

The production and preliminary investigation of Burulin, a new skin test reagent for *Mycobacterium ulcerans* infection

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(Received 22 June 1974)

SUMMARY

The preparation of a skin test antigen from *Mycobacterium ulcerans* by ultrasonic disintegration and filtration is described. The reagent, called Burulin, was tested in Africa in normal school children, and in patients with leprosy, tuberculosis or *M. ulcerans* disease. Those with tuberculosis or *M. ulcerans* disease were simultaneously tested with Tuberculin PPD. Burulin was found to be highly specific for patients in the reactive stage of *M. ulcerans* disease, and there was no cross-reaction in patients with other mycobacterioses. On the other hand, the majority of patients with *M. ulcerans* disease reacting to Burulin also produce positive reactions to Tuberculin PPD.

INTRODUCTION

The disease caused by *Mycobacterium ulcerans* occurs mainly in tropical countries (Revill, Morrow, Parson & Kiryabwire, 1972). Most of the reported cases come from the endemic areas in Uganda and Zaire. The populations in these areas are also subject to leprosy, tuberculosis and mycobacterial injection abscesses (Vandepitte, Desmyter & Gatti, 1969).

The source of *M. ulcerans* is probably environmental (Uganda Buruli Group, 1971; Barker, 1972) and the disease is thought to occur at the site of inoculation. There is an incubation period of about 2 months. The initial lesion is a bland, acellular necrosis of the subcutaneous tissue which is associated with a negative tuberculin reaction (Uganda Buruli Group, 1969). Later a specific cellular response develops and this is accompanied by conversion to tuberculin positivity. The duration of the initial non-reactive phase is variable and ulceration may occur in either stage. Occasionally a lesion may ulcerate and heal in the non-reactive phase (personal observation W. D. L. R.). Subclinical infection with *M. ulcerans* probably occurs.

The diagnosis is made on clinical grounds, histopathological appearance and isolation of the organism. Whilst clinical and histological diagnosis can be made with a high degree of confidence in early lesions, both become less reliable with older lesions.

A specific skin-test reagent would be of value in the investigation of infection

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with *M. ulcerans*. It might be useful in the diagnosis of late and healed cases and in determining the progress and planning the treatment of individual patients. By its use additional information might be obtained about the prevalence of sub-clinical infection and its effect on the development of tuberculin positivity in a population.

Because of the very poor growth of *M. ulcerans* in liquid media and on simple solid media, the production of an Old Tuberculin or a PPD has not been possible. This paper describes the production and investigation of a skin test antigen prepared by methods based on those used previously (Stanford, 1973) in the preparation of antigen for stimulating antibody production in rabbits.

MATERIALS AND METHODS

Production of antigen

Strains of *Mycobacterium ulcerans* known to grow well on Löwenstein-Jensen medium at 32° C. were selected as producer strains. For the first batch of reagent two strains (nos. 297 and 298, originally called *M. buruli*) isolated from cases of active disease in Uganda were used, and for subsequent batches one of these (no. 297) was replaced by a strain from Zaire (no. 408). Each of the strains had been isolated 2 or more years before this study and had been maintained on Löwenstein-Jensen medium by subculturing every few months. All three strains were known to be antigenically typical of the species (Stanford, 1973).

To produce a batch of reagent each strain was heavily inoculated on eight large slopes each with a surface area of approximately 35 cm.² of Löwenstein-Jensen medium and incubated at 32° C. until good growth was obtained. This took from 5 to 8 weeks. Organisms were harvested by scraping them gently from the surface of the medium and suspending them in M/15 phosphate buffer (pH 6·8). After washing several times the organisms were suspended in 15 ml. of buffer to which was added an equal volume of Whitemor oil. This mixture was violently shaken by hand to produce a partial emulsion and left at 4° C. overnight to separate. In this process almost all the mycobacteria pass into the oily phase (Mudd, 1925), leaving the remaining particles of egg medium in the aqueous phase to be discarded. The oily suspension of organisms was washed 3 or 4 times with distilled water and then carefully separated by pipetting the oil layer into a 40 ml. glass bottle containing 15 ml. of heptane. The thinned suspension was then centrifuged for 30 min. at 2500 rev./min. and most of the supernatant oil was discarded. The deposit was washed once with heptane, twice with acetone, twice with distilled water and then the organisms were suspended in 25 ml. of phosphate buffer. The clean suspension was placed in a 50 ml. water-jacketed treatment vessel and subjected to ultrasonic disintegration with an amplitude of 8–10 μm . for 15 min. in an M.S.E. 100 W. ultrasonic disintegrator with a probe of 2 cm. diameter.

The resultant mixture of residual whole organisms, broken cell walls and cytoplasm was then centrifuged at 10,000 rev./min. for 30 min. to remove most of the particles. The supernatant was filtered serially through one 0·8 μm ., one 0·45 μm . and two 0·22 μm . sterile membrane filters and a small sample was removed for

protein estimation. The centrifuged deposit was used as a control by ultrasonication a second time in 2 ml. of buffer to produce antigen for analysis by immunodiffusion (Stanford, 1973).

An ultraviolet spectroscopic method was used for protein estimation without correction for nucleic acid content; bovine serum albumin standard solutions were used. Readings were taken at 215 and 225 nm. for both test samples and albumin controls. The concentration of protein was calculated as follows:

$$E_{215} - E_{225} \frac{\text{test}}{\text{control}} \text{ mg./ml.}$$

The reagent was then diluted to suitable test concentrations with the following diluent: phenol, 5 g.; sodium chloride, 5 g.; potassium di-hydrogen phosphate, 1.81 g.; di-sodium hydrogen phosphate dihydrate, 3.56 g.; glycerol, 80 ml.; distilled water, to 1 l.

Four separate batches of the reagent referred to as 'Burulin' were prepared. The first batch was small and was used to investigate the feasibility of producing subsequent larger batches for more extensive studies in the field.

Preliminary assay of batches

Batch I

This small batch was prepared at an estimated protein concentration of 500 $\mu\text{g./ml.}$ It was dispensed in 0.5 ml. quantities in heat-sealed glass ampoules. A small number of volunteers (including some of the authors) from the laboratory staff who were known to have varying degrees of tuberculin positivity were tested by the Mantoux method, and all were negative. The remainder of the batch was tested on patients with *M. ulcerans* disease in Uganda where it was found that a dose of 2 $\mu\text{g.}$ (i.e. 0.1 ml. of a 1/25 dilution) protein produced an area of induration similar to that produced by 0.1 $\mu\text{g.}$ Tuberculin PPD (Merk, Sharp and Dohme) in tuberculin-positive patients. This dose of Burulin failed to produce any response in 34 patients with tuberculosis.

Batches II, III and IV

These three larger batches were prepared at an estimated protein concentration of 20 $\mu\text{g./ml.}$ as a result of the observations made with batch I. Batch II was dispensed in 0.2 ml. quantities and batch III in 1 ml. quantities in sealed glass ampoules. Batch IV was dispensed in 1 ml. quantities in multidose vials.

Each of the three batches was pre-tested both in volunteer laboratory staff, including some tested with batch I, and also in small groups of Ugandan patients with *M. ulcerans* disease. No reactions occurred in the laboratory staff. In patients, each new batch of reagent was compared with the preceding one by testing each simultaneously in the same patient. Batches I, II and III produced similar reactions but batch IV produced larger reactions with surrounding oedema and required a further tenfold dilution. Subsequent tests in Uganda were performed with diluted batch IV Burulin, but all the tests performed in Zaire used batch IV at its original concentration.

Table 1. Showing groups of persons receiving various batches and doses of Burulin

	Burulin reagent		
	Batch I, II, III, dose 2 μ g.	Batch IV, dose 2 μ g.	Batch IV, dose 0.2 μ g.
Normal schoolchildren	—	589 Zairean (Fig. 1)	132 Ugandan (Fig. 1)
Leprosy patients	—	50 Zairean (Fig. 2)	40 Ugandan (Fig. 2)
Tuberculosis patients	34 Ugandan (Fig. 3)	—	40 Ugandan (Fig. 4)
<i>M. ulcerans</i> patients	190 Ugandan (Figs. 6-8)	25 Ugandan and Zairean (Fig. 5)	15 Ugandan (Fig. 5)

Skin testing in the field

Whichever reagent was used 0.1 ml. was injected intradermally on the flexor aspect of the forearm. The exact site was recorded so that any subsequent testing could be done at different sites. Reactions were read 72 hr. after injection by measuring the transverse diameter of induration or by taking the mean of the transverse and longitudinal diameters, depending on the reader.

Ugandan patients with tuberculosis, leprosy or *M. ulcerans* infection were tested at the same time with both Burulin and Tuberculin PPD (0.1 μ g. Merck, Sharp and Dohme PPD in the earlier studies and 0.02 μ g. RT23 PPD (with Tween 80) in later studies). A group of Ugandan schoolchildren and all persons tested in Zaire received Burulin alone.

The Ugandan schoolchildren came from an area of North East Busogo where *M. ulcerans* infection has not been recorded. The Zairean schoolchildren came from three villages in Bas Zaire, in one of which *M. ulcerans* infection has been endemic for some years (Anderson, 1965). However, since the distribution of reaction sizes was the same for all three villages they are treated here as a single group. The ages of the children in both countries varied from 5 to 13 years.

Table 1 shows the number of persons in each group subdivided according to batch and dose of Burulin.

RESULTS

Skin-test results are illustrated as graphs in Figs. 1-8. The cumulative percentage of reactors is recorded on the ordinate and the diameter of induration on the abscissa. Except in Fig. 7, initial tests only are included. Where results of Burulin and tuberculin are compared (Figs. 3, 4 and 6) the tests were done simultaneously.

Normal schoolchildren

Fig. 1 shows the results of Burulin tests on (a) Ugandan children with 0.2 μ g. batch IV and (b) Zairean children with 2 μ g. batch IV. Only 2 out of 132 Ugandan

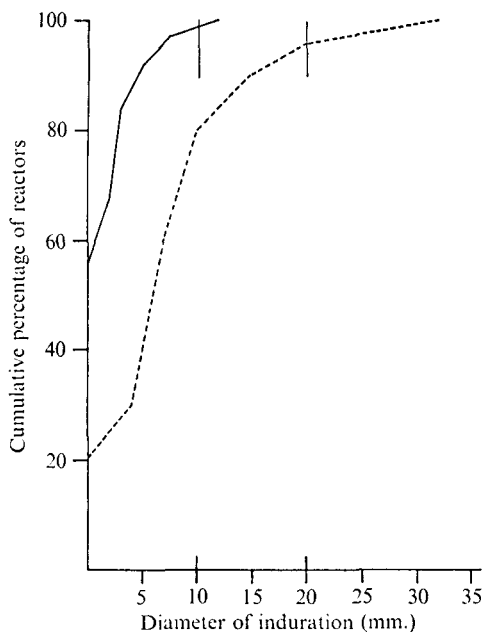


Fig. 1

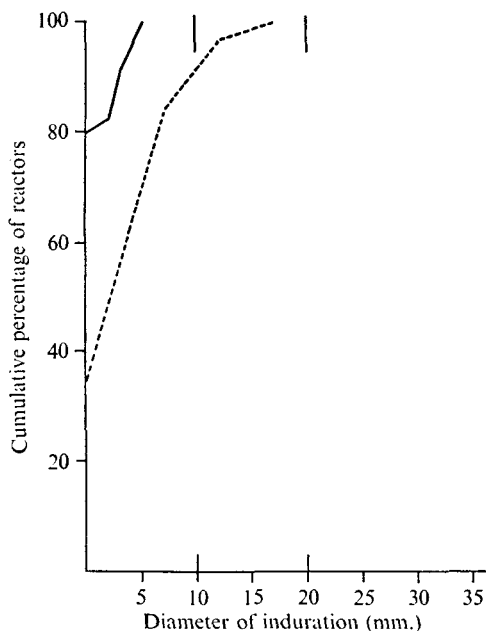


Fig. 2

Fig. 1. Graph of the results obtained by testing two groups of schoolchildren with different concentrations of Burulin. The solid line represents 132 Ugandan children tested with 0.2 $\mu\text{g.}$ of batch IV, and the broken line 589 Zairean children tested with 2 $\mu\text{g.}$ of batch IV. The ordinate is the cumulative percentage of persons producing the mean diameters of induration shown in mm. on the abscissa. The two short vertical lines cutting the curves indicate reactions of 10 and 20 mm. which are the minimum reactions taken as positive for the two reagent concentrations.

Fig. 2. Graph of the results obtained by testing two groups of leprosy patients with different concentrations of Burulin. The solid line represents 40 Ugandan patients tested with 0.2 $\mu\text{g.}$ batch IV, and the broken line 50 Zairean patients tested with 2 $\mu\text{g.}$ batch IV. Other details are as described in the legend to Fig. 1.

children, tested with the lower concentration, produced reactions of 10 mm. diameter or more, and 56% of these children showed no reaction at all. Twenty-three (i.e. 4%) of the 589 Zairean children produced reactions of 20 mm. and 20% of them failed to react at all.

Leprosy patients

Fig. 2 shows the results of Burulin tests on both Ugandan and Zairean patients. The same reagents were used as with the normal schoolchildren. Of the 40 Ugandan patients tested with the lower concentration, 80% failed to respond at all and none produced reactions exceeding 5 mm. With the higher concentration used in Zaire over 30% of the 50 patients failed to react at all and none gave a reaction as great as 20 mm. The type of leprosy was recorded for both groups of patients, but there was no significant difference in Burulin reactivity between lepromatous and tuberculoid patients. It is interesting to note that both groups of leprosy patients react less strongly to Burulin than do the normal schoolchildren. (The readers were the same.)

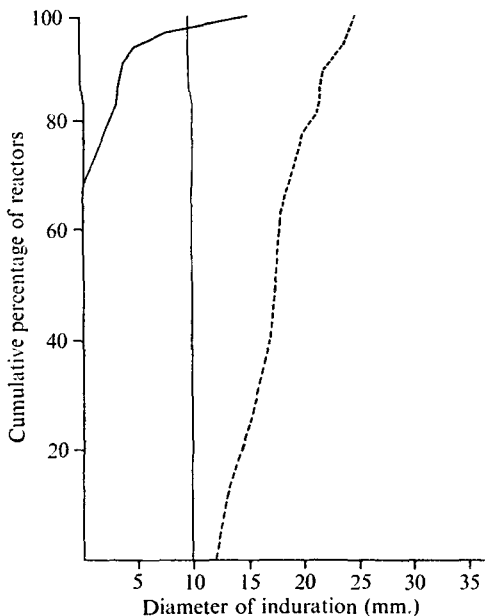


Fig. 3

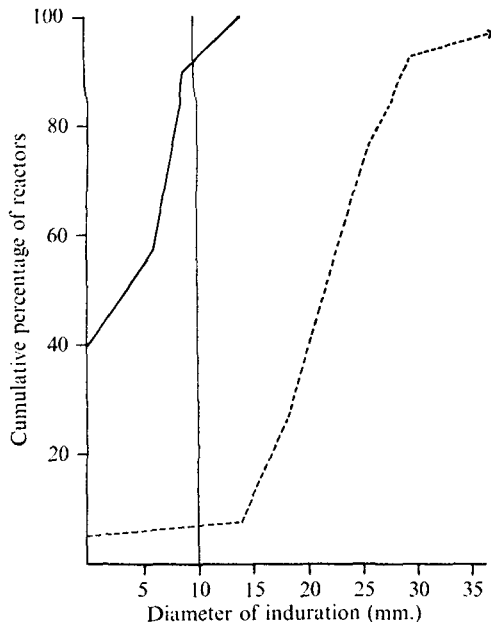


Fig. 4

Fig. 3. Graph of the results obtained by testing 34 tuberculosis patients in Uganda with 2 μg . Burulin batches I and II (solid line) and 0.1 μg . Tuberculin PPD, Merck, Sharp and Dohme (broken line). The ordinate is the cumulative percentage of persons producing the transverse diameters of induration shown in mm. on the abscissa. Results of 10 mm. or more are taken as positive for both Burulin and tuberculin.

Fig. 4. Graph of the results obtained by testing 40 cases of tuberculosis in Uganda with 0.2 μg . Burulin batch IV (solid line) and 0.02 μg . Tuberculin PPD RT23 (broken line). Further details are as described in the legend to Fig. 3 except that the abscissa is of mean diameters of induration.

Tuberculosis patients

Fig. 3 compares the results of tests with 2 μg . Burulin batch I with 0.1 μg . Tuberculin PPD (Merck, Sharp and Dohme) in 34 Ugandans with active pulmonary tuberculosis. Though all the patients had reactions of 12 mm. or greater to Tuberculin, only one had a reaction greater than 10 mm. to Burulin. The exceptional patient came from an area where *M. ulcerans* disease was endemic.

Fig. 4 shows the results in 40 other Ugandan patients with tuberculosis tested with 0.2 μg . Burulin batch IV and with 2 i.u. of Tuberculin PPD batch RT23. (The reader was also different.) Whilst reactions to both reagents tended to be slightly greater than those shown in Fig. 3, over 90% of Burulin reactions were less than 10 mm.

Patients with Mycobacterium ulcerans infection

Fig. 5 shows the results of Burulin tests on Ugandan and Zairean patients with *M. ulcerans* infection. The two concentrations of batch IV are compared. Of those receiving the lower concentration of Burulin (15 Ugandan patients), 80% produced reactions of 10 mm. or more and none failed to react entirely. Of the 25 Ugandan

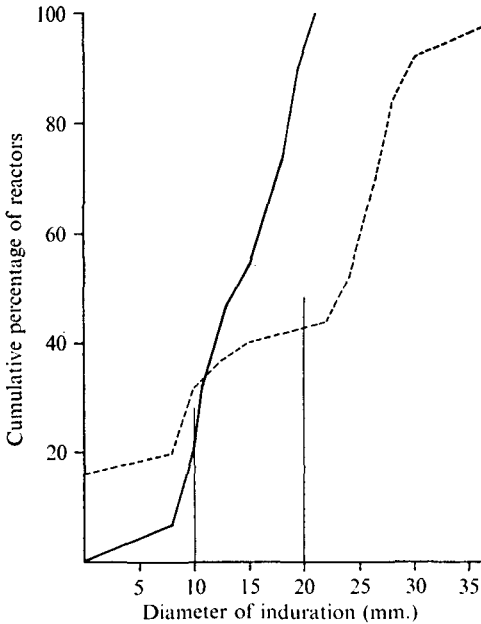


Fig. 5

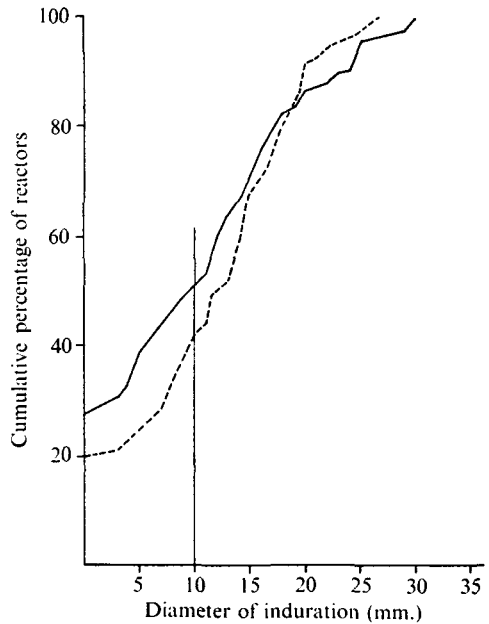


Fig. 6

Fig. 5. Graph of the results obtained by testing two groups of patients with *M. ulcerans* disease with different concentrations of Burulin. The solid line represents 15 Ugandan patients tested with 0.2 μg . batch IV, and the broken line 25 patients from both Uganda and Zaire tested with 2 μg . batch IV. Reactions in Ugandan patients were recorded as transverse diameters and in Zairean patients as mean diameters. Other details are as described in the legend to Fig. 1.

Fig. 6. Graph of the results obtained by testing 190 patients with *M. ulcerans* disease in Uganda with 2 μg . Burulin batches II and III (solid line) and with 0.02 μg . Tuberculin PPD RT23 (broken line). Other details are as described in the legend to Fig. 3.

and Zairean patients receiving the high concentration 14 (56%) produced reactions of 20 mm. or more and 4 (16%) did not react at all.

Fig. 6 shows the results of initial tests with 2 μg . Burulin batches I, II, III and with Tuberculin PPD in 190 Ugandan patients with *M. ulcerans* disease. The distribution of the size of reaction to Burulin closely follows that to tuberculin. Half of the patients had Burulin reactions of 10 mm. or greater. Fig. 7 shows the results of retesting weeks or months later 45 of these patients who had Burulin reactions less than 10 mm. Thirty-four (76%) later produced reactions greater than 10 mm. and seven others (16%) produced some increased response. Fig. 8 shows a dissociation of the Burulin results between those patients whose tuberculin reactions were less than 10 mm. (73 patients) and those with tuberculin reactions of 10 mm. or greater (117 patients). Of the former 90% had Burulin reactions less than 10 mm. whereas 65% of the latter had Burulin reactions of 10 mm. or greater.

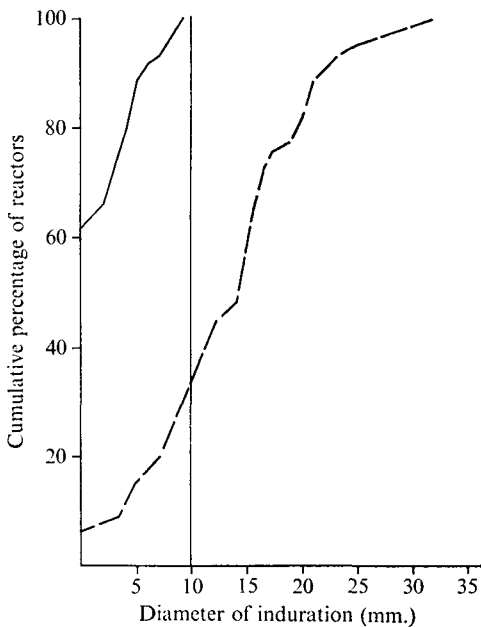


Fig. 7

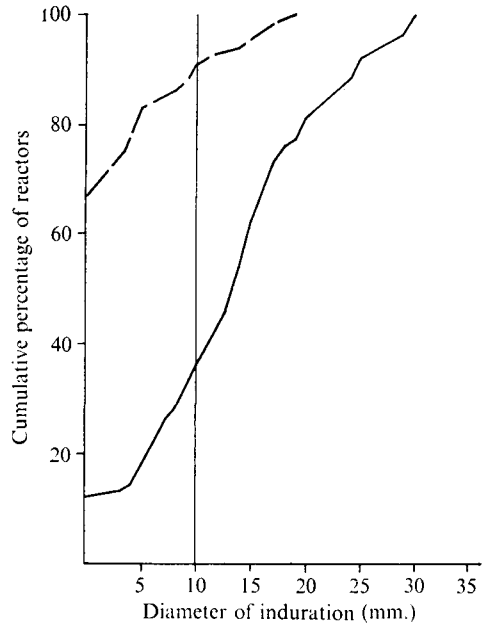


Fig. 8

Fig. 7. Graph showing the results of initial Burulin tests (solid line) in 45 Ugandan patients with *M. ulcerans* disease categorized as Burulin-negative (diameter of reaction less than 10 mm.), and the results of retesting (broken line) weeks or months later. For those patients tested more than twice the most recent result is incorporated in the retest curve. Other details are as described in the legend to Fig. 3.

Fig. 8. Graph showing the Burulin test results on Ugandan patients with *M. ulcerans* disease categorized according to their reactions to Tuberculin PPD RT23 (a reaction of 10 mm. or more was taken as positive). The broken line represents the Burulin test results for 73 tuberculin-negative patients and the solid line those for 117 tuberculin-positive patients.

DISCUSSION

Ever since Koch's description of Old Tuberculin there has been an obvious need for increasingly specific skin-test reagents. The development of purified protein derivatives (PPDs) enabled improved standardization by quantitative analysis of protein rather than the biological method used for Old Tuberculin. Also the assessment of results was simplified because the toxic component of Old Tuberculin was eliminated. However, specificity was not noticeably improved.

PPDs from a range of different mycobacteria have produced interesting results, but these have been difficult to interpret because of high background non-specificity. Despite many attempts to improve specificity and reports of success with a variety of chemical extracts (almost always of *Mycobacterium tuberculosis*) none of them have been fully assessed in man and they have not become generally available.

The present method was adopted because the cultural characters of *M. ulcerans* did not enable an Old Tuberculin or a PPD to be prepared. The major disadvantages of the method are the lengthy process used to free the organisms of traces of egg protein, and the poor method used for protein estimation. Subsequent to the

present study a strain of *M. ulcerans* capable of growing on non-antigenic media has been developed and a much improved method of protein assay has been adopted. Although insufficient of the four batches of Burulin described remains for reassessment of protein concentration, comparative studies with later batches of similar reagents suggest that the doses used for the first three batches were nearer to 0.2 μg . than to the estimated 2 μg . Nevertheless, despite standardization difficulties sufficient information has been obtained for preliminary assessment of Burulin.

Of the four batches assessed the first three produced very similar results at the same concentration, but to equal these batch IV required a further tenfold dilution. Even after dilution batch IV tended to produce oedema which made measurement of induration more difficult. The explanation of these differences remains conjectural. All four batches were checked by immunodiffusion analysis during the course of their production and were found to conform as expected. Although difficult to prove retrospectively, there was no apparent contamination of producer cultures. The most likely explanation lies in the method used for protein estimation which failed to allow for the presence of nucleic acids now known to be present in large and variable amounts in our reagents.

For ease of interpretation we selected a minimum diameter of induration which we called positive. Ten mm. combines ease of measurement with lack of severe reaction and makes the reactions directly comparable with those to the Tuberculin PPPDs. With the standard dilution of batches I, II and III and the lower concentration of batch IV, most normal schoolchildren and patients suffering from tuberculosis or leprosy are Burulin-negative. When using the high concentration of batch IV a cut-off point of 20 mm. induration produced similar results.

The results obtained in active tuberculosis (Figs. 3, 4) indicate that there is virtually no cross-reactivity between Burulin and RT23 PPD in this disease (Figs. 1, 4). With the same material (batch IV, 0.2 μg . dose) patients with leprosy in Uganda showed responses depressed even below those of the schoolchildren (Fig. 2). With the tenfold higher concentration of the same batch of Burulin leprosy patients in Zaire also showed depressed responses in comparison with the Zairean schoolchildren (Figs. 1, 2).

The results obtained from 190 patients with *M. ulcerans* disease who were simultaneously tested with both Burulin and Tuberculin PPD batch RT23 show cross-reactivity between the reagents. Fig. 8 shows the distribution of Burulin reactions for positive and negative reactors to RT23 PPD as separate lines. This form of presentation has been used rather than the conventional scatter diagram because an immunosuppressive effect possibly complicates the picture. It can be seen that there is a close correlation between negative reactors to both reagents and between positive reactors to both reagents. These results are in contrast to those obtained in the tuberculosis and leprosy patients.

The three groups of patients with *M. ulcerans* disease (Figs. 5, 6) varied in percentage of positive reactors to Burulin from 50 to 80 %, depending on the numbers of early cases included in the groups. Unlike tuberculosis in which the great majority of patients are tuberculin-positive on presentation, patients with *M. ulcerans* infection often develop demonstrable delayed hypersensitivity late in

the disease. Despite this delay we believe that the majority of patients eventually become skin-test positive and in a number of cases we were able to demonstrate this (Fig. 7). Other studies (unpublished observations) indicate that in many cases of *M. ulcerans* disease humoral antibodies become detectable at about the same time that Burulin and Tuberculin reactions become positive.

In conclusion a skin test antigen has been prepared which has proved to be of high specificity for *M. ulcerans* disease.

We would like to thank Dr W. M. Meyers of Kivuvu Leprosarium, Zaire, for his assistance to two of the authors while they were in that country and for permission to carry out tests on his patients. In Uganda Drs A. Patel and W. Blenska were kind enough to help us in testing Burulin on their patients with tuberculosis or leprosy, and we are also grateful to the Chief Medical Officer of the Uganda Ministry of Health. This study was financially supported by the National Fund for Research into Crippling Diseases and the Wellcome Trust, to whom we are very grateful.

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