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Seroepidemiological Studies of Leprosy in Northern Malawi Based on an Enzyme-linked Immunosorbent Assay Using Synthetic Glycoconjugate Antigen[1]

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The problem of recognizing past or present infection with the leprosy bacillus—as distinct from clinical disease—is one of the greatest challenges facing leprosy research today ([11]). Answers to many important questions—such as the sources of infection in human communities, the duration of the incubation period, and the identification of risk factors for infection and for disease—hinge upon the recognition of infection ([12]). In addition to improving our understanding of the natural history of leprosy, an ability to recognize infection might have important implications for leprosy control, particularly if it permitted effective prophylactic treatment prior to onset of infectiousness or clinical disease.

It has been hoped that serological tests might provide a measure of infection in populations in endemic areas. Among the various serological methods which have been tried are tests based on the indirect fluorescent antibody method on absorbed sera (FLA-ABS), radioimmunoassays, competition assays with isotope or enzyme-labeled monoclonal antibodies, and enzyme-linked immunosorbent assays (ELISA) with different antigens ([3]). Of all these methods, the ELISA is most appropriate for large scale use. In addition, the ELISA based upon specific phenolic glycolipid antigens of *Mycobacterium leprae* is the only assay thus far standardized among laboratories (Engers, H. D. Summary of IMMLEP-sponsored phenolic glycolipid workshop, June 1985, unpublished report 1985, IMMLEP program, World Health Organization, Geneva).

The literature on serological tests in leprosy is devoted almost entirely to applications of assays to small panels of sera from selected case and control groups. We have commented elsewhere on the difficulty of interpreting such data ([9]). There are very few examples of the application of such tests in epidemiological studies ([1, 5, 6, 10, 20]). These studies have reported very different results, e.g., from 6% ([5]) to 92% ([1]) seropositive among "contacts" of leprosy cases. None of the published investigations has provided data on the detailed distribution of (apparent) *M. leprae*-specific antibodies in an endemic population.

This paper presents results of seroepidemiological studies carried out in a leprosy-endemic population in Northern Malawi. The studies are based on an ELISA employing an *M. leprae*-specific antigen used in the World Health Organization (WHO) workshop (Engers, H. D. Summary of IMMLEP-sponsored phenolic glycolipid workshop, June 1985, unpublished report 1985, IMMLEP program, World Health Organization, Geneva). The work arose as part of the Lepra Evaluation Project (LEP).

METHODS

The structure and methods of the LEP are described in detail elsewhere ([17]). In this context, we note that the project is a total population longitudinal survey in Karonga District, Northern Malawi (Fig. 1). The population is rural, and numbered approximately 125,000 in 1984. The sera described in this report were collected during the first LEP survey (1980–1984). More than 112,000 persons were included in this sur-

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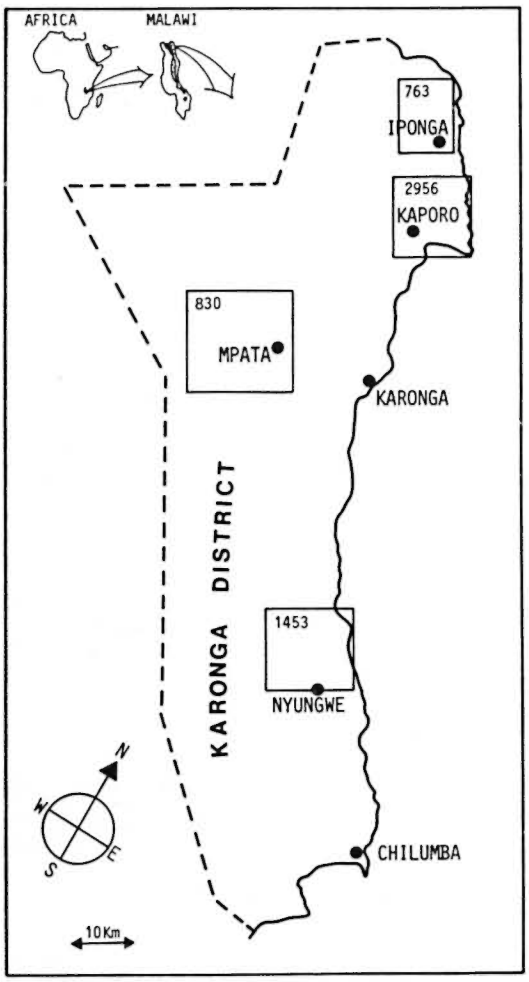

FIG. 1. Map of Karonga District, Northern Malawi, showing areas from which study sera were collected. Numbers of sera from each area shown in rectangles.

vey. Leprosy examinations were performed by trained paramedical workers (leprosy control assistants = LCAs). In addition to examining for leprosy, LCAs recorded the BCG scar status of each individual. All newly found suspects were examined by a medical officer (JMP). Biopsies were obtained from most suspects, and confirmation of the diagnosis of leprosy was based on clinical, bacteriological and histopathological information ([16]). Only cases with a high level of certainty (N or M group) ([16]) are included in this paper.

Capillary blood was collected by LCAs from more than 26,000 persons during the first survey. These individuals represented total populations (with the exception of a small number of refusers) of several specific areas covered by the survey teams. The blood was obtained by finger-prick, and dropped onto sheets of Whatman no. 3 chromatography paper. Three spots, each at least 16 mm in diameter, were normally collected from each person on a single 5″ × 3″ sheet of paper, on which was written the name, personal identification number, date, and household number. The papers were air-dried in wooden boxes and then stored in zip-seal plastic bags in paraffin fridges in the field. They were transported weekly to project headquarters in Chilumba, and there stored for periods of up to several months in freezer compartments of electric refrigerators. The papers were later shipped to London in insulated cold boxes, and stored at −20°C at the London School of Hygiene and Tropical Medicine (LSHTM) until tested.

For the purposes of this study, specimens collected in four different parts of the project area were withdrawn from the freezers and tested by ELISA at LSHTM. The test protocol is described in detail elsewhere ([9]). In brief, a 16-mm-diameter disc (containing approximately 50 μl blood) was punched from the blood-impregnated paper and eluted in buffer. An equivalent of 1:20 serum dilution was incubated in antigen-coated and uncoated wells of a microtiter plate. Following washing, a peroxidase-labeled anti-human-IgM was incubated in the wells. After another wash, the peroxidase substrate (OPD-H_2O_2) was added. Synthetic glycoconjugate antigen was used, having been generously supplied by Drs. R. Gigg and R. J. W. Rees (National Institute of Medical Research, London). The results were read as absorbance at 492 nm. A correction factor was applied to compensate for plate-to-plate variation in results recorded for the reference sera. This paper reports results expressed as specific activity (antigen coated well − uncoated well).

Serological data were linked via personal identification numbers to information in the main project files, and analyzed on computers at LSHTM and the University of London. Standardized prevalence or positivity ratios were calculated by conventional "indirect" methods (as the observed/ex-

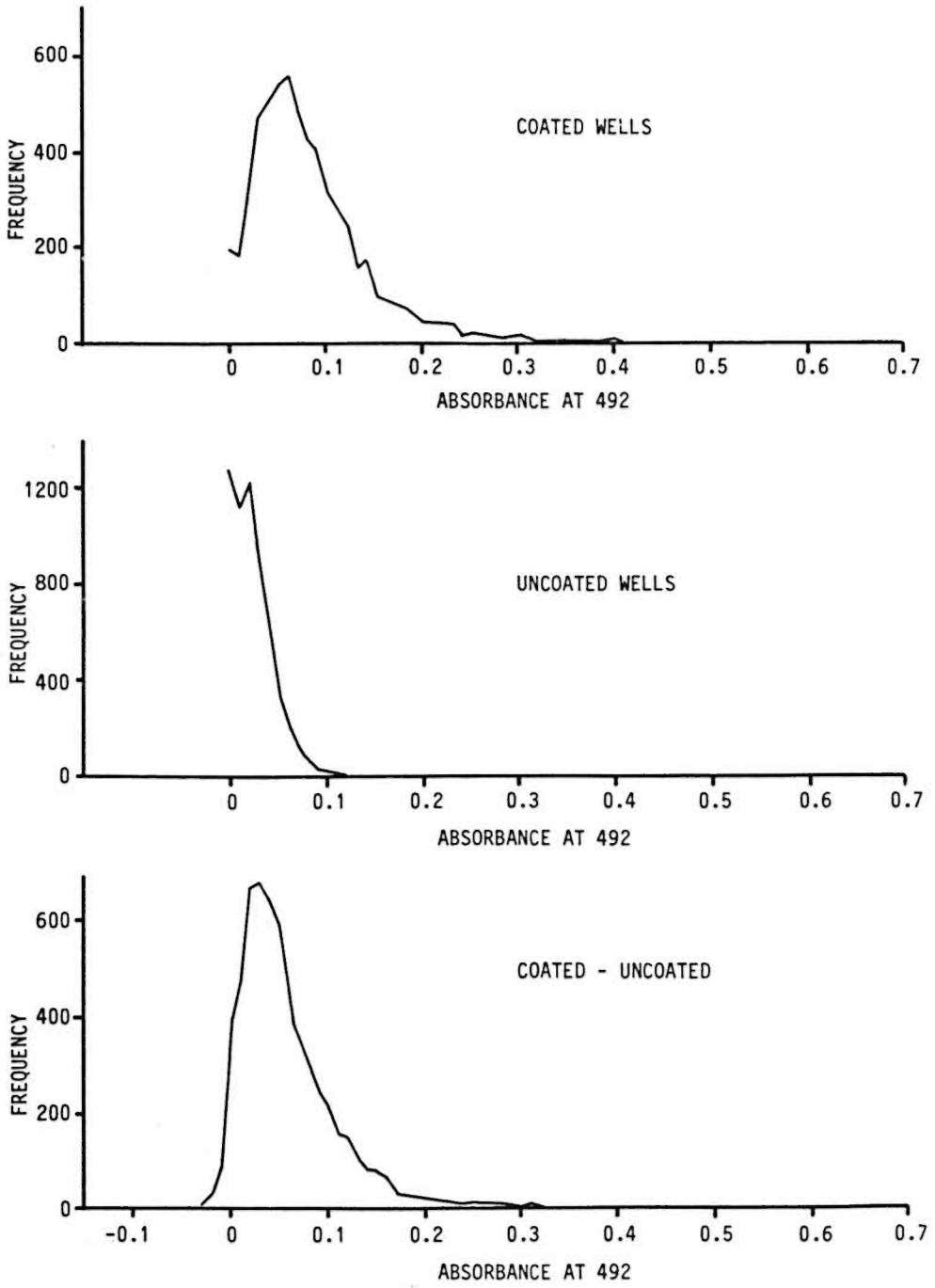

FIG. 2. Frequency distributions of ELISA results (coated plate, uncoated plate, coated − uncoated plate) on all 6002 sera included in this study.

pected ratio after applying age-specific rates in the total population of each sex to age-specific numbers in each group to calculate the expected value) ([4]). Two-stage catalytic models were fitted by least squares to age-specific prevalence data in order to estimate (age-independent) seroconversion and seroreversion rates ([15]).

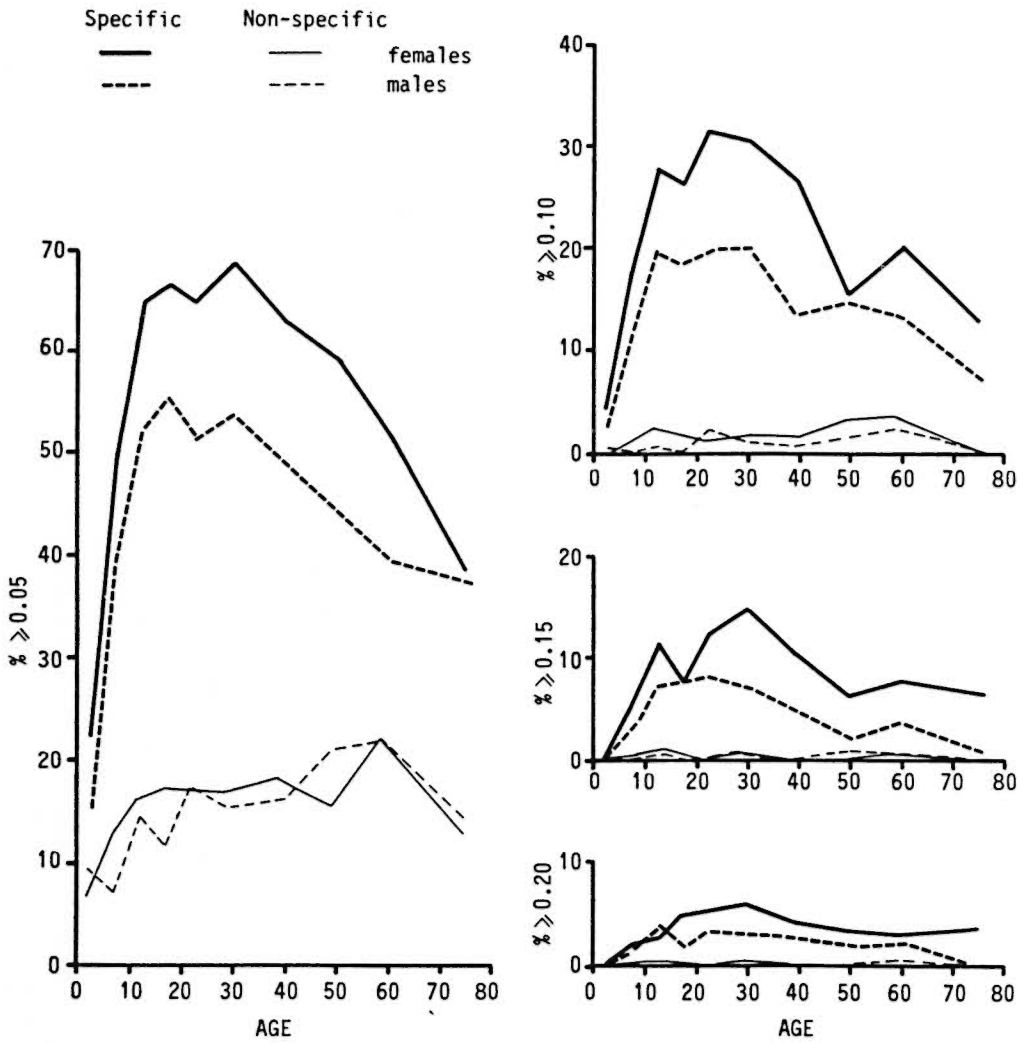

FIG. 3. Percentage "positive" to ELISA test, by age and sex, using different criteria for seropositivity: A = 0.05; B = 0.10; C = 0.15; D = 0.20. Bold lines refer to "specific" antibodies (coated − uncoated wells); thin lines refer to "nonspecific" antibodies (uncoated wells only).

RESULTS

Sera from 6002 individuals were examined in this study. These came from four well defined areas within Karonga District, as shown in Figure 1: Iponga (763); Kaporo (2956); Mpata (830) and Nyungwe (1453).

The frequency distributions of all ELISA results are shown in Figure 2 for coated and uncoated plate wells separately, and for the differences between them. This difference should represent IgM antibodies specific for *M. leprae* and is the measure presented in all subsequent figures and tables in this paper. The distribution of antibody titers is seen to be skewed to the right, but with no sign of bimodality such as might suggest an unambiguous cut off between "negative" and "positive."

Figure 3 shows the proportion "seropositive" by age, for each sex, using different criteria for positivity (bold lines). Here we see that, regardless of the criterion used, the prevalence rate of seropositivity rises rapidly to a plateau between the ages of 20 and 30, and then falls. It is consistently higher among females than among males, at all ages. Within these groups, there was no evidence of differences in seropositivity rates between individuals with and without BCG

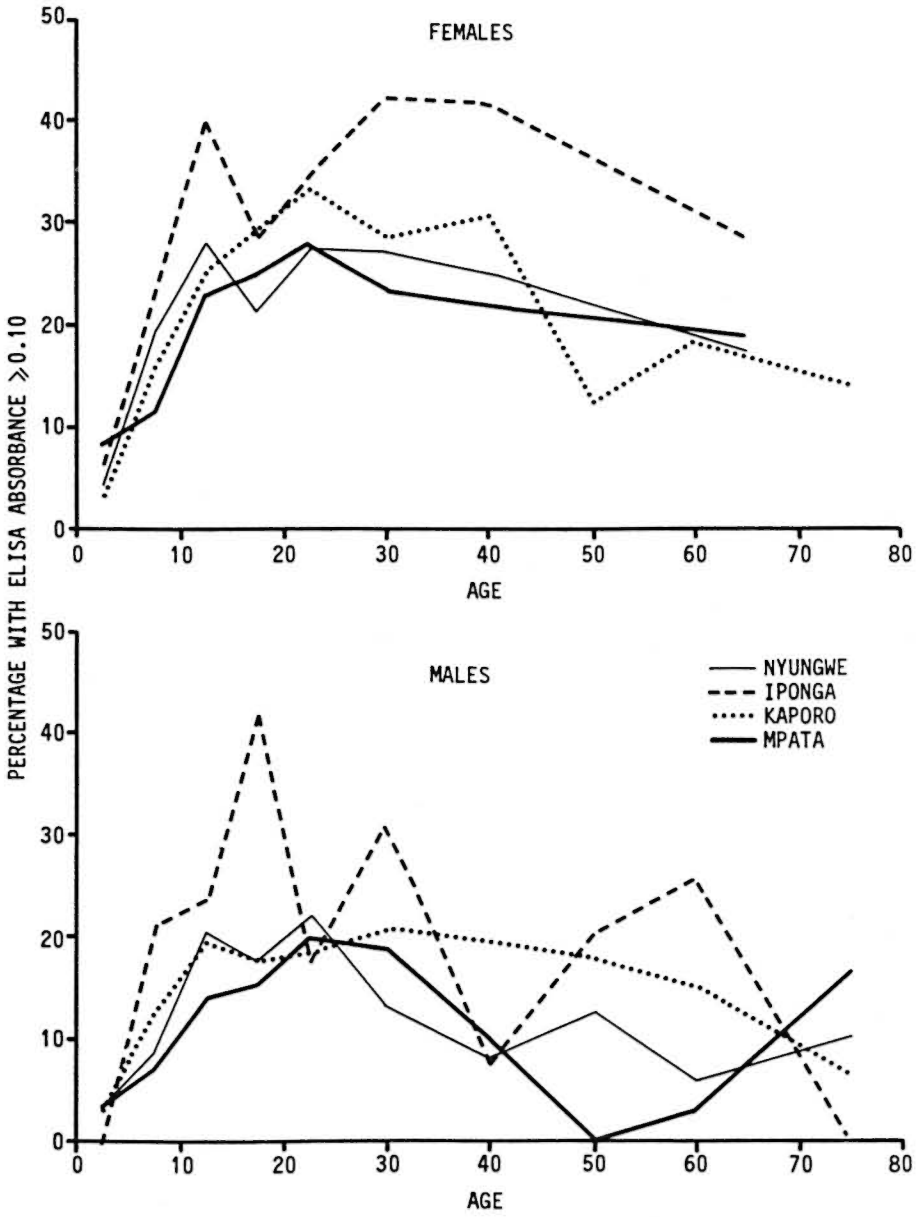

FIG. 4. Percentage positive to ELISA test (≥0.10) by age, sex and area within Karonga District.

scars. Also shown in Figure 3 (thin lines) are the proportions of sera which gave positive results in the uncoated wells. Although there is evidence for a rise in such nonspecific antibody up to age 10, and for higher nonspecific antibody among young females compared to young males, proportions "positive" do not follow the age pattern observed for specific antibody (bold lines).

The data are presented separately by area in Figure 4. The age and sex trends shown in Figure 3 are seen to occur in all four areas, though with less regularity due to smaller numbers of sera. The seropositivity rates appear marginally higher in the Iponga group than in the others. This is born out in Table 1, showing age-standardized positivity rates for the four areas. For both males and females the order is the same: Iponga (highest) > Kaporo > Nyungwe > Mpata.

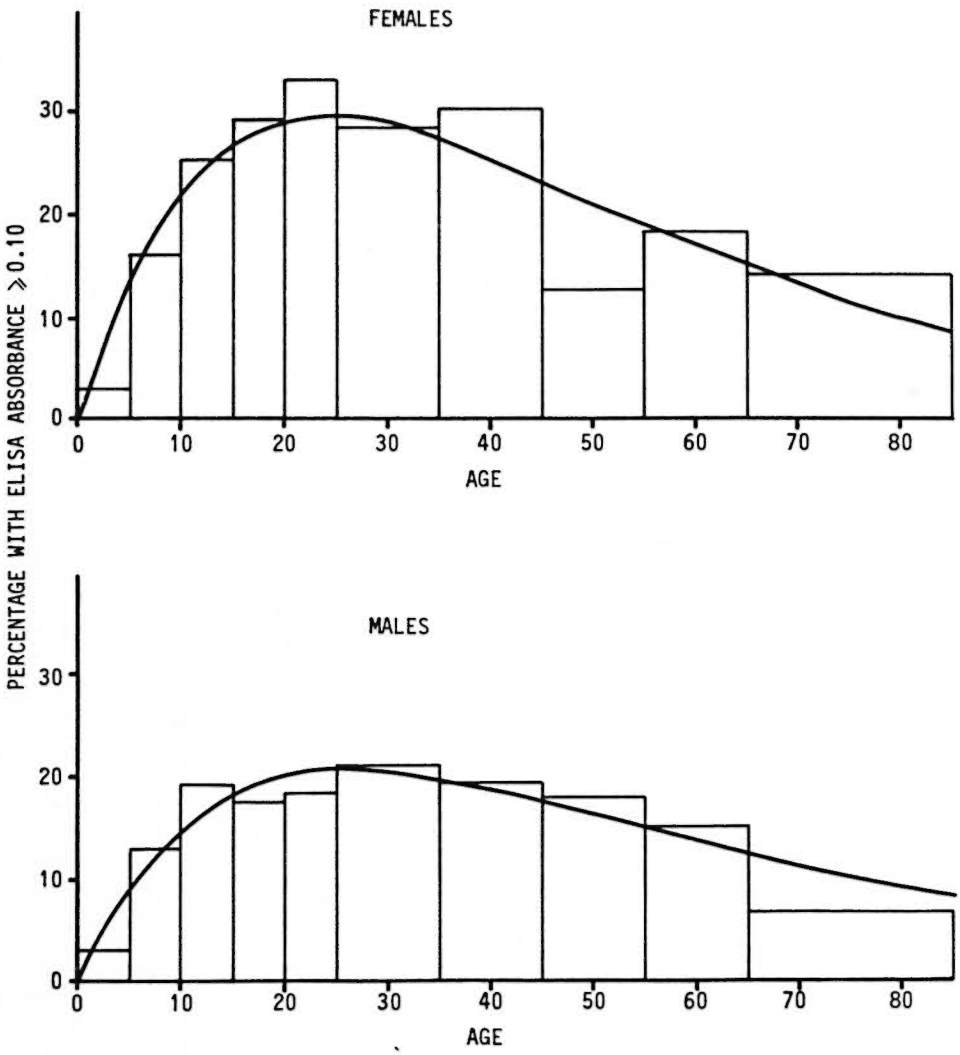

FIG. 5. Age distribution of seropositivity (ELISA ≥ 0.10) in males and females in the Kaporo area, showing fit of two-stage catalytic model (Table 2).

Also shown in this table are leprosy prevalence rates in the four areas, using standardized prevalence ratios to control for age. (These statistics are based upon the total numbers of persons and of cases residing in the four areas illustrated in Figure 1, and not just on those from whom blood was collected.) The highest prevalence ratios are observed for Iponga, for both sexes. As far as the other areas are concerned, the prevalence trend matches that for seropositivity for females, but not for males.

Figure 5 shows two-stage catalytic models fit to the age-specific seropositivity data for Kaporo region. Kaporo was chosen for this illustration since it had the largest sample size. Optimal fit is obtained for females with an α (incidence rate per year of conversion to seropositive) of 0.034 and β (rate of reversion to seronegative) of 0.050. For males the optimal parameters were 0.023 and 0.061, respectively. These parameters are tabulated for all four areas and both sexes in Table 2.

Figure 6 shows cumulative relative frequency distributions of ELISA results for noncases (a) and cases (b–e). Cases are here grouped into those which are: b) paucibacillary and treated; c) paucibacillary and untreated; d) treated multibacillary with a bac-

TABLE 1. *Seropositivity rates (ELISA ≥ 0.10) by age, sex, and area.*

Area	Age (yr)								Standardized positivity ratio[a]	Standardized leprosy prevalence ratio[a]
	0–4	5–9	10–14	15–19	20–24	25–34	35–44	45–		
					Females					
Iponga	0.057	0.225	0.400	0.286	0.347	0.423	0.415	0.286	1.36	1.30
Kaporo	0.029	0.162	0.255	0.296	0.333	0.286	0.304	0.169	1.00	0.980
Nyungwe	0.042	0.191	0.281	0.214	0.271	0.270	0.150	0.172	0.867	0.926
Mpata	0.082	0.114	0.227	0.250	0.279	0.235	0.219	0.188	0.863	0.883
					Males					
Iponga	0.000	0.210	0.237	0.412	0.176	0.314	0.079	0.190	1.36	1.17
Kaporo	0.030	0.130	0.195	0.177	0.184	0.210	0.197	0.140	1.06	0.930
Nyungwe	0.033	0.091	0.205	0.178	0.221	0.132	0.082	0.095	0.867	1.03
Mpata	0.036	0.071	0.140	0.154	0.200	0.188	0.100	0.055	0.765	1.04

[a] Age-standardized positivity ratios (observed/expected) and leprosy prevalence ratios (observed/expected) calculated by indirect method using total population of all areas as standard ([4]).

terial index (BI) = 0; and e) multibacillary with a BI of >0. The order of the distributions is of interest, indicating similar low ELISA results for noncases and paucibacillary cases. Higher antibody levels were observed in multibacillary cases, in particular those still bacteriologically positive. It may be noted that this figure takes no account of age differences between the case groups.

Table 3 shows the relationship of the ELISA result to case and contact status, controlling for age and sex differences. Cases are separated into four groups, as in Figure 6. Contacts are defined as individuals living in the same household as a confirmed case at the time the serum specimen was collected. Individuals in household contact with more than one case are categorized according to the case which was presumptively most infectious (i.e., e > d > c > b, using the categories stipulated above). Two different criteria for seropositivity are used: 0.1 and 0.2. Because of the differences in age distribution between the several groups, an age-standardized positivity rate has been calculated for each, using the noncase noncontact group as standard. There is evidence for higher seropositivity rates among cases than among noncases, but no evidence for higher rates among household contacts of cases than among individuals not living in the same household with a case.

DISCUSSION

This study provides detailed data on the seroepidemiology of leprosy in an unselected population. It was carried out in order to investigate the pattern of infection with *M. leprae*, in the hope of gaining insights beyond those provided by the more easily observed pattern of clinical disease. Patterns have become evident but, given the novelty of such data, we must question critically to what extent they reflect the natural history of the leprosy bacillus in this population.

The survey was based upon sera eluted from spots of capillary blood dried on absorbent paper. This method of collecting

TABLE 2. *Alpha (α) (seroconversion to positive) and beta (β) (seroconversion to negative) parameters derived by fitting two-stage catalytic model to age-specific seropositivity (ELISA ≥ 0.10) rates, by sex and area ([15]).*

Area	Females	Males
Iponga	α 0.037	α 0.038
	β 0.030	β 0.062
Kaporo	α 0.034	α 0.023
	β 0.050	β 0.061
Nyungwe	α 0.033	α 0.027
	β 0.061	β 0.091
Mpata	α 0.025	α 0.022
	β 0.046	β 0.094

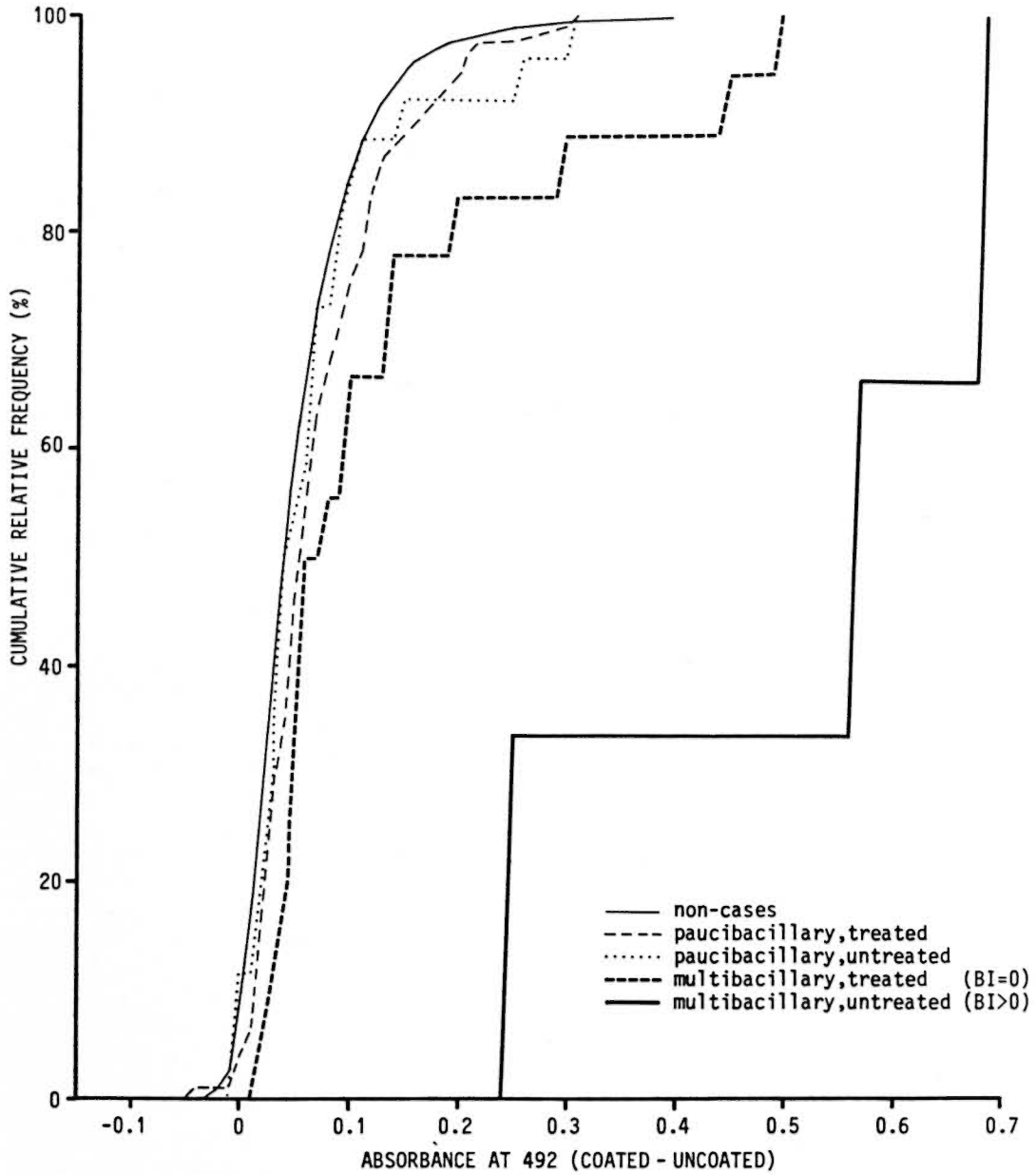

FIG. 6. Cumulative relative frequency of ELISA values in noncases, treated paucibacillary cases, untreated paucibacillary cases, treated multibacillary cases, and untreated multibacillary cases.

samples proved well suited to the field conditions, and a high correlation has been shown between phenolic glycolipid-I-ELISA results obtained on such specimens when compared with duplicate venipuncture sera ([2], and Burgess, unpublished data). On the other hand, these comparative studies were carried out on relatively small numbers of specimens prepared under ideal laboratory conditions. The assay employed in this investigation was based on a standardized antigen, and its results had been shown to be consistent with those of other laboratories when applied to venipuncture specimens in a multi-center workshop organized by the Immunology of Leprosy (IMMLEP) group of WHO (Engers, H. D., Summary of IMMLEP-sponsored phenolic glycolipid

TABLE 3. *Age-standardized seropositivity ratios (observed/expected) calculated for leprosy cases, contacts, and noncontacts in Karonga District, Northern Malawi.*

	No. tested		Age-standardized positivity[a] ratios			
			ELISA ≥ 0.1 = positive		ELISA ≥ 0.2 = positive	
	Females	Males	Females	Males	Females	Males
Noncontacts	2552	2524	0.987	0.983	0.975	0.828
Contacts of:						
Treated PB	235	208	0.840	1.03	0.391	1.47
Untreated PB	131	115	1.29	0.921	1.95	1.29
Treated MB	48	32	1.18	0.621	0.704	0
Untreated MB	11	8	0.851	0.998	0	0
All contacts	425	363	1.02	0.957	0.925	1.24
Cases:						
Treated PB	47	44	1.47	1.23	1.63	2.83
Untreated PB	15	11	0.768	1.06	1.68	3.63
Treated MB	8	10	1.10	4.57	3.78	14.3
Untreated MB	1	2	3.26	7.89	16.8	75.0
All cases	71	67	1.31	1.76	2.17	5.73
Total	3048	2954				

[a] Seropositivity defined as ELISA result of ≥0.10 or ≥0.20.

workshop, June 1985, unpublished report 1985, IMMLEP program, World Health Organization, Geneva).

The distribution of antibody concentration is unimodal (Fig. 2), thus any criterion for positivity is arbitrary. For this reason the absolute prevalence of seropositivity cannot be obtained. On the other hand, by exploring different criteria for positivity, we observe consistent relative patterns. Given the absence of any valid and independent measure of infection, we cannot be sure of the sensitivity or specificity of the assay used. Previous experience with this assay, using venipuncture sera from the same population, suggested that the 0.1 and 0.2 absorbance values as positivity criteria would identify approximately 70% and 10% of individuals with paucibacillary disease, respectively (9), but the data in Figure 6 show that the test's sensitivity in this large scale survey was far inferior to this (25% and 5%, respectively) in these two groups. More importantly, in this study we are looking at a general population in an effort to identify infection—for which no absolute reference exists. As far as specificity is concerned, past experience with the assay on sera from nonendemic populations suggests that a positivity criterion of 0.1 would give specificity in the range of 60% to 90%, depending on whether the individuals were or were not infected with some mycobacterium other than *M. leprae*, and that the 0.2 criterion should give almost 100% specificity for *M. leprae* infection (9). Lower specificity may have been obtained in this survey, given that the population is exposed to a wide variety of environmental mycobacterial infections (Ponnighaus and Fine, unpublished data). On the other hand, the consistently higher age-standardized positivity ratios observed in cases with the 0.2 in comparison with the 0.1 criterion (Table 3), and the lack of association of test results with a history (based on scar) of BCG vaccination, provide evidence of some specificity in this context. Beyond this we can do little more than recognize that the higher the criterion used for positivity, the lower the sensitivity, and the higher the specificity, of the assay as an indicator of *M. leprae* infection.

Given these provisos, we see several clear patterns in the data. First, the proportion seropositive is consistently higher among females than among males (Fig. 3). We have questioned whether the higher antibody concentrations could reflect lower hematocrits among females (and hence larger quantities of serum per unit volume of blood in the spots). This seems unlikely, particularly given that the female excess is observed at all ages, even in the very young. If the difference is indeed true, we may ques-

tion whether it reflects a higher exposure, an enhanced response, a more durable response or higher antibody avidity among females. Given that *M. leprae* is thought to be transmitted from person to person, it appears unlikely that females should be more exposed to infection at all ages. On the other hand, there are several reports in the literature indicating that IgM levels are typically higher among females than among males, perhaps reflecting an X-linked determinant ([13, 14, 19]). This may provide the simplest explanation for our observation. The female excess is particularly interesting in this example insofar as clinical leprosy is more frequent among females than among males in this population ([18]). However, the female excess of clinical leprosy in this population is restricted entirely to paucibacillary disease; whereas multibacillary disease, which is associated with higher antibody levels, is more common in males. It will be of interest to see if the female excess in "*M. leprae*-specific" IgM antibody positivity observed in noncases in Malawi is found also in populations in which paucibacillary leprosy occurs more commonly in males than in females.

A consistent age trend is observed, in both sexes, regardless of positivity criterion (Figs. 3–5). We begin by noting that this trend, with a peak in young adulthood, bears a striking resemblance to patterns of overall IgM that have been reported in several human populations ([7, 8]). On the other hand, the absence of such a trend in the uncoated wells suggests that it is attributable to antibody adhering specifically to the glycoconjugate antigen. There are four general circumstances which may lead to a rise and subsequent fall of antibody titers with age. First, if the exposure responsible for seroconversion had only recently been introduced into the population, and if seropositivity once acquired were permanent, then the age pattern of seropositivity would reflect the age distribution of exposure. This explanation seems unlikely in our case, since there is evidence that leprosy has been present in this population for at least many decades. Second, if a population has for many years been exposed to infection, and if seropositivity once acquired is permanent, then a rise-and-fall pattern would indicate either selective mortality of positives or else a cohort effect (i.e., individuals born 15–30 years previously were more intensely exposed than were individuals born either before or after that time). We see no reason to suppose that a selective mortality, or such a cohort phenomenon, has occurred. Third, if seropositivity is time-limited, and individuals who have reverted to seronegativity, having once been positive, are as likely to "reconvert" to positive as are individuals who have never yet been positive, then a rise and fall of seropositivity with age can arise as a direct reflection of age differences in risk of seroconversion. According to this explanation, the seropositivity peak between 15 and 30 years of age would imply that these age groups are most exposed to infection. Fourth, if exposure has remained constant over time and similar in all age groups, then a rise-and-fall pattern is predicted if seropositivity is time-limited, and if individuals who have reverted to seronegativity are less likely to convert to positive than are individuals who have never yet converted to positive. This fourth explanation is represented by the two-stage catalytic model applied in Figure 5 and Table 2, which assumes that the rates of conversion (α) and reversion (β) are constant at all ages and that no one "reconverts" after first reverting from positive to negative. The model is observed to provide reasonably close fits to the data. It should be emphasized that the close fit does not mean that the model's assumptions are necessarily correct but only that they are consistent with observed trends. Beyond this, the α and β statistics in Table 2 are of interest insofar as they suggest that the major difference between the sexes lies in the reversion rates, which are consistently higher among males than among females. This suggests that the female excess might be due not to a higher "risk" of females to seroconvert, but to a higher probability of seroreversion among the seropositive males.

Although the reason for the age trend is not clear, its presence has important implications for seroepidemiological studies, in that it means that age must be considered in the comparative analysis of such data.

The IgM antibodies observed in this study provide hints, but no clear picture, of the underlying pattern of *M. leprae* infection in this population. Area differences in sero-

positivity (either as standardized positivity ratios [Table 1] or as α statistics [Table 2]) were similar for males and females, and showed a correlation with leprosy prevalence in Iponga and in females, but not in males, in Kaporo, Nyungwe and Mpata. On the other hand, there was no evidence of higher seropositivity among household contacts than among individuals not living in the same household of a known case, even with the most specific (0.2) positivity criterion (Table 3). Not even the youngest age groups showed any evidence of higher antibody levels among contacts. Given that the antigen used in this assay is thought to be specific for *M. leprae,* and given the evidence for an association between this test and clinical leprosy (Table 3, Fig. 6, and [3, 9, 10]), the inability to discriminate contacts from noncontacts in this study is surprising as well as disappointing. It may mean just what the result appears to say—that this assay, at least on specimens such as those used in this study, is not a sensitive indicator of past or present *M. leprae* infection in this population. If so, this may reflect the low antigen load or the predisposition to paucibacillary-type immune responses (high cell-mediated, low humoral antibody) in this population. Alternatively, it could reflect that household contact status is not a good discriminator of infection in this population, perhaps because transmission is very intense, and virtually the entire population is infected.

These results provide little support for optimistic claims which have appeared in the literature concerning the potential value of ELISA tests based upon phenolic glycolipid and synthetic glycoconjugate antigens for field studies or the control of leprosy ([3]). In this context, it should be emphasized that none of the other published studies of leprosy serology has provided so rigorous an assessment as this, in terms of having a representative population sample, independent clinical assessment, and detailed analysis by age and sex as well as by contact status.

The data described here were collected in a single survey, and thus reflect the prevalence of seropositivity in this population during the early 1980s. Follow-up studies, currently in progress, will ultimately provide information on the predictive value of serology in terms of risk of future disease.

SUMMARY

A total of 6002 blood samples from total population samples in four separate areas within Karonga District, Northern Malawi, were tested for anti-*Mycobacterium leprae* antibody using an ELISA based on synthetic glycoconjugate antigen. Results are presented using different criteria for seropositivity. Regardless of the criterion used, the proportion of individuals classified as "positive" rose to a peak at 20–30 years of age and then fell, and it was higher at all ages in females than in males. There was no difference in seropositivity levels between individuals with or without BCG scars. Although leprosy cases, in particular those with positive smears, had higher antibody levels than nonleprosy cases, analysis of age-standardized data revealed only weak evidence for a correlation between the prevalence rates of clinical leprosy and of seropositivity within the four areas. There was no evidence for higher seropositivity levels in household contacts of leprosy cases compared to noncontacts. The implications of these results for the epidemiology of leprosy in this population are discussed.

RESUMEN

Usando un ensayo inmunoenzimático (ELISA) con un antígeno glicoconjugado sintético, se buscaron anticuerpos contra *Mycobacterium leprae* en un total de 6002 muestras de sangre de individuos de 4 áreas separadas, dentro del Distrito de Karonga en Malawi del Norte. Los resultados se presentan usando diferentes criterios de seropositividad. Independientemente del criterio usado, la proporción de individuos clasificados como "positivos" fue mayor en el grupo de 20 a 30 años de edad y después disminuyó. La proporción de positivos fue más alta en mujeres que en hombres en todas las edades. No hubieron diferencias en la seropositividad entre los individuos vacunados con BCG y los no vacunados. Aunque los casos de lepra, en particular aquellos con baciloscopías positivas, tuvieron niveles más elevados que los casos no leprosos, el análisis de datos estandarizados de acuerdo a la edad, revelaron sólo una débil evidencia de correlación entre los grados de prevalencia de un tipo clínico particular de lepra y la seropositividad dentro de las cuatro áreas. No hubieron evidencias de mayores niveles de seropositividad en los contactos familiares de pacientes con lepra cuando se compararon con no contactos. Se discuten las implicaciones de estos resultados en los estudios epidemiológicos de la lepra en esta población.

RÉSUMÉ

On a recherché un anticorps contre *Mycobacterium leprae,* au moyen d'un ELISA basé sur un antigène

glucoconjugué synthétique, dans un total de 6002 échantillons de sang, provenant de la population de 4 régions différentes du district de Karonga, au Malawi du Nord. Les résultats sont présentés selon différents critères de séropositivité. Nonobstant le critère utilisé, la proportion d'individus classés comme positifs s'élève avec l'âge jusqu'à un pic situé aux environs de 20 à 30 ans, pour retomber ensuite. Cette proportion était supérieure à tous les âges chez les femmes par rapport aux hommes. On n'a observé aucune différence dans les taux de séropositivité entre les individus selon que ceux-ci présentaient ou non des cicatrices de BCG. Quoique les cas de lèpre, et particulièrement ceux qui ont des frottis positifs, aient présenté des taux d'anticorps supérieurs à ceux décelés chez les individus témoins, l'analyse des données normalisées pour l'âge n'a révélé qu'une corrélation faible entre les taux de prévalence de la lèpre clinique et les taux de séropositivité dans ces 4 régions. On n'a pas observé de taux de séropositivité supérieurs chez les contacts domiciliaires de malades de la lèpre par rapport aux individus qui n'étaient pas contacts. Les implications de ces résultats pour l'épidémiologie de la lèpre dans cette population sont discutés.

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