SHORT ARTICLES

AN IMMUNODIFFUSION ANALYSIS OF STRAINS OF *MYCOBACTERIUM ULCERANS* ISOLATED IN AUSTRALIA, MALAYA, MEXICO, UGANDA AND ZAIRE

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STRAINS of a slow-growing psychrophilic *Mycobacterium* have been isolated from chronic cutaneous, frequently ulcerated lesions in people living in Australia (MacCallum *et al.*, 1948), Malaya (Pettit, Marchette and Rees, 1966), Mexico (Aguilar, Iturribarria and Middlebrook, 1953), New Guinea (Read, 1967), Zaire (Van Oye and Ballion, 1950) and several other central and west African states. The first strains isolated in Australia were thoroughly investigated, recognised as a new species and named *Mycobacterium ulcerans* by MacCallum *et al.* in 1948. Subsequently, strains isolated from patients living in Buruli country of Uganda were considered to differ sufficiently from *M. ulcerans* to merit separate species status and were called *M. buruli* Clancey (1964). Strains isolated at Kasongo in Zaire were referred to as *M. kasongo* by Gatti *et al.* in 1965, although they did not intend to propose it as a new species. In general, most authors have considered their own strains to be *M. ulcerans* wherever they were isolated, but no comparative study of strains from a number of countries in which the disease occurs has been made. The chief difficulty in undertaking a taxonomic study of these organisms is their plethora of negative reactions in the majority of tests that are applied to slow-growing mycobacteria. For the present study, immunodiffusion analysis, a method of high species-differentiative value amongst mycobacteria, has alone been used.

MATERIALS AND METHODS

The strains. There were four strains from Australia, including NCTC no. 10417 (type strain of *Mycobacterium ulcerans*) and NCTC no. 10013; two strains from Malaya; one strain from Mexico; 17 strains from Uganda, including NCTC no. 10445 (type strain of *M. buruli*); and 11 strains from Zaire.

Methods. Antigens were prepared from each strain and antisera were raised to seven of them (NCTC no. 10013 and two other Australian strains; NCTC no. 10445 and another Ugandan strain; two Zairian strains). Two 60-ml bottles each containing 30 ml of Lowenstein-Jensen medium sloped to give a large surface area were inoculated with each strain and incubated at 32°C until good growth was obtained; this time varied from 4 to 8 wk. The organisms were carefully scraped from the medium and suspended in 5 ml of normal saline. These suspensions were then treated for 15 min. in an M.S.E. 100-watt ultrasonic disintegrator set at a peak distance of 8 μ m, and the resulting material was used without further treatment in immunodiffusion tests. Because of the contamination with egg protein from the medium, strains used for raising antisera were treated as follows before ultrasonic disintegration. To each suspension of organisms in saline was added an equal volume of a mixture of 1 part heptane and 1 part Whitemor oil, and this was shaken until an emulsion formed. The emulsions were stood at 4°C overnight to separate and the suspensions of organisms in oil were washed several times in distilled water. The suspensions were centrifuged at 3000 r.p.m. for 20 min. and the supernatant oil was discarded. The deposited organisms were washed twice in acetone, resuspended in 5 ml of normal saline

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and treated in the ultrasonic disintegrator as before. In each case, two rabbits were immunised with intramuscular injections of 0.5 ml of antigen extract emulsified with 0.5 ml of a mixture of 8.5 parts Bayol F and 1.5 parts Arlacel A. Each rabbit received six injections at weekly intervals.

Immunodiffusion tests were carried out as previously described (Stanford and Beck, 1968). Antigens of representative strains from each country were tested with each antiserum and antigens of all strains were tested with the best two antisera (those to NCTC no. 10445 and one of the Zairian strains). Liquid Lowenstein-Jensen medium was used in one of the tests to check that none of the reactions obtained was due to contaminating egg protein. Antigens of a number of other species of mycobacteria were tested with these sera, and representative antigen extracts of the present study strains were tested with antisera to other mycobacterial species available from previous studies.

RESULTS

Except for minor differences in concentration of individual antigens, each antiserum raised to the study strains produced the same pattern of immune precipitates with antigen extracts of those strains. The best two antisera produced a pattern of 12 precipitates with

	Individual antigens							Specific antigens*			
	1	2	3	4	5	7	8				
Mycobacterium ulcerans	■	■	■	■	■	■	■	■	■	■	■
Other slow-growing mycobacteria	■	■	■	■	■	■	■	■	■	■	■
Fast-growing mycobacteria	■	■	■	■	■						

* Numbers not yet assigned

FIGURE.

all 35 extracts. Seven of these precipitates were also formed when the antisera were tested with extracts of *M. avium* A (Stanford and Muser, 1969), *M. kansasii* and *M. nonchromogenicum*, and five of the same precipitates were formed with *M. ranae*, *M. thermoresistibile* and *M. smegmatis*. When representative extracts of the study strains were tested with antisera to *M. avium* A and *M. nonchromogenicum*, seven precipitates formed, whereas these same sera produced 13 and 15 precipitates respectively with extracts of the organisms to which they were raised. When tested with antisera to fast-growing mycobacteria, only four antigens could be demonstrated in extracts of representative study strains. When liquid Lowenstein-Jensen medium was used as antigen with the antisera to the study strains, a single diffuse precipitate formed that was not represented in the pattern formed with extracts of the study strains.

The results are shown in the figure.

DISCUSSION

Methods different from those previously described (Stanford and Beck, 1968; Stanford and Gunthorpe, 1971) had to be employed because the study strains grew very poorly on liquid media and hardly at all on simple media. The use of Mudd's (1925) observation of the lipophilic nature of mycobacteria to purify the organisms was necessary because in initial experiments rabbits died during immunisation from anaphylaxis caused by egg protein.

The results show that the 35 study strains coming from five countries all belong to a single species. By the rule of chronological priority, *Mycobacterium ulcerans* MacCallum, Tolhurst, Buckle and Sissons is the correct name for the species and *M. buruli* Clancey should be considered as a later synonym. The comparative studies with other species of mycobacteria show *M. ulcerans* to conform to the general pattern of slow-growing

mycobacteria. It shares five antigens with both fast- and slow-growing species, two extra antigens with slow-growing species and five antigens are species specific. These results differ from the only previous immunodiffusion study of *M. ulcerans* (Lind and Norlin, 1963), in which a single strain (NCTC no. 7816) was studied and found to be virtually identical with *M. avium*. Subsequent studies on this strain showed it to be *M. avium* of the *intracellulare* subspecies and not representative of *M. ulcerans*. This strain has been withdrawn from the National Collection of Type Cultures.

Earlier studies on Australian strains by means of the complement-fixation test (Fenner and Leach, 1952) established *M. ulcerans* as a species antigenically different from other mycobacteria. The non-serological studies of Pattyn and his colleagues (Pattyn *et al.*, 1964; Pattyn, 1965) identified a number of Zairian strains with *M. ulcerans* from Australia, and showed them to differ from *M. tuberculosis*, *M. avium* and some other named mycobacteria.

SUMMARY

Thirty-five strains of slowly growing mycobacteria received as *Mycobacterium ulcerans* and *M. buruli*, including the type strains, have been subjected to immunodiffusion analysis and compared with other mycobacterial species. The strains come from cases of ulcerative mycobacteriosis occurring in Australia, Malaya, Mexico, Uganda and Zaire. They were all found to belong to a single distinct species for which *M. ulcerans* MacCallum *et al.* is the correct name and *M. buruli* Clancey is a later synonym. The species was found to possess the antigens typical of other slow-growing mycobacteria and to have five antigens specific to itself alone.

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