INTERNATIONAL JOURNAL OF LEPROSY

Volume 55, Number 4
Printed in the U.S.A.

Influence of Environmental Mycobacteria on the Prevalence of Leprosy Clinical Type[1]

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In susceptible individuals, the type of disease which develops within the clinical spectrum of leprosy appears to be determined by the degree of cell-mediated immune response following infection. There is good correlation between diminution of this ([21]) toward the lepromatous pole of the disease ([29]) which is also evident from the increasing numbers of *Mycobacterium leprae* found in tissue. This apparent tolerance to the presence of the organisms can be reversed by stimulation with crossreactive antigenic exposure to other mycobacteria ([6]).

Humoral response also results from infection with *M. leprae* ([22]), and increased levels of serum immunoglobulins, mainly IgG and IgM, are detectable in patients whose disease develops toward the lepromatous pole ([20]). The species-specific *M. leprae* antigen phenolic glycolipid-I ([3]) also invokes an IgG and IgM response detectable using the sensitive ELISA ([7]). Shared-antigen-induced immunoglobulins can also be detected using whole mycobacterial cells and other bacteria with similar proteins ([9]).

Although shared and species-specific antibodies appear to afford little or no protection against the proliferation and dissemination of *M. leprae*, it is possible that prior exposure to environmental mycobacteria might influence the type of leprosy which an individual develops.

This paper presents evidence for this phenomenon using an ELISA to detect antibody levels against 16 environmental mycobacteria in sera from leprosy patients and controls from two areas in Zimbabwe.

[1] Received for publication on 29 December 1986; accepted for publication in revised form on 7 July 1987.

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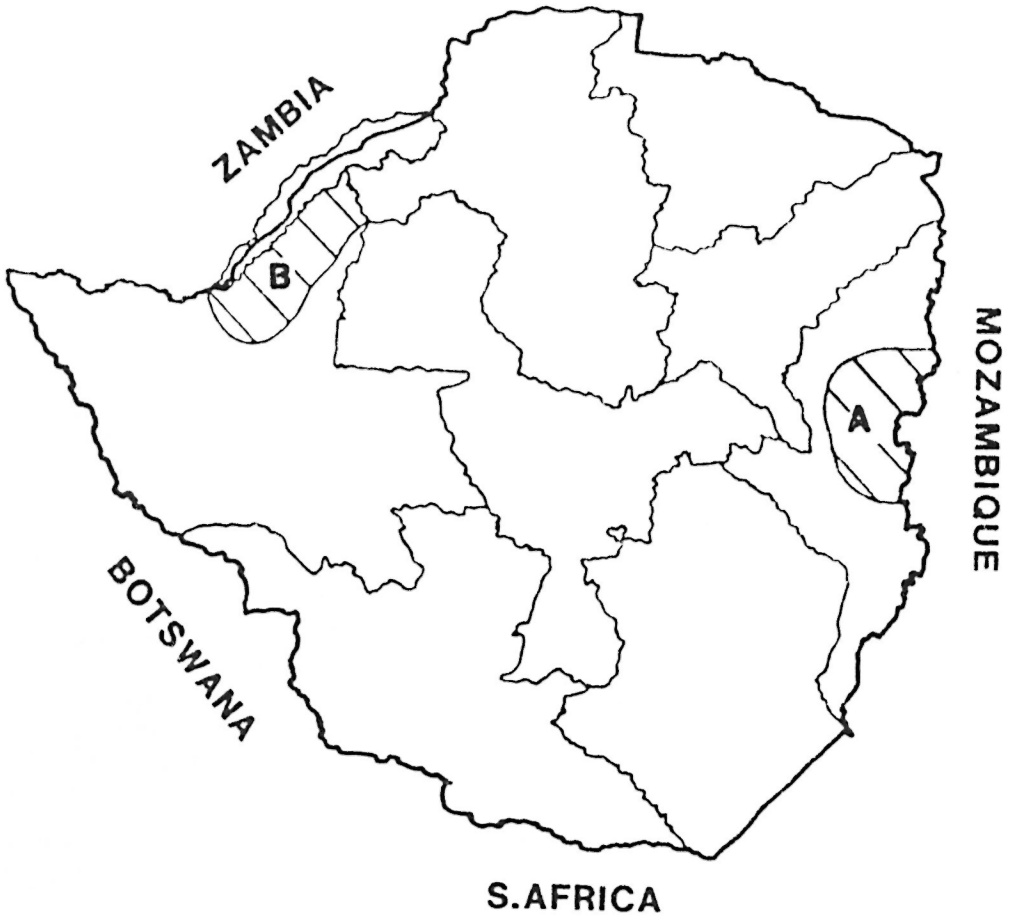
MATERIALS AND METHODS

Selection of study areas

Zimbabwe is situated in southern Africa and is bordered by Zambia, Mozambique, South Africa, and Botswana. The country covers an area of 390,757 km^2 and is divided into eight provinces (Fig. 1). The latest census in 1982 ([19]) estimates the total population as 7,546,071, and the prevalence of leprosy is between 1 and 2 cases per thousand ([18]).

A liberation war severely disrupted the health services between 1973 and 1979, and many leprosy patients were unable to continue treatment under the existing National Leprosy Control Programme. In 1983 a new control program was established adopting the World Health Organization therapy recommendations for leprosy. At the time our investigation was concluded, 3150 patients were again under active control. All cases are assessed clinically and bacteriologically and, for the purposes of receiving treatment, they are divided into paucibacillary and multibacillary groups. For this investigation, paucibacillary cases are included as tuberculoid and multibacillary cases as lepromatous.

An analysis from central statistics shows the national ratio of tuberculoid-to-lepromatous leprosy as 1.94:1 (N = 3150). A breakdown of the tuberculoid-to-lepromatous ratio on a provincial level shows that most provinces are similar to the national figure except for Manicaland, where the ratio is 0.69:1 (N = 161). Topographically, Manicaland (Area A) differs from the remainder of Zimbabwe, being higher in altitude, cooler, and wetter. For comparison, Matebeleland North (Area B) was selected. This province has a tuberculoid-to-lepromatous ratio of 2.12:1 (N = 399), and is lower in altitude, hotter, and drier. There are also other marked differences between the two areas, including soil and vegetation type (Fig. 1).



AREA B		AREA A
Generally below 3000 ft	**Altitude**	4000–6000 ft with mountain peaks rising to 8514 ft (Mt. Nyangani)
Mean = 22.5–25°C Range = 22.5–35°C	**Temperature**	Mean = 15–22.5°C Range = 15–32.5°C (with night temperatures falling below 0°C)
1700+ hr in dry season (May–Oct.) 1300–1400 hr in wet season (Nov.–Apr.)	**Sunshine**	1500+ hr in dry season (May–Oct.) <1200–1400 hr in wet season (Nov.–Apr.)
400–800 mm	**Rainfall**	400–1400 mm
45%–50% in dry season 75%–80% in wet season	**Humidity**	50%–70% in dry season 65%–85% in wet season
Mainly weakly developed including Lithosol and Regosol groups plus Siallitic group and Calcimorphic type	**Soil type**	Basically Kaolinitic and including Orthoferallitic, Para-ferallitic, and Fersiallitic groups plus Calcimorphic type and Lithosol group weakly developed in southern area
Mainly savanna woodland	**Vegetation**	Wide ranging, from medium altitude closed forest through moist montaine *Macarang-Albizia* forest, *Brachystegia spitiformis* and *Julbernardia globifora* thicket, savanna woodland and tree savanna
16–30 × 10^6 gallons per square mile	**Surface water**	8–80+ × 10^6 gallons run-off per square mile

Isolation of environmental mycobacteria

Numerous methods have been described for the isolation of mycobacteria from environmental sources ([31]). Several of these were assessed but none was found ideal, mostly due to the low pick-up rate caused by harsh decontamination procedures. It was also possible that mycobacteria adsorbed to soil particles were lost during centrifugation or sedimentation.

A method was developed which provided a much higher isolation rate and was convenient for handling under field conditions since no major items of equipment are required for the pre-incubation steps. Tween 80 was incorporated into the primary suspension fluid to release mycobacteria from soil particles, and benzylkonium chloride was used as the decontamination agent.

Method.

a) Add approximately 5 g soil to 15 ml 0.1% Tween 80 in distilled water previously sterilized by pressure millipore filtration.

b) Store at room temperature for 1–7 days with frequent agitation.

c) Allow to settle for 24 hr and decant the supernatant into a sterile tube.

d) To the supernatant add an equal amount of 0.16% (w/v) benzylkonium chloride and mix well.

e) Leave for 24 hr with occasional agitation.

f) Inoculate 4 drops into Löwenstein-Jensen medium and incubate in 5% CO_2 at 35°C.

The soil samples were collected randomly but generally from moist, shady locations within 500 meters of human habitation. A total of 250 samples (125 from each area) was processed, and 35 isolates of acid-fast bacilli were obtained from Area A and 24 from Area B (Table 1).

Of the isolates selected for the study, five were unidentifiable, two were species isolated from one area only, and the remainder were common to both areas. Seven isolates used were *M. intracellulare* which were selected because there are many agglutination types of this species dependent on surface antigens and contact with different serotypes would be likely to induce antibody of different specificities in man.

TABLE 1. *Identification and area of isolation of environmental mycobacteria obtained from 250 soil samples (125 from each study area).*

Identification	Area	
	A	B
M. intracellulare	15	15
M. fortuitum	1	0
M. gordonae	12	6
M. phlei	1	0
M. scrofulaceum	2	2
Unidentified rapid grower	3	1
Unidentified slow grower	1	0
Total	35	24

Identification of mycobacteria

Isolates were identified where possible using criteria including: rate of growth, pigment formation, colonial characteristics, niacin production, nitrate reduction, catalase activity, Tween hydrolysis and arysulfatase production ([26]), and thin-layer lipid chromatography ([15]). All except six were slow growers (>7 days) (Table 2).

Preparation of antigen

The isolates were transferred to 200 ml volumes of Sauton's liquid medium ([30]) and incubated at 35°C with occasional agitation until maximum growth was obtained. Once this was achieved, the antigen was prepared.

Method.

a) Centrifuge at 4000 rpm for 4 hr at 2°C.

b) Remove supernatant and sterilize by passing through a 0.2 μ membrane filter.

c) Aliquot into 20 ml amounts, freeze to −70°C, and lyophilize. Store at −20°C.

d) For use, reconstitute with 1 ml phosphate buffered saline (PBS).

Serum

Forty random serum samples from leprosy patients and ten samples from healthy

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FIG. 1. Provinces of Zimbabwe and topographic features of study areas. Area A is in Manicaland Province; Area B in Matabeleland North.

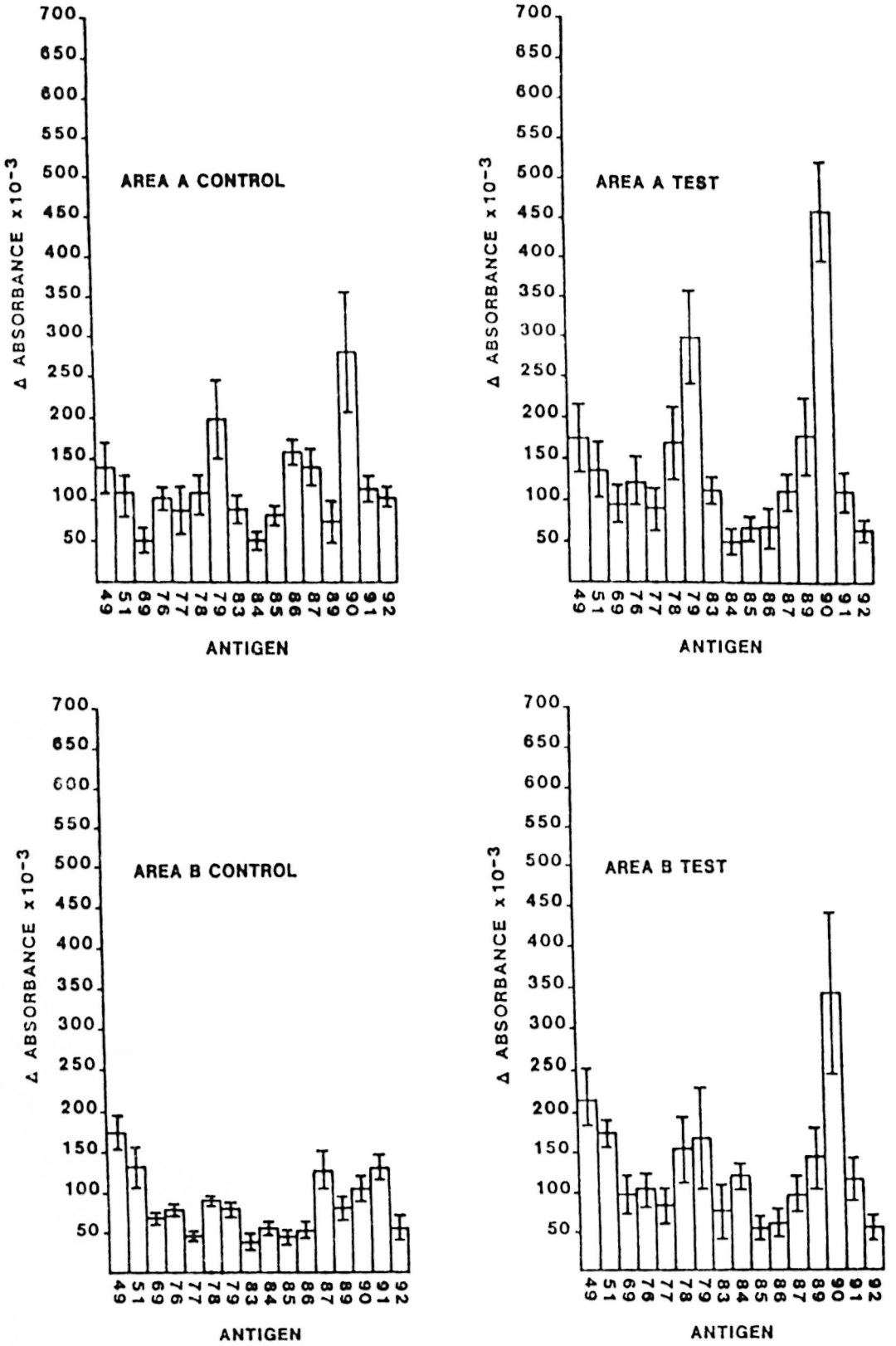

FIG. 2. Means (vertical columns) and standard deviations (bars) for control and test sera from Areas A and B.

controls were collected from each area and stored in aliquots without preservative at −20°C. For use, aliquots were thawed only once for testing. None of the leprosy patients or healthy controls had received BCG vaccination, and none had a history of tuberculosis. The samples from patients from Area A included 8 paucibacillary (TT-BT) and 32 multibacillary (BB-LL) cases; from Area B, 37 paucibacillary and 3 multibacillary cases.

ELISA

All serum specimens were tested against all 16 antigen preparations. The antigen was diluted 1:100 with 0.2 M carbonate buffer, pH 9.2, and 50 μl added to paired wells of a flexible, polyvinyl chloride microtiter plate. Duplicate paired wells received 50 μl of buffer alone and served as controls. After incubation for 16 hr at 2°C, the plates were washed with 0.5% bovine serum albumin in phosphate buffered saline (BSA-PBS), and the wells were blocked with 5% BSA-PBS. Serum was diluted 1:200 with PBS. The enzyme system used was peroxidase-conjugated anti-human IgG (Fc fragment specific) diluted 1:300 with PBS, and orthophenylene diamine was used as the substrate. The absorbance values were read at 492 nm. Delta absorbance was calculated by subtracting the mean of the duplicate control wells from the mean of the duplicate test wells.

Standardization for each ELISA run was achieved using two duplicate wells containing M49 and M90 antigens against a control serum sample previously shown to have a high titer against both antigens. Plates showing a 5% deviation from expected values for these controls were repeated.

RESULTS

The mean and standard deviations for delta absorbance values for control and test sera against each antigen are shown in Figure 2. Statistical analysis between values obtained from test and control sera from each of the two areas for each antigen was carried out using a two-tailed Student's *t* test (Table 2). When a significant difference was found, the antigens were grouped according to whether the higher mean values were obtained from test or control sera against homologous or heterologous antigen (Table 3). Analysis of the results showed marked deviation in response to some of the antigens between the two areas and also, in some cases, indicated differences between control and test sera from the same area (Table 2).

TABLE 2. *Identification of mycobacteria used for preparation of antigen and two-tailed* t *test p values between control sera from one area and control sera from the other area and between test sera from one area compared to test sera from the other area.*

Antigen	Identification	Control sera	Test sera
M49	*M. intracellulare*[a]	0.244	0.038
M51	*M. intracellulare*[a]	0.168	0.002
M69	Unidentified rapid grower	0.210	0.818
M76	*M. fortuitum*	0.015	0.093
M77	Unidentified rapid grower	0.046	0.524
M78	*M. phlei*	0.248	0.434
M79	*M. gordonae*	0.005	0.000
M83	*M. intracellulare*[a]	0.001	0.004
M84	*M. intracellulare*[a]	0.519	0.000
M85	*M. scrofulaceum*	0.001	0.111
M86	*M. intracellulare*[a]	0.000	0.352
M87	*M. intracellulare*[a]	0.651	0.187
M89	*M. intracellulare*[a]	0.713	0.044
M90	Unidentified slow grower	0.003	0.001
M91	Unidentified rapid grower	0.170	0.561
M92	Unidentified rapid grower	0.002	0.217

[a] Serotype unknown.

Where significant p values were obtained, antigens could be arranged into five groups on the basis of higher mean test or control values being found in serum from the same area as isolation or from the opposing area (Table 3). Interpretation of Table 3 shows the possibility of enhancement or suppression of immune response which may relate to the prevalence of leprosy types between Area A and Area B.

The two antigens (M77, M92) in Group 1 were both isolated from Area A, where a higher prevalence of lepromatous leprosy occurs. Since significant delta absorbance values were obtained from control sera in this area when compared to the test sera, this could indicate protective enhancement of immune response in the controls not present in the test group.

Three antigens (M49, M51, M89) occur in Group 2, where significant values occurred with the test sera but not with the

TABLE 3. *Antigenic groupings.*

Group[a]	Antigen	Identification	Area isolated	Area of higher mean value
1	M77	Unidentified rapid grower	A	A
	M92	Unidentified rapid grower	A	A
2	M49	*M. intracellulare*	B	B
	M51	*M. intracellulare*	B	B
	M89	*M. intracellulare*	A	A
3	M76	*M. fortuitum*	A	A
	M79	*M. gordonae*	A	A
	M83	*M. intracellulare*	A	A
	M90	Unidentified slow grower	A	A
4	M85	*M. scrofulaceum*	B	A
	M86	*M. intracellulare*	B	A
5	M84	*M. intracellulare*	A	B
6	M69	Unidentified rapid grower	A	
	M78	*M. phlei*	B	
	M87	*M. intracellulare*	B	
	M91	Unidentified rapid grower	B	

[a] Group 1 includes organisms producing significantly higher values (and tendency towards significance) in control sera only from the same area of isolation.

Group 2 includes organisms producing significantly higher values in test sera only from the same area of isolation.

Group 3 includes organisms producing significantly higher values in both control and test sera from the same area of isolation.

Group 4 includes organisms producing significantly higher values in control sera only from the opposite area of isolation.

Group 5 includes organisms producing significantly higher values in test sera only from the opposite area of isolation.

Group 6 includes organisms producing no significant values in either control or test sera.

control sera. The first two antigens were from the predominantly tuberculoid Area B; the other was from the lepromatous Area A. Results from this group suggest either a suppressive effect on the immune response of the test group in both areas, thus interfering with protective immunity when challenged with *M. leprae*, or the presence of antibody to shared antigen. This latter possibility is worth questioning since some studies have shown that many patients with tuberculoid leprosy have no detectable antibody against mycobacterial antigens ([24, 27, 37, 39]). However, these results were obtained using gel diffusion and were in contrast to the high antibody values obtained using a radioimmunoassay ([12]), which is a much more sensitive technique.

Group 3 shows remarkable correlation between control and test sera from Area A against antigens M76, M83, and M90. In each case, the mean control value is lower than the mean test value. This may imply that suppression of the immune response is greater in the test series, a high proportion of whom had developed lepromatous leprosy.

In Group 4, two antigens (M85, M86) gave higher significant values in the control sera of the area opposite to their isolation. The values of the test sera from both areas against these antigens were unremarkable. This is difficult to interpret but may result from crossreactive suppression of antibody expression in both sets of test sera and the control sera from the area of isolation, similar to the hypersensitivity suppression seen with mixed slow- and rapid-grower reagents used in Nepal and Bombay ([25, 32]).

Only one antigen (M84) falls in Group 5, and this showed a higher significant value in the test sera from the predominantly lepromatous Area A although it was isolated in Area B. The most likely explanation for this is a shared crossreactivity with antigen of *M. leprae*.

Antigens M69, M78, M87, and M91 produced no significant variation between control and test sera from either area and, presumably, have a more ubiquitous distribution or multiple shared antigenicity.

DISCUSSION

Studies and antigenic characterization of *M. leprae* have shown that in addition to a polysaccharide antigen ([10]), two antigens shared with *M. smegmatis* ([23]) and a soluble antigen extracted from leprosy nodules ([1]), at least 20 distinct antigenic components can be demonstrated. Detection of so many lines of precipitation, however, appears difficult to reproduce, and other workers have been able to produce only seven. Some reference lines of *M. leprae* are not specific for that species, including antigen 7 which crossreacts with BCG antigen 60 and with antigen 38 of *M. lepraemurium* ([13]).

Most of the investigations cited were undertaken using antisera raised in experimental animals. When sera from leprosy patients were examined using conventional or more sensitive techniques involving radioimmunoassay, SDS-polyacrylamide gel electrophoresis immunoperoxidase, immunoblotting and monoclonal antibodies, more antigen determinants have been found ([5, 11, 14, 16, 17]). Considerable crossreactivity was also noted between these antigens and nonmycobacterial genera ([28]).

Whether the large amounts of antimycobacterial antibodies reported in the sera from cases of lepromatous leprosy ([21]) are the result of marked humoral response to the massive amount of *M. leprae* antigen or the result of interaction following priming by prior exposure to other mycobacteria is not clear.

"Tuberculin"-type antigens prepared from many cultivable mycobacterial species have been used as skin-test reagents in numerous studies on human volunteers. The range of responses correlates with geographic conditions and the resident flora of "environmental" mycobacteria. In healthy individuals living in a rural area of the U.S.A., it was shown that the percentage of positive skin-test reactions to mycobacterial organisms found in the same area increased with age, whereas reactions against organisms from other areas did not ([4]). A similar study among Ugandan adults also showed some correlation between reactivity to the mycobacterial reagents and the frequency of mycobacteria in the environment ([33]). Children living in Libyan desert conditions where few mycobacteria were found reacted less frequently to a range of tuberculins prepared from 12 different species when compared to other children living in close proximity to farm animals ([34]). These studies not only demonstrate some specificity of immune recognition but also geographic variation in the range of species of mycobacteria capable of environmental survival.

There may also be similar recognition following exposure to primary pathogenic mycobacteria, including *M. leprae* and *M. tuberculosis*. A survey using lepromin skin-testing antigen in Venezuela, a country with high leprosy endemicity, and Chile, a nonendemic country, showed marked differences in responses both in children and adults which might be due to a specific sensitization or to crossreaction with *M. tuberculosis* ([8]).

The influence of the Listeria- and Koch-type immune responses, following priming with environmental mycobacterial antigen and the subsequent effects on BCG vaccination and exposure to *M. leprae* ([35]), has been suggested as the apparent cause for failure ([2]) or success ([36]) of BCG vaccination against leprosy.

Evidence for suppression of the immune response has also been shown using skin tests in leprosy patients in Nepal and Bombay ([25, 32]). When pooled reagents prepared from fast-growing mycobacteria were mixed with reagents from pooled slow-growers, suppression of a hypersensitivity response occurred. Similar suppression occurred when the fast- and slow-growing mixture was administered to one arm and the slow-growing reagent alone was used on the other arm.

The hypersensitivity reaction produced by using skin-test antigens is largely due to a cell-mediated immune response. The suppressor mechanism caused by the administration of a slow-growing mycobacterial antigen with concomitant administration of a fast-growing antigen may be similar to the cell-mediated deficit against *M. leprae* which occurs in leprosy. If this were the case, it would be reasonable to assume that exposure to an individual antigen or to a mixture of antigens from environmental sources may mediate the clinical response in leprosy patients by producing degrees of deficit from partial to total.

The present investigation makes use of soluble antigen derived from environmental sources in two areas of Zimbabwe that are widely diverse in topography and where opposing prevalence of the two polar forms of leprosy occurs. Measurement of immunoglobulin levels in sera from leprosy patients from these two areas showed that the predominant antibody response was of the IgG class. Comparison of the results from paucibacillary and multibacillary cases showed little or no variation, suggesting that the *M. leprae* antigen load did not influence the production of antibody to other mycobacteria, although some crossreactivity does occur.

It should be emphasized that infection

with mycobacteria other than *M. leprae* and *M. tuberculosis* in Zimbabwe is not uncommon. A two-year study conducted at a chest hospital showed that of 4876 strains of acid-fast organisms isolated from sputa, 161 were other than tubercle bacilli ([38]).

The results presented from this investigation show that contact with "environmental" mycobacteria appears to influence the prevalence of leprosy type by modification of the immune response to *M. leprae*. Further studies need to be undertaken in areas where one polar form of the disease predominates over the other to confirm this.

Further investigation of the effects of antibody stimulation following administration of experimental antileprosy vaccines may reveal that prior stimulation or suppression of humoral response caused by exposure to "environmental" mycobacteria may necessitate regional changes in antileprosy vaccine formulation.

SUMMARY

A comparison was made of antibody levels to 16 environmental mycobacteria in leprosy patients and healthy controls. Significant differences in response were found between patients and controls from an area of Zimbabwe with predominantly lepromatous leprosy when compared to an area where more tuberculoid cases were found. The results obtained suggest that exposure to some environmental mycobacteria may influence the type of leprosy developed by susceptible individuals.

RESUMEN

Se hizo una comparación de los niveles de anticuerpos contra 16 microbacterias ambientales en dos grupos de pacientes con lepra y sus controles sanos. Se encontraron diferencias significativas en los resultados cuando los pacientes y controles de un área de Zimbabwe con lepra predominantemente lepromatosa se compararon con los de un área con predomino de lepra tuberculoide. Los resultados obtenidos sugieren que la exposición a algunas micobacterias ambientales puede influir en el tipo de lepra desarrollado por los individuos susceptibles.

RÉSUMÉ

Chez les malades de la lèpre et chez les sujets témoins en bonne santé, on a procédé à une comparaison des taux d'anticorps à 16 mycobactéries de l'environnement. On a observé des différences significatives entre les malades et les témoins, dans une région du Zimbabwe où la lèpre lépromateuse était prédominante, par rapport à une région où le nombre de cas tuberculoides était plus élevé. Les résultats obtenus suggèrent que l'exposition aux mêmes mycobactéries de l'environnement peut avoir une influence sur le type de la lèpre qui se développe chez les individus susceptibles.

Acknowledgments. The authors wish to thank Dr. P. A. Jenkins of the United Kingdom Public Health Service Mycobacteriology Reference Laboratory for his assistance in the identification of some of the organisms used in this study.

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