J. Hyg., Camb. (1975), **75**, 57
Printed in Great Britain

Multiple skin testing in leprosy

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(*Received* 9 *January* 1975)

SUMMARY

Groups of patients with lepromatous and tuberculoid leprosy and hospital staff from six leprosaria in East Africa and 'non-contact' groups of villagers or staff from general hospitals have been skin tested with 10 reagents. These were prepared by ultrasonic disintegration from *M. tuberculosis*, *M. duvalii*, *M. chelonei* and 7 other species identified in the Ugandan environment. Comparisons were made of the percentages of positive reactors in each study group for each reagent. The 'specific' defect of lepromatous patients was found to apply to a variable extent to six of the species tested, but not to *M. tuberculosis*, *M. avium* or *M. 'A*'*. The defect applied most noticeably to *M. nonchromogenicum* and *M. vaccae*, suggesting that they are more closely related to *M. leprae* than are the other species tested. The reagent Chelonin produced unexpected and anomalous results in the lepromatous group. It is suggested that this was due to an unusually slow clearing of Arthus' reaction.

INTRODUCTION

It has long been recognized that a deficiency in immunity is associated with lepromatous leprosy and it has more recently been demonstrated that a degree of this deficiency also occurs in tuberculoid patients (Bullock, 1968). Studies on leprosy contacts have shown that the lymphocytes of healthy people may transform markedly in the presence of whole leprosy bacilli (Godal & Negassi, 1973), indicating that subclinical infection occurs and probably conveys some degree of protection. Whether the immune deficit present in the disease is a predisposing factor in its development, or the result of the infection, remains uncertain. It has been suggested that the macrophage of the lepromatous patient is inherently unable to destroy leprosy bacilli (Beiguelman, 1967). Alternatively the apparent macrophage defect may reflect the absence of a circulating clone of thymic (T) lymphocytes reactive to the leprosy organism. In polar cases of lepromatous (LL) leprosy this clone may never have existed; it may have been totally ablated by antigen overload or its activity may have been suppressed perhaps in a manner

Table 1. *Details of the organisms used for the production of the skin test antigens used in this study*

Collection No.	Organism	Source	Skin test antigen
813	*M. tuberculosis*	Clinical isolate	Tuberculin
R527	*M. avium*	Soil, Uganda	Aviumin
R528	*M. sp.* '*A**'	Soil, Uganda	'*A**'-in
R62/81	*M. gordonae*	Soil, Uganda	Gordonin
R507	*M. nonchromogenicum*	Soil, Uganda	Nonchromogenicin
R29/812	*M. engbaekii*	Soil, Uganda	Lactin
R859/877	*M. vaccae*	Soil, Uganda	Vaccin
R191/197	*M. fortuitum*	Soil, Uganda	Ranin
70	*M. duvalii*	Type strain NCTC 358	Duvalin
124/446	*M. chelonei*	Injection abscesses	Chelonin

analogous to that which apparently occurs in the early phase of *M. ulcerans* infection. Amongst sub-polar lepromatous cases which have the ability to upgrade, that is move towards the tuberculoid end of the disease spectrum, some residuum of an effective T-cell clone must exist either as an antigen-resistant precursor or possibly as potent cells trapped in the lymph nodes.

Whatever the explanation it seems likely that reactivity to other organisms closely related to the leprosy bacillus might also be impaired; in fact there is already some evidence for this (Godal, Myrvang, Stanford & Samuel, 1974). As an extension of this it might be postulated that the amount of incompetence in the handling of an organism by leprosy patients could be used as a measure of the relation between that organism and the leprosy bacillus. To assess this possibility tuberculins have been prepared from ten species of mycobacteria and these have been used to skin-test groups of leprosy patients, leprosaria staff and others in East Africa.

Somewhat similar studies carried out by Pinto, Arseculeratne & Welianga (1973) in Ceylon failed to demonstrate differences in response between the two immunological extremes of leprosy. However, they used PPD's of five species of mycobacteria, only one of which (*M. fortuitum*) was a fast growing species.

MATERIALS AND METHODS

The skin test antigens used in this study were prepared from the organisms listed in Table 1. With the exception of *M. tuberculosis*, *M. duvalii* and *M. chelonei* all the mycobacteria used were isolated from the Ugandan environment (Stanford & Paul, 1973); six of these were of named species and one was of a new slow growing species, referred to as *M.* '*A**'.

For the preparation of each reagent 100 ml. quantities of Sauton's medium in 1 l. flat bottles were inoculated with mycobacteria grown on Löwenstein Jensen medium. The cultures were incubated at 32° C. for several weeks and the growth on them was used to inoculate 800 ml. quantities of Sauton's medium in Thompson flasks.

Table 2. *The number of people tested with each of the antigens in each of the study groups from each of the leprosarium districts*

Place	Group	Tuberculin	Aviumin	A*-in	Gordonin	Nonchromogenicin	Lactin	Vaccin	Ranin	Duvalin	Chelonin	Total	
Addis Ababa (Ethiopia)	L+BL	23	23	—	23	25	22	25	23	23	—	187	
	T+BT	17	15	—	15	15	16	16	17	15	—	126	
	Contact	29	29	—	28	28	28	28	30	29	—	229	
	Non-contact	16	16	—	13	12	13	12	16	16	—	114	
Buluba/Nyenga (Uganda)	L+BL	22	9	21	11	11	11	11	22	10	10	138	
	T+BT	31	19	29	16	16	16	16	29	16	16	204	
	Contact	16	5	15	27	27	30	28	14	5	4	171	
	Non-contact	35	47	35	33	33	38	29	39	—	33	322	
Kumi (Uganda)	L+BL	9	8	12	11	—	—	9	11	16	9	85	
	T+BT	38	35	14	11	—	—	38	11	15	38	200	
	Contact	26	27	36	31	—	—	28	37	35	32	252	
	Non-contact	27	27	28	24	—	—	25	28	28	27	214	
		T	T	T	A	A	A	A	A	A	T	A	T
Alupe (A)	L+BL	17	17	17	17	17	16	17	13	13	17	93	68
Tumbe (T)	T+BT	15	15	15	17	17	17	17	21	21	15	110	60
(Kenya)	Contact	26	26	26	18	18	18	18	33	32	26	137	104
	Non-contact	20	20	20	—	—	—	—	—	—	20	—	80

When good growth had been obtained, the organisms were harvested by centrifugation at 20,000 *g* for 15 min. and the medium was discarded. The organisms were washed twice with 2 vol. M/15 phosphate buffer (pH 6·8) and suspended in approximately 50 ml. of the same buffer. These suspensions were then treated for 15 min. in an M.S.E. 100 watt ultrasonic disintegrator with the wave peak distance set at 8–9 μm. Extracts produced were centrifuged at 70,000 *g* for 30 min. and the supernatants were serially filtered through three sterile membrane filters, one with a pore size of 0·45 μm. and two with pore sizes of 0·22 μm. Small amounts of the concentrated sonicated extracts were tested for confirmation of identity against rabbit antisera to the species under test in an immunodiffusion system. The resultant filtrates were tested for sterility on Löwenstein Jensen medium and protein concentrations were assayed by the method of Lowry, Rosebrough, Farr & Randall (1951). Each preparation was then diluted to a concentration of 2 μg. protein (100 units)/ml. with sterile borate buffered saline (M/15 borate, pH 8·0) containing (g./l.) $Na_2B_4O_7.10H_2O$, 3·63; H_3BO_3, 5·25; NaCl, 6·19 and Tween 80 at a concentration of 0·0005 %. The diluted antigenic preparations were dispensed through a sterile 0·22 μm. membrane filter into sterile 1 ml. tuberculin vials. Essentially similar reagents have been used in earlier studies (Pritchard, Stanford & Paul, 1974; Stanford, Revill, Gunthorpe & Grange, 1975).

Multiple skin testing was carried out on leprosy patients and staff at six leprosaria in East Africa. Wherever possible, non-leprosy contacts consisting either of villagers or staff at non-leprosy hospitals in the same areas were also tested. The leprosaria visited were ALERT (All Africa Leprosy and Rehabilitation Training Centre) at Addis Ababa, Ethiopia; Buluba and Nyenga near Lake Victoria in Uganda; Kumi in the Teso district of Uganda; Alupe near Busia in West Kenya and Tumbe on the Kenyan coast (see Table 2).

For skin testing 0·2 μg. (0·1 ml.) of reagent was injected intradermally into the front surface of the forearm using Gillette Scimitar 1 ml. syringes fitted with size No. 20 needles. Four tests were performed per person, two on each arm with the injection sites at least $1\frac{1}{2}$ in. apart, using any four of the skin test antigens (see Table 2). Reactions were read after 72 hr. by measuring the longitudinal and transverse diameters of induration in mm.

All the injecting and reading was done by the same person (R.C.P.). Information about age, previous BCG vaccination and contact with leprosy or the presence of the disease was recorded for each person. Results for staff and patients below 19 years have been omitted as they formed an unevenly balanced small minority group. The majority of patients were on treatment, and those receiving immunosuppressive reagents have been omitted from this study. The clinical diagnosis of the state of the disease of the leprosy patients was taken as that given by the doctors in charge.

RESULTS

In a preliminary analysis the results for each reagent were found to be very similar for patients categorized as lepromatous (L) or borderline lepromatous (BL) on the one hand and those categorized as tuberculoid (T) or borderline

Table 3. *Percentages of persons producing positive reactions to each of the skin test reagents in each of the study groups from each of the leprosarium districts*

Place	Group	Tuberculin	Aviumin	A*-in	Gordonin	Nonchromogenicin	Lactin	Vaccin	Ranin	Duvalin	Chelonin
Addis Ababa	L+BL	44	39	—	30	8	9	8	4	4	—
(Ethiopia)	T+BT	65	53	—	40	13	19	25	12	7	—
	Contact	100	76	—	61	36	39	29	23	31	—
	Non-contact	94	74	—	77	8	0	0	31	43	—
Buluba/Nyenga	L+BL	73	44	67	0	0	9	9	14	20	90
(Uganda)	T+BT	77	5	48	50	38	44	19	7	6	56
	Contact	94	20	80	37	37	73	25	29	20	75
	Non-contact	97	15	74	39	21	47	24	38	—	76
Kumi	L+BL	78	75	67	18	—	—	11	9	6	89
(Uganda)	T+BT	61	40	64	36	—	—	29	18	47	79
	Contact	88	56	56	61	—	—	36	16	29	63
	Non-contact	92	89	93	79	—	—	24	36	50	78
		T	T	T	A	A	A	A	A	A	T
Alupe (A)	L+BL	77	35	0	18	6	0	12	8	23	94
Tumbe (T)	T+BT	87	53	0	12	6	12	12	29	10	67
(Kenya)	Contact	81	65	77	50	33	33	17	33	9	69
	Non-contact	100	60	60	—	—	—	—	—	—	75

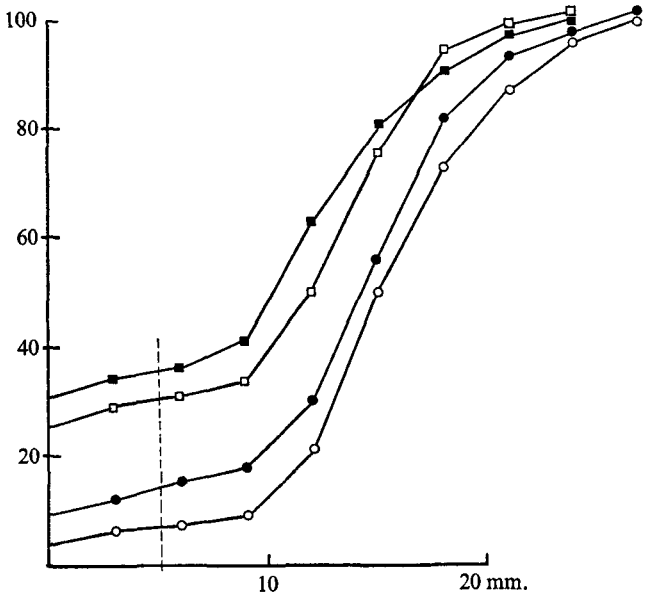

Fig. 1. Graph of the reactions to Tuberculin of persons in the four study groups. The ordinate is the cumulative percentage of people producing the mean diameters of induration shown on the abscissa. Each point represents the mean of the percentages for the individual leprosarium districts. ■, lepromatous leprosy patients; □, tuberculoid leprosy patients; ●, 'Contact' group; ○, 'non-contact' group.

tuberculoid (BT) on the other. Thus the patients were divided into only two groups (L + BL) called lepromatous and (T + BT) called tuberculoid. Since both the numbers of persons in each of the groups tested with each of the reagents and the prevalence of individual mycobacterial species in the environment or of tuberculosis in the local population differed from place to place, the percentages of persons giving various sizes of reaction also differed. For simplicity a certain minimum reaction size has been chosen as 'positive' and results are shown in Table 3 as percentages of persons producing positive reactions. In fact 5 mm. or more was selected as 'positive' since in the cases of the reagents to which a high percentage of persons reacted this diameter fell on the initial 'flat' part of the sigmoid distribution curve (Fig. 1). To correct for the differences in numbers tested and for differences in the frequency of contact with the various mycobacterial species, graphs have been prepared showing the means of the results for each study group. Examples of these are shown in Figs. 1, 2 and 3. The mean percentages positive and the means of the average positive reaction sizes for each of the groups tested with each of the reagents are shown in tables 4 and 5.

In none of the groups were the positive reactions to the majority of reagents evenly distributed. The majority were either negative to all four reagents or produced two or more positive responses. The explanation of this pattern does not apparently lie in cross-reaction between the different reagents since patterns of reactivity occurred with differing frequency in the different places and differed from individual to individual. Also the distribution of immunization with BCG

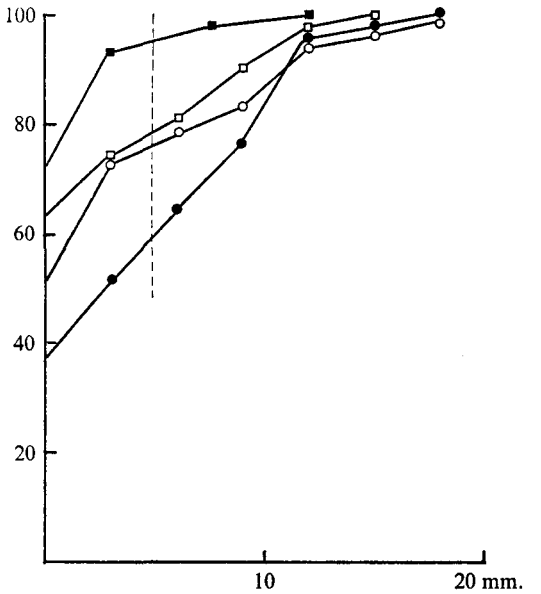

Fig. 2. Graph of the reactions to Lactin of persons in the four study groups. The ordinate is the cumulative percentage of people producing the mean diameters of induration shown on the abscissa. Each point represents the mean of the percentages for the individual leprosarium districts. Key as for Fig. 1.

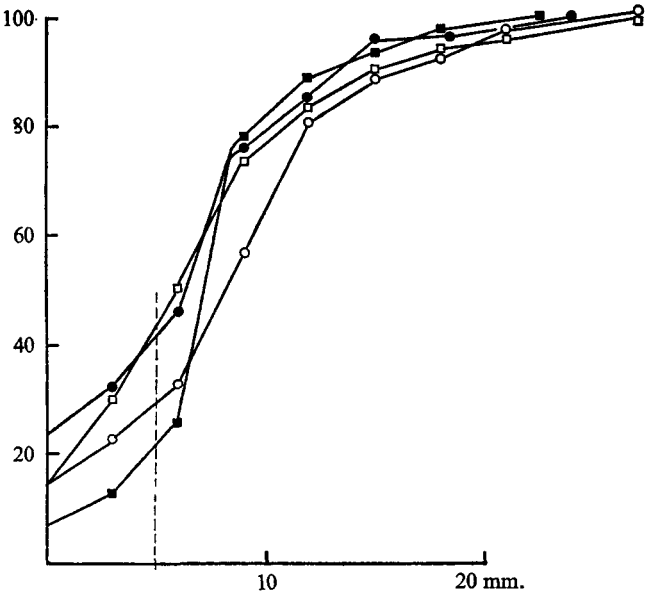

Fig. 3. Graph of the reactions to Chelonin of persons in the four study groups. The ordinate is the cumulative percentage of people producing the mean diameters of induration shown on the abscissa. Each point represents the mean of the percentages for the individual leprosarium districts. Key as for Fig. 1.

https://doi.org/10.1017/S0022172400047069 Published online by Cambridge University Press

Table 4. *The means of the percentages of positive reactions to each of the skin test reagents in each leprosarium district for each study group*

	L + BL	T + BT	Contact	Non-contact
Tuberculin	68	73	91	96
Aviumin	48	38	54	60
A*-in	45	37	71	76
Gordonin	17	35	52	65
Nonchromogenicin	5	19	35	15
Lactin	6	25	48	24
Vaccin	10	21	27	16
Ranin	9	17	25	35
Duvalin	13	18	22	47
Chelonin	91	67	69	76

differed widely among the groups. It was 7% amongst the leprosy patients, 29% in the contact group and 70% in the non-contact group.

DISCUSSION

If we interpret a positive skin test as evidence of the ability of the individual to develop delayed hypersensitivity to the organism from which the reagent was prepared, and the percentage of positive reactors to a reagent amongst the healthy population as an index of the allergenicity of the organism and the frequency with which it is encountered, then we have a system for the study of the relation of other mycobacteria to the leprosy bacillus. Of course, this is only the case if the reagents used have some specificity. The evidence we have, only a small amount of which has been published so far (Pritchard *et al.* 1974; Stanford *et al.* 1975) or is included in this paper, indicates that our reagents have a considerable degree of specificity. Table 4, which gives the means of the percentage of positive reactors to each reagent in each leprosarium district for each study group, provides information to which this system can be applied. With the exception of Chelonin which will be discussed later, the percentage of positive reactors was higher in the contact (staff) group than among the leprosy patients in every case (see Figs. 1, 2 and 3). Although the non-contact groups were in general badly matched, it is interesting to note that with the exception of Nonchromogenicin, Lactin and Vaccin, the percentage of contacts producing positive reactions to each reagent is lower than that of the non-contacts.

The effect of differing percentages of persons immunized with BCG among the study groups could only explain the instances where the contact group and the tuberculoid patient group contain fewer positive reactors than does the non-contact group. The inequality of BCG administration could not explain differences between results for tuberculoid and lepromatous patients or those instances where most positive reactors are amongst the contact or tuberculoid groups.

If we now turn to the differences between the two groups of leprosy patient. and again leave Chelonin for discussion later, reactions fall into 2 categoriess Tuberculin, Aviumin and A*-in show little difference between the groups and the

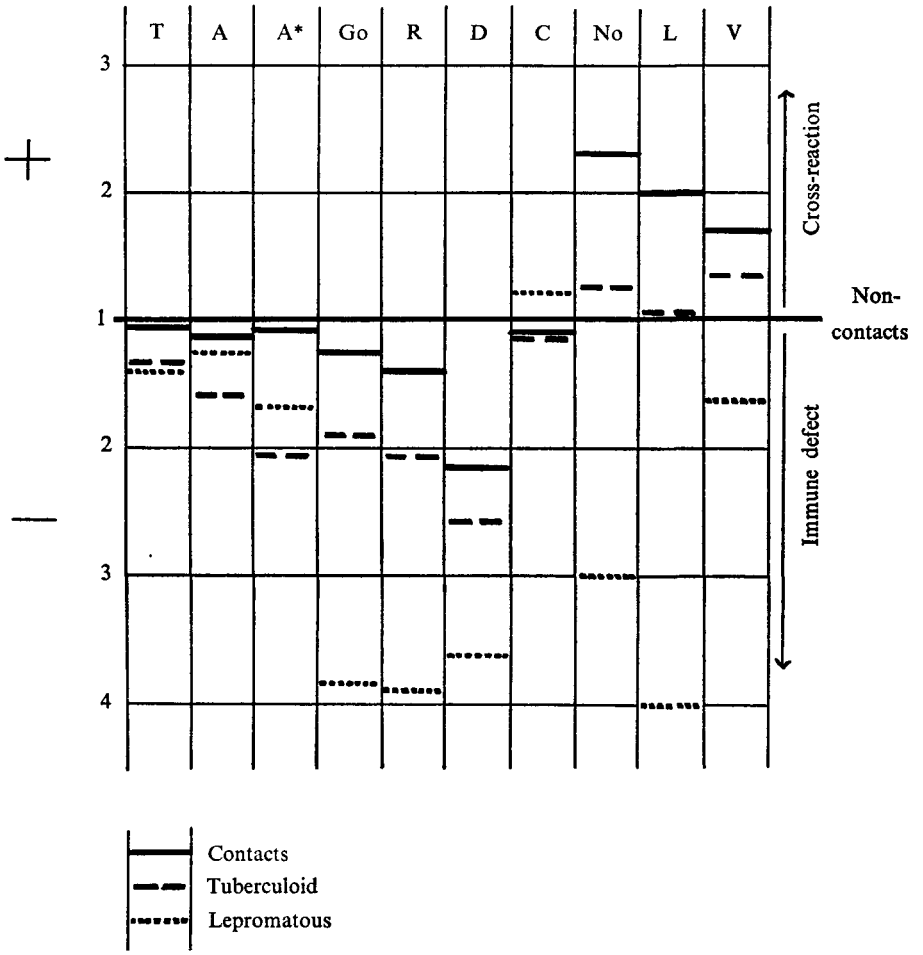

Fig. 4. Diagram illustrating the effect of leprosy on reactions to 10 mycobacterial skin test antigens. The percentage of positive reactors to each of the reagents used are expressed as +ve (1+) or −ve (1−) ratios of the patient and contact groups to the 'non-contact' group (taken as 1). Negative ratios for the 'contact' and tuberculoid patient groups may be due to inequality of distribution of BCG vaccination, but the other differences are related to leprosy. Key to skin-test antigens: T, Tuberculin; A, Aviumin; A*, A*-in; Go, Gordonin; R, Ranin; D, Duvalin; C, Chelonin; No, Nonchromogenicin; L, Lactin; V, Vaccin.

results for Gordonin, Nonchromogenicin, Lactin, Vaccin and Ranin show at least a twofold difference (see Figs. 1 and 2). The results for Nonchromogenicin and Lactin are of particular interest since there is at least a threefold difference between the results for lepromatous patients and those for tuberculoid patients (Fig. 4). These together with Vaccin are the only three reagents for which the contact (staff) group results are higher than those of the non-contact group and the percentage of positives amongst the tuberculoid patients is equal to or greater than the percentage of positives amongst the non-contacts.

https://doi.org/10.1017/S0022172400047069 Published online by Cambridge University Press

Table 5. *The average reaction size (calculated from the mean diameters of induration in mm.) of positive reactors in each of the study groups*

	L + BL	T + BT	Contact	Non-contact
Tuberculin	14·2	14·0	15·7	16·2
Aviumin	15·1	12·9	12·1	11·7
A*-in	11·2	11·0	11·3	11·1
Gordonin	7·9	10·5	10·5	11·0
Nonchromogenicin	7·3	7·9	8·9	6 8
Lactin	9·0	9·8	9·6	11·0
Vaccin	8·2	10·6	9·7	8·7
Ranin	9·5	9·0	9·4	8·5
Duvalin	9·4	9·5	9·0	9·3
Chelonin	9·0	11·1	11·1	11·0

The evidence presented indicates that the immune deficiency of lepromatous leprosy patients does not extend to Tuberculin, Aviumin and A*-in to any great degree but is less specific than was thought hitherto. The immune deficiency to *M. leprae* also applies to a variable degree to *M. gordonae*, *M. nonchromogenicum*, *M. engbaekii*, *M. vaccae*, *M. fortuitum* and *M. duvalii*. (At this point it should be noted that most of these species are of fast growing mycobacteria.) Additionally the larger number of positive reactors to Nonchromogenicin, Lactin and Vaccin amongst the contact group as compared with the admittedly poorly matched non-contact group suggests some cross-reactivity. The amount of such cross-reactivity is not very great since the percentage of positive reactors is low in each case and there is close similarity between the mean positive reaction diameters (Table 5) of all the groups. Nevertheless our evidence suggests that these species are more closely related to *M. leprae* than are the others tested.

Mycobacterium vaccae is a fast growing species which is not known to be pathogenic for man or animals. First isolated from the surroundings of cattle (Bönicke & Juhasz, 1964) it is found in occasional milk samples. In our study of mycobacteria from the Ugandan environment (Stanford & Paul, 1973) *M. vaccae* was only found in the swampy regions around Lake Kyoga (areas similar to those in which the studies of Kinnear Brown & Stone (1966) were performed), although skin test results with Vaccin suggest it is more widespread. *M. nonchromogenicum* (Tsukamura, 1965) and *M. engbaekii* (Korsak & Boisvert, 1972) were considered at the beginning of this study to be separate species, the first slow growing and the second fast growing. However, subsequent immunodiffusion analyses have shown them to be variants of a single species which should be placed in the fast growing subgenus. This discovery has provided our study with an unexpected but valuable internal control since the results obtained with the reagents prepared from the two variants are in good agreement. *M. nonchromogenicum* is a common environmental organism which is not known to cause disease in man or other animals. The relatively low levels of delayed hypersensitivity to this organism despite the frequency of contact with it suggests that it is only feebly allergenic – perhaps a further correlation with *M. leprae*.

The Chelonin results for tuberculoid patients and the contact and non-contact

groups are similar to those obtained with other reagents, but the results for the lepromatous group are grossly anomalous (see Fig. 3). Chelonin was tested on leprosy patients in three of the hospitals and although only a small number of patients were tested the results are almost identical in each place (Table 3). One possible explanation is that the lepromatous patients have received many more injections than most people and since *M. chelonei* is a common cause of injection abscesses in Africa, a number of them might have experienced such infections. Such an explanation would seem improbable. An alternative explanation might be that the reactions recorded as delayed hypersensitivity were in fact persisting Arthus' phenomena in some ways comparable with Erythaema nodosum leprosum and the giant reaction to tuberculin PPD. The finding of easily detectable antibody to one of the antigenic components believed to be specific to *M. chelonei* in a pool of sera from lepromatous patients in Addis Ababa and in two out of two sera from individual lepromatous patients in Malaysia adds some credence to the latter hypothesis. Obviously further studies must be made on this unexpected finding.

We are grateful to the many people who helped us in this study. Among them in Ethiopia were Dr G. Kronvall, Armauer Hansen Research Institute, Addis Ababa, and Dr F. Ross, All Africa Leprosy and Rehabilitation Training Centre, Addis Ababa. In Uganda, Professor S. J. Farsey, Department of Preventive Medicine, Mulago Hospital, Dr H. Oliver, Mengo Hospital and the staff of Nsambya Hospital, Kampala; Dr W. Blenska, Buluba Hospital, near Iganga and Dr J. Landheer, Kumi Leprosy Centre, Kumi, Teso. In Kenya Dr P. W. Kent, East African Tuberculosis Investigation Centre, Nairobi; Dr A. J. de Rijk, Alupe Leprosy Hospital; Dr Otsyula, East African Leprosy Research Centre, Busia and Dr C. Waaldijk, Msambweni District Hospital, Tumbe. Dr B. Hartman of the University Hospital, Groningen, The Netherlands also helped us in Kenya. Dr I. Sutherland of the M.R.C. Statistical Research and Services Unit, London, was kind enough to advise on working out the results. We are also grateful to the Ministries of Health of Ethiopia, Kenya and Uganda for permission to carry out our studies.

We should like to thank the Medical Research Council, The National Fund for Research into Crippling Diseases and the Wellcome Trust for financial help towards this project.

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