist well into therapy and present a chronic antigenic stimulus to the host.

Using the AM assay, the pattern of change in antibody titer during ENL reactions was not distinguishable from that seen in uncomplicated cases. The other studies which examined serum specimens during ENL reactions also found no consistent variation in antibody levels when compared with those of uncomplicated cases ([3, 5, 12]). Circulating and fixed immune complexes are involved in the pathogenesis of ENL ([20]) and it is noteworthy that only those patients with the highest antibody levels, and presumably greater bacterial loads, experienced this complication.

The pattern observed in the three patients with reversal reaction was quite distinct from that seen in the other six patients. Each manifested a triphasic pattern with an initial fall in antibody preceding the clinical development of the reversal reaction, followed by a sharp rise coincident with the appearance of symptoms, and finally a second decline, occurring several weeks to months after the onset of the reaction. These variations in antibody level did not correlate with changes in steroid dosage, and the peak levels occurred at a time when the clinical symptoms of the reactional state were well controlled. This triphasic pattern may be correlated with the rapid improvement in effective cell-mediated immunity against *M. leprae* ([19]), with resultant release of antigens, which is the hallmark of the reversal reaction.

Little data are available from prior studies on the variation of humoral response to other antigens during reversal reactions. Melsom, *et al.* ([11, 12]) reported that one of the patients involved in their study developed increasing antibody titers to *M. leprae* antigen 7 during a reversal reaction, but the patient was subsequently shown to have active tuberculosis. Abe, *et al.*, using a fluorescent leprosy antibody absorption test, studied two patients with reversal reactions ([3]), one of whom manifested a sharp increase in IgG antibody titer at the onset of the reversal reaction, followed by a decline in titer three and six months later. This was distinct from the pattern they observed in ENL or in uncomplicated cases. Further cases will have to be studied to confirm these preliminary findings, but the marked contrast between the behavior of the antibody response to AM in our three patients with reversal reactions when compared to that of the six patients with other clinical courses appears to be significant.

Further studies with more patients will be of interest to determine whether the level of antibody to AM in a pretreatment serum allows prediction of the relative risk of developing a reactional state. In this study, two of three patients with pretreatment ELISA values greater than 1.5 developed ENL, and all three of the patients with values of 0.7–1.1 developed reversal reactions (The Table, Figs. 1 and 2). In contrast, the three patients with pretreatment serum ELISA values less than 0.35 experienced no reactional states during more than one year of follow up. Since the antibody level to AM correlates directly with the estimated bacillary load, this implies that patients with the fewest organisms are the least likely to develop reactional states. Patients with moderate numbers of organisms may be the most likely to develop reversal reactions, and those with the greatest numbers may be the most susceptible to developing ENL. If ongoing research confirms the utility of this assay in defining subgroups at high risk for the development of reactional states, it may facilitate the design of studies aimed at decreasing the incidence of these unpleasant and dangerous complications.

SUMMARY

An enzyme-linked immunosorbent assay was used to measure antibody to mycobac-

terial arabinomannan in serial serum specimens obtained over the initial 12–31 months of therapy from nine patients with leprosy. The antibody level in pretreatment sera was directly proportional to the quantity of *Mycobacterium leprae* present in each patient as assessed by six-site scrapings (r = 0.75). The three patients with the lowest antibody levels (OD 0.1–0.3) had uncomplicated courses and their levels declined slowly with treatment. Three patients with intermediate antibody levels (OD 0.7–1.1) each experienced a reversal reaction during therapy; serial antibody titers in all three followed a triphasic pattern over the course of the reaction. The two patients who developed erythema nodosum leprosum during therapy had extremely high levels of antibody initially (OD > 1.5), which fell slowly with time and which were unaffected by the reactional state. The pretreatment antibody level to arabinomannan reflects the amount of *M. leprae* present and may have predictive value for the development of reactional states.

RESUMEN

Se usó un inmunoensayo enzimático para medir la concentración de anticuerpo contra una arabionomanana micobacteriana en especímenes seriados de suero obtenidos durante los 12 a 31 meses iniciales de terapia en 9 pacientes con lepra. El nivel de anticuerpo en los sueros de pretratamiento fue directamente proporcional a la cantidad de *Mycobacterium leprae* presente en cada paciente según se determinó por el análisis bacteriológico en 6 sitios de abrasión (r = 0.75). Los 3 pacientes con los niveles más bajos de anticuerpos (DO 0.1–0.3) evolucionaron sin mostrar complicaciones y sus niveles decayeron lentamente con el tratamiento. Tres pacientes que tuvieron niveles intermedios de anticuerpos (DO 0.7–1.1) presentaron algún tipo de reacción reversa durante la terapia; en todos los casos, los títulos seriados de anticuerpos siguieron un patrón trifásico durante el curso de la reacción. Los dos pacientes que desarrollaron eritema nodoso leproso durante la terapia tuvieron niveles de anticuerpo extremadamente elevados al inicio (DO > 1.5), mismos que decayeron lentamente con el tiempo sin ser afectados por el estado reaccional. El nivel de anticuerpos contra arabinomanana antes de iniciarse el tratamiento refleja la cantidad de *M. leprae* presente y puede tener un valor predictivo del desarrollo de estados reaccionales.

RÉSUMÉ

Une évaluation par une méthode d'immunosorbant, associée à une enzyme, a été utilisée pour mesurer un anticorps arabinomannan mycobactérien dans des séries d'échantillons de serum obtenus au cours de la période initiale de la thérapeutique chez neuf malades atteints de lèpre, période qui s'étendait sur 12 à 13 mois. Le taux d'antibiotiques dans le sérum prélevé avant traitement était directement proportionnel à la quantité de *Mycobacterium leprae* qui était présente chez chaque malade, ainsi qu'on pouvait l'estimer par des prélèvements cutanés effectués en six endroits (r = 0,75). Les trois malades présentant les taux d'anticorps les plus faibles (OD 0,1–0,3) présentaient une évolution non compliquée et leur taux montrait une diminution lente avec le traitement. Trois malades présentant des taux d'anticorps intermédiaires (OD 0,7–1,1) avaient chacun souffert d'une "réaction reverse" au cours du traitement. Chez chacun de ces trois malades les titres d'anticorps en séries avaient présenté un profil en trois phases au cours de la réaction. Les deux malades qui ont développé un érythème noueux lépreux au cours de la thérapeutique présentaient des taux extrêmement élevés d'anticorps au début (OD < 1,5), taux qui sont retombés lentement avec le temps, et qui n'ont pas été affectés par l'état réactionnel. Le taux d'anticorps arabinomannan avant traitement reflètait la quantité de *M. leprae* qui était présente, et pourrait avoir une valeur de prédiction quant au développement d'états réactionnels.

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