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Multiple skin testing of Kenyan schoolchildren with a series of new tuberculins

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SUMMARY

This study on Kenyan schoolchildren aims to elucidate the effect of contact with environmental mycobacteria on the development of specific delayed hypersensitivity. A series of 12 skin test reagents was employed; eleven of them were prepared from extracts of living mycobacteria and the last was the P.P.D. RT 23. Eight of the new tuberculins were prepared from mycobacteria recovered from the East African environment. A total of 8641 tests were carried out on 4320 children between the ages of 6 and 17 years in four townships. Two of these townships were in fertile agricultural areas and two were in the desert. Just over 80% of the children had received BCG immunization.

The results obtained showed that increasing age, geographical locality and BCG immunization all had a profound effect, and socioeconomic background had some effect, on the pattern of reactivity to the various reagents. The rationale behind the use of the series of new tuberculins and the results obtained with them are discussed in relation to the interacting effects of the factors complicating these results.

INTRODUCTION

Increasing knowledge of the interactions between men and mycobacteria indicates that contact with environmental species with low or perhaps no pathogenicity may profoundly affect susceptibility to the more pathogenic species. This acts both at the individual level and at the level of population protection, perhaps greatly influencing distribution of disease. Available evidence suggests that the range of mycobacterial species which take part in limiting prevalence of tuberculosis may be different from those limiting prevalence of leprosy.

There are a number of possible avenues of approach to this problem. One of them is analysis of mycobacteria in the environment and many such studies have been carried out (Gordon, 1937; Beerwerth & Schürmann, 1969; Stanford & Paul, 1973; Reznikov & Leggo, 1974). Another is to measure the amount of sensitization of populations to skin test reagents prepared from environmental mycobacteria (Ogunbi, 1969; Edwards *et al.* 1969; Pinto, Arseculeratne, Uragoda & Hemawardene,

1972). A third is through the study of the reactions of patients with mycobacterial diseases (Pinto, Arseculeratne & Welianga, 1973; Pinto, Arseculeratne, Uragoda & Hemawardene, 1973; Stanford, Revill, Gunthorpe & Grange, 1975; Paul, Stanford & Carswell, 1975), and a fourth is through studies of susceptibility of animals to experimental infections. Most such studies have been limited by the use of bacterial isolation techniques that are inadvertently selective for certain species or by the use of P.P.D.s with specificities too low to be of value.

The present study on Kenyan schoolchildren attempts to elucidate the effect of contact with environmental mycobacteria on the natural acquisition of specific delayed hypersensitivity. A series of new skin test reagents that have seemed remarkably specific in our previous studies are employed (Pritchard, Stanford & Paul, 1974; Stanford *et al.* 1975; Paul *et al.*, 1975).

MATERIALS AND METHODS

The skin test antigens used in this study were the same as those used in a previous study (Paul *et al.* 1975) with the addition of Neoaurumin. With the exception of *M. tuberculosis*, *M. duvalii* and *M. chelonei*, all the mycobacteria used for the preparation of these skin test reagents were isolated from the Ugandan environment (Stanford & Paul, 1973); seven of these were of named species and one was of a new slow growing species referred to as *M.* '*A**'.

The preparation of these skin test reagents has been described previously (Paul *et al.* 1975). The diluted antigenic preparations (2 μg. protein/ml.) were dispensed into sterile 1 ml. tuberculin vials and kept at 4° C. whenever possible until required for use.

Multiple skin testing was carried out on schoolchildren in 4 areas of Kenya. These were Kitale, Kericho, Lodwar and Marsabit. The children were arbitrarily divided into 2 age groups, 6–10 years and 11–17 years. Injections of 0·2 μg. protein (0·1 ml. of reagent) were given intradermally into either the front or back surface of the forearm using Gillette Scimitar 1 ml. syringes fitted with size No. 20 needles. Two tests were performed per child, one on each arm. Reagents were tested in pairs arranged so that each reagent would be paired with each of the others at some point in our study. Approximately 65 children were tested with each of the 66 possible pairs. Reactions were read after 72 hr. by measuring the longitudinal and transverse diameters of the indurations in mm. In Kitale and Kericho, all the injecting and reading (blind) was done by O.M. and J.L. In Lodwar and Marsabit all the injecting and reading (not blind) was done by R.C.P.

RT 23 P.P.D. obtained from the Statenseruminstitut, Copenhagen was administered as doses of 0·04 μg. protein (2 units).

Information about age and previous BCG vaccination was recorded for each child.

Table 1. *Table of the sources of the skin test antigens and the numbers of persons tested with them*

Collection No.	Organism	Origin	Skin test antigen	No. of tests 0·2 μg. protein BCG	non-BCG
R527	*M. avium*	Soil, Uganda	Aviumin	578	110
R528	*M. sp. 'A*'*	Soil, Uganda	A*-in	590	150
124/446	*M. chelonei*	Injection abscesses	Chelonin	593	153
70	*M. duvalii*	Type Strain NCTC 358	Duvalin	538	166
R62/81	*M. gordonae*	Soil, Uganda	Gordonin	562	145
R29/812	*M. engbaekii*	Soil, Uganda	Lactin	624	135
R507	*M. nonchromogenicum*	Soil, Uganda	Nonchromogenicin	586	133
R191/197	*M. fortuitum*	Soil, Uganda	Ranin	578	155
813	*M. tuberculosis*	Clinical isolate	Tuberculin	552	137
R859/877	*M. vaccae*	Soil, Uganda	Vaccin	523	81
R872/922	*M. neoaurum*	Soil, Uganda	Neoaurumin	578	127
	M. tuberculosis	Clinical isolate	P.P.D. (RT 23)	675*	172*
			Total	6977	1664

* 0·04 μg. protein administered.

RESULTS

The total number of children tested was 4320, of which 2509 were aged between 6 and 10 years inclusive and 1811 were aged between 11 and 17 years. 1273 children were tested at Kericho, 2212 in Kitale, 537 in Marsabit and 298 in Lodwar. Taken all together, 81% of the children had been previously immunized with BCG. Numbers of children tested with each reagent are shown in Table 1.

As in our previous study using the same batch of reagents (Paul *et al.* 1975) a mean diameter of induration of 5 mm. or more has been taken as 'positive' in every case. A complete picture of reactivity to the different reagents expressed as percentages of children producing positive reactions is given in Fig. 1. Reactivity to the reagents varies from 66% for Tuberculin to 8% for Lactin. There were numerous zero reactors to each of the reagents and the largest recorded mean diameter of induration was 30 mm., to both Tuberculin and P.P.D. (RT 23). The mean positive reaction sizes for each reagent are shown in Table 2 in two columns according to BCG status. Examples of the distribution of mean diameters of induration for individual reagents are shown in Fig. 2. Percentages of positive reactors in each age group and in the BCG immunized and non-immunized groups are shown in Table 3. These results for each centre are expressed diagrammatically in Fig. 3.

Most children were tested in government schools and many came from poor families. However, two schools, one at Kericho and one at Kitale, drew their pupils from homes of a higher socioeconomic level. Thus to some extent it was possible to compare children from the same towns but with different home backgrounds. These results are shown in Table 4. (Kericho Highland and Kitale Primary are the schools concerned.)

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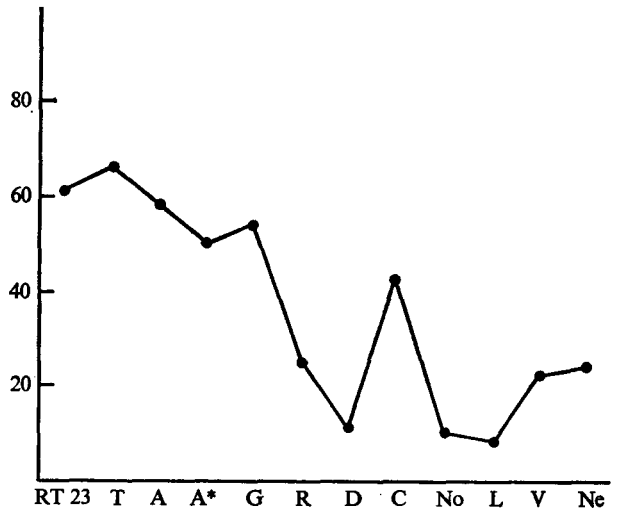

Fig. 1. Diagram showing the overall percentages of positive reactors (5 mm. or more) to each of the skin test reagents. These are easily identifiable from Table 1.

Table 2. *Table of the averages of the mean diameters of induration of all positive reactors*

	BCG	
Skin test antigen	Immunized	Non-immunized
Aviumin	10·8	9·0
A*-in	9·6	8·3
Chelonin	8·0	7·8
Duvalin	8·3	7·8
Gordonin	10·3	9·4
Lactin	9·8	8·0
Nonchromogenicin	9·4	7·8
Ranin	9·0	9·2
Tuberculin	12·3	10·6
P.P.D. (RT 23)	12·8	13·8
Vaccin	9·3	8·7
Neoaurumin	9·4	9·4

DISCUSSION

Mycobacteria have several categories of antigens capable of inducing antibodies in rabbits (Stanford, 1973). The evidence suggests that a similar categorization could be applied to antigens inducing cell mediated immunity although it is not certain whether precisely the same antigens are effective in each case. Thus group i antigens (shared by all mycobacteria and nocardiae) are of little importance compared with groups ii (shared by slow growing species of mycobacteria) or iv (species specific) antigens in the elicitation of delayed hypersensitivity. There is insufficient evidence as yet concerning the role of group iii antigens (shared by fast growing species).

Old Tuberculins and P.P.D.s show some specificity and much cross-reactivity;

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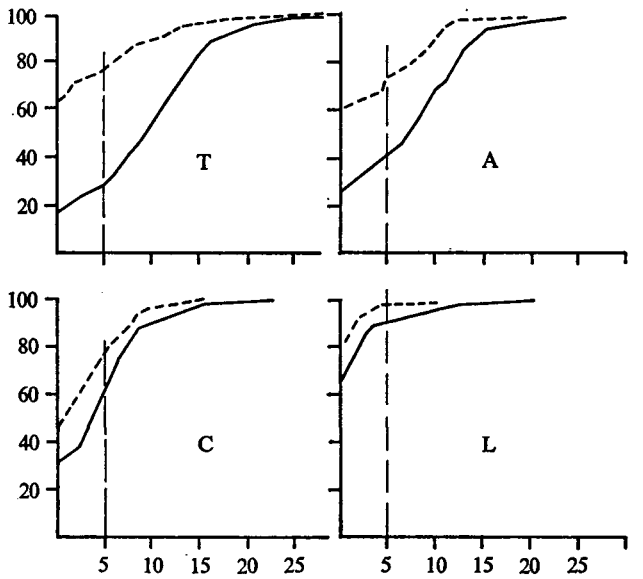

Fig. 2. Graphs showing percentage distribution of reaction sizes for four reagents. T = Tuberculin, A = Aviumin, C = Chelonin and L = Lactin, for children grouped accordingly to BCG status. The ordinates are cumulative percentages of persons producing reactions equal to or less than the point indicated by the graph. The abscissae are mean diameters of induration in mm. The vertical broken lines indicate the minimum sizes of reaction taken as positive. ———, immunized with BCG; ------, not immunized with BCG.

Table 3. *Table of percentages of children grouped according to age and BCG status producing positive reactions (5 mm. or more) to each of the skin test reagents*

Skin test antigen	All ages		6–10 years		11 + years	
	BCG	Non-BCG	BCG	Non-BCG	BCG	Non-BCG
Aviumin	62	34	48	18	72	62
A*-in	58	19	43	15	74	39
Chelonin	45	29	38	26	52	35
Duvalin	13	5	8	3	17	9
Gordonin	59	34	53	35	68	29
Lactin	9	2	2	0	17	7
Nonchromogenicin	11	6	10	4	11	17
Ranin	28	13	18	8	42	24
Tuberculin	76	24	72	24	82	23
P.P.D. (RT 23)	73	16	67	13	83	36
Vaccin	24	14	16	10	30	26
Neoaurumin	25	22	18	17	32	35

thus the principle of the differential tuberculin test as it is applied to cattle or man is to compare the sizes of reactions to P.P.D.s produced from two species of slow growing mycobacteria (generally *M. tuberculosis* and *M. avium*). The cross-reactivity might be attributed to delayed hypersensitivity to group ii antigens present in both P.P.D.s and the specificity to hypersensitivity to group iv antigens.

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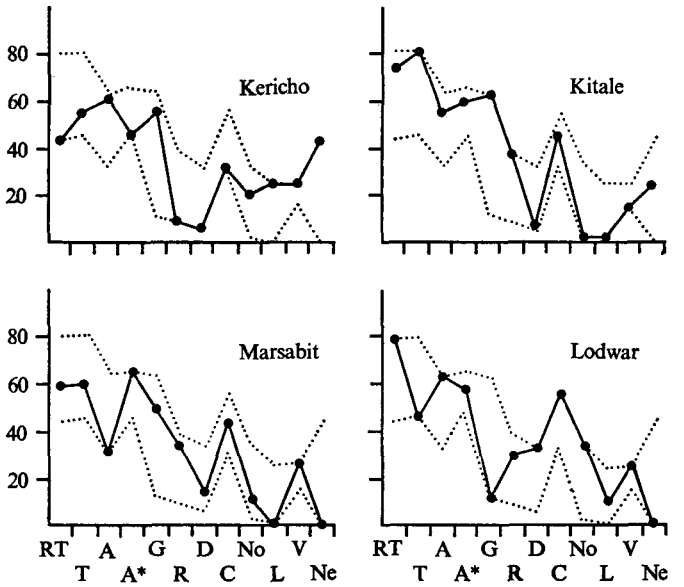

Fig. 3. Diagrams showing the percentages of positive reactions (5 mm. or more) to each of the skin test reagents at each centre (solid line). The dotted lines indicate the maximum and minimum percentages positive achieved in any one centre. The skin test reagents should be identified from Table 1.

Table 4. *Table showing the differences in percentages of positive reactors among children grouped according to age, coming from different socioeconomic backgrounds in two Kenyan townships*

	Kericho				Kitale			
	Highland school		Other schools		Primary school		Other schools	
Skin test antigen	6–10 years	11+ years	6–10 years	11+ years	6–10 years	11+ years	6–10 years	11+ years
Aviumin	38	—	35	85	—	76	46	62
A*-in	22	63	42	—	22	—	44	79
Chelonin	25	40	15	20	—	—	42	50
Duvalin	—	3	5	10	3	3	8	10
Gordonin	47	67	53	57	—	—	56	69
Lactin	—	30	2	60	0	3	2	4
Nonchromogenicin	15	27	14	27	2	—	5	2
Ranin	7	0	2	13	—	—	26	51
Tuberculin	44	75	53	58	67	—	75	87
P.P.D. (RT 23)	—	—	31	56	28	—	62	87
Vaccin	12	0	64	46	3	—	16	18
Neoaurumin	27	55	25	65	0	—	23	33

— indicates that no children in the particular school were tested with this antigen.

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Infections with fast growing mycobacteria do not give rise to positive reactions to these P.P.D.s.

Immunodiffusion studies of ultrasonicated suspensions of living mycobacteria have shown that such extracts are rich in group iv antigens and relatively poor in group ii antigens (unpublished observation, J.L.S.). Thus skin test reagents prepared from these extracts might show greater specificity for delayed hypersensitivity to species specific antigens. Our earlier studies on cattle (Pritchard *et al.* 1974), *Mycobacterium ulcerans* infection (Stanford *et al.* 1975), and leprosy (Paul *et al.* 1975) have shown this to be the case and the present studies provide further evidence of this specificity.

Fig. 3 shows the distribution of positive reactions to each of the new antigens and P.P.D. (RT 23) at each of the four centres. It can be seen that with each reagent there is considerable variation from place to place. Leaving aside the reactions to our Tuberculin and P.P.D. (RT 23) the degree of reactivity to each reagent is thought to indicate the effectiveness of contact with environmental species. Reactivity to Aviumin might be a consequence of subclinical infection. However, such infection is not likely to be a prerequisite for the development of delayed hypersensitivity to non-pathogenic species. The necessary combination of contact between mycobacterium, macrophage and lymphocyte might be achieved in the tonsil, through the intestinal mucosa, through cuts, abrasions, etc., or through the lungs. Effectiveness of contact is probably partly a measure of frequency of the individual species in the environment and partly a function of their relative allergenicity.

At Kericho, Kitale and Marsabit the results for P.P.D. (RT 23) are very similar to those for our Tuberculin, but at Lodwar far more people react to the P.P.D. than to the Tuberculin. The results for Aviumin are very similar (between 55 and 63 %) at Kericho, Kitale and Lodwar, but much lower (32 %) at Marsabit. There was less clearcut variation between the results for A*-in at the 4 centres (46–65 %). The reactivity to Gordonin is much reduced (to 12 %) at Lodwar in comparison with the other centres (49–63 %). Reaction to fastgrowers was greatest at Lodwar particularly due to Nonchromogenicin and Duvalin and least at Kitale and Marsabit. However, there was considerable variation between the centres for individual reagents.

We believe that these differences reflect the geographical variation between the centres. Both Kericho and Kitale are typical African townships in fertile hilly grassland regions between five and seven thousand feet above sea level. Kericho, which has the highest rainfall, is a major centre for tea and sugar cane cultivation, and lies about 30 miles east of Lake Victoria. Kitale, which is mainly a maize growing region, lies 100 miles north-east of Lake Victoria to the east of Mount Elgon. Marsabit is a rather poorer township than Kericho or Kitale lying 100 miles east of the southern tip of Lake Rudolf, on a partly forested plateau about 3500 ft. above sea level and set 1000 ft. above the surrounding desert. At the time of the study there had been very little rainfall for over a year and dust storms were common. Many of the children tested at Marsabit came from the surrounding desert regions. Lodwar lies 30 miles west of Lake Rudolf at 1500 ft. above sea level

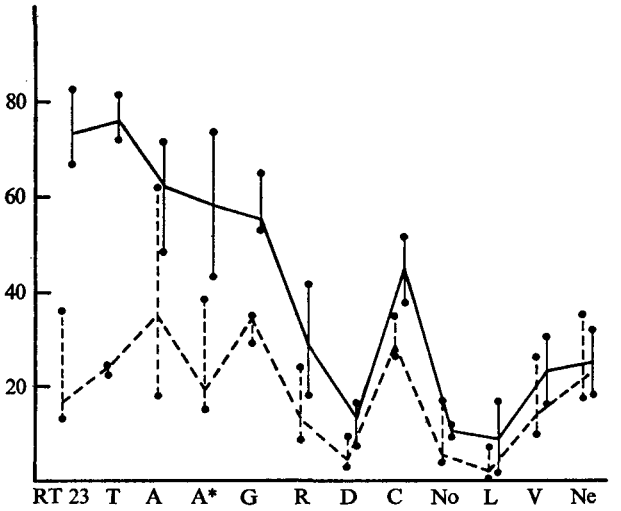

Fig. 4. Diagram showing the percentages of positive reactors (5 mm. or more) to each of the skin test reagents for children from all centres, grouped accordingly to their BCG status. The solid line shows the results for those immunized with BCG and the broken line shows the results for those who have not received BCG. The vertical lines link the results for children aged 6–10 years (lower end) and aged 11+ (upper end). The skin test reagents should be identified from Table 1.

in a desert region on the banks of the dried up river Turkwel. The people are herdsmen living in close association with their goats and chickens in much poorer conditions than those found at the other townships.

Thus the results shown in Fig. 3 for Kericho, Kitale and Marsabit may indicate differing contact with mycobacterial strains associated with the inanimate environment. The results for Lodwar would be bizarre if one only considered the inanimate surroundings since Lodwar is a very arid and hot place. In fact the low results for Gordonin and Neoaurumin reflect these arid conditions and the high results for Aviumin and some fast growers probably reflect poor sanitary conditions and close contact with animals. Rather similar results were obtained at the township of Amudat in Karamoja, Uganda, where the living conditions approximate to those of Lodwar.

As well as geographical conditions, age, BCG immunization and socioeconomic conditions are major factors affecting the results. Increasing age (Fig. 4) has a particularly large effect on delayed hypersensitivity to Aviumin, presumably due to increased contact with *M. avium* either in the school or home environment. This increase between primary and secondary schoolchildren has been found previously in studies using 'avian' P.P.D. in Britain and the United States. (British Tuberculosis Association, 1965; Bleiker, 1968; Stewart, Dixon & Curtis, 1970.)

Returning to the present study, increasing age also greatly increased the percentage of reactors to P.P.D. (TR 23) and A*-in, but had much less effect on sensitivity to our Tuberculin or Gordonin. Increase in age considerably increased sensitivity to most of the fast growers.

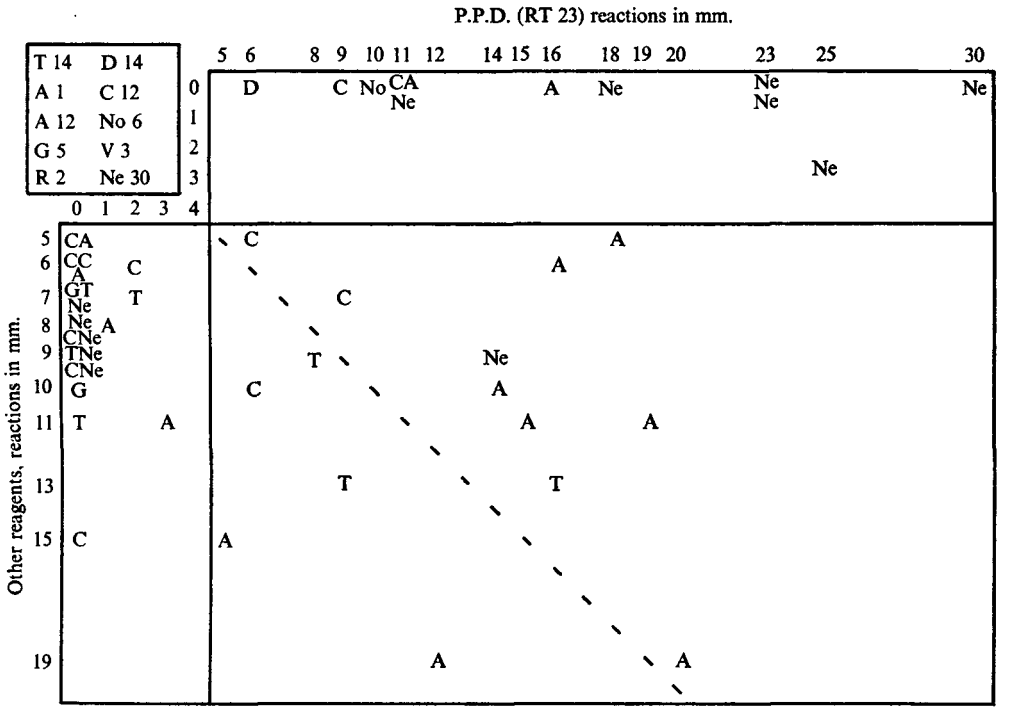

Fig. 5. Scatter diagram showing the distribution of positive reactions of non-BCG immunized children who received P.P.D. (RT 23) as one of their skin tests. Numbers of negative reactions to both tests are shown in the box at the upper left corner of the diagram.

As expected BCG immunization had its greatest effect (Fig. 4) on delayed hypersensitivity to P.P.D. (RT 23) and our Tuberculin (Fig. 2), increasing the percentage of positive reactors by 54 and 50 % respectively. Its next most marked effect was on the other slow growers, A*-in 37 %, Aviumin 32 % (Fig. 2) and Gordonin 25 %. The effect of BCG on the percentage of positive reactors to the reagents prepared from fast growing species varied from 16 % for Chelonin (Fig. 2) to 4 % for Neoaurumin. Discussion of the mechanism of these effects of BCG is left for later publication since only a part of the evidence required is presented here.

We have only been able to examine the effects of differing socioeconomic backgrounds at two centres, Kitale where one of the schools was private and Kericho where one school in particular drew its pupils from homes of a higher social status. Although the results are incomplete, the evidence available showed that fewer children aged 6–10 produced positive reactions to A*-in and Vaccin if they came from the better off families, than those from poorer homes. This probably reflects wearing clothes from an early age and other restrictions on contact with the environment (Table 4).

If we are right in considering our Tuberculin to be more specific than P.P.D. (RT 23), one would expect more positive reactors to the latter reagent than to the

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former. In fact the percentages of reactors to these reagents are very similar when the results for all places are pooled (Fig. 1), but there are differences between the centres (Fig. 3). At Kericho, Kitale and Marsabit there were slightly more positive reactors to our Tuberculin (11, 6 and 1% respectively), whereas at Lodwar there were considerably more reactors to P.P.D. (RT 23) than to our Tuberculin (35% more). Fig. 5 is a scatter diagram showing the reactions of non-BCG immunized persons who received P.P.D. (RT 23) as one of their tests. (Results of BCG immunized persons have been omitted because of the varying augmentation effects on reaction to the different reagents.) These results indicate that P.P.D. (RT 23) may cross-react with Aviumin sensitivity. It is interesting that individuals showing this cross-reaction all came from Lodwar. There is no indication of cross-reaction with Gordonin, Ranin or Chelonin in agreement with our previous results of skin testing of cattle (Pritchard *et al.* 1974), or of cross-reaction with any of the other reagents. *Mycobacterium avium* is known to be one of the most variable of mycobacterial species, and it seems possible that the sero-types present at Lodwar, most likely of animal origin, may differ from those at other centres which are probably mainly soil strains.

As in the case of our studies on Leprosy (Paul *et al.* 1975) the identity between *M. engbaekii* (the source of Lactin) and *M. nonchromogenicum* was discovered after the skin tests were performed. Thus a comparison of the results for the two reagents acts as an interesting and unexpected control. Considering that the two strains used to produce the reagents belong to different variants within *M. nonchromogenicum*, the results are remarkably similar, indirectly validating our results for the other reagents.

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