AN IMMUNODIFFUSION ANALYSIS OF *MYCOBACTERIUM LEPPRAEMURIUM* MARCHOUX AND SOREL

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MYCOBACTERIUM LEPPRAEMURIUM Marchoux and Sorel, was originally described as a non-cultivable acidfast organism responsible for an epizootic among wild rats in Odessa by Stefansky (1903) and later in the same year in Britain (Dean, 1903). This organism is like *M. leprae* Hansen, in its inability to grow in non-living culture systems so far devised. The disease in rodents caused by *M. leppraemurium* is somewhat like lepromatous leprosy in man, but unlike the disease caused by *M. leprae* when injected into mice (Shepard, 1960; Rees, Weddell and Pearson, 1969). Although most cases of *M. leppraemurium* infection have been discovered in rats and mice, occasionally cats have been infected and one possible human infection is recorded (Marchoux, 1922). Over the 70 years since the organism was discovered, numerous attempts have been made to grow it in non-living media and recently some success has been reported (Nakamura, 1972). The lack of cultivability, except in tissue culture (Wallace, Elek and Hanks, 1958; Rees and Wong, 1958), has held up bacteriological study of the species, although much is known about its pathology in rodents. Recently some studies have been made by immunodiffusion of organisms laboriously extracted from heavily infected tissues (Fukui *et al.*, 1966; Kwapinski *et al.*, 1972; Navalkar, 1972). The first of these studies showed *M. leppraemurium* to have a closer antigenic relationship with *M. avium* than with other mycobacteria, but this has not been further elucidated.

In this paper, six strains of *M. leppraemurium* have been studied by immunodiffusion analysis in comparison with each other and with seven different species of culturable mycobacteria.

MATERIALS AND METHODS

Strains. These were received in the form of spleens and livers of rats or mice heavily infected with *Mycobacterium leppraemurium*. All had been maintained for some years by passage from animal to animal. No. 1, the "Douglas" strain, was maintained for many years in rats and mice and was supplied by Dr R. J. W. Rees. No. 2, the "Hawaii" strain (Brinckerhoff, 1910) was maintained in mice and supplied by Dr B. S. Tepper. No. 3 was a strain maintained in rats and supplied by Dr P. C. Wong. No. 4 was a German strain originally isolated from a rat, but maintained in mice and was supplied by Dr R. J. W. Rees. No. 5 was a British strain originally isolated from a cat, but maintained in mice. It was also supplied by Dr R. J. W. Rees, as was no. 6, a Dutch strain isolated from a cat in 1964 and maintained in mice.

Preparation of reagents. The method used was that described by Stanford and Beck (1968) and Stanford and Gunthorpe (1971), with certain modifications. Antigen extracts were

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prepared from each of the strains and antisera were prepared against strains 1 and 3. Approximately 20 g of the heavily infected spleens and livers were homogenised for 3 min. in 100 ml of distilled water by means of an MSE "Atomix". The material obtained was filtered through a piece of muslin before centrifugation for 1 min. at 1000 r.p.m. The cloudy supernatant was then centrifuged for 20 min. at 10,000 r.p.m. and the supernatant discarded. The deposit was washed and resuspended in 10 ml of normal saline. With the exception of half of each of the suspensions prepared from strains 1 and 3, which were retained for the production of antisera, the suspensions were treated for 15 min. in the MSE 100-watt Ultrasonic Disintegrator with the peak distance set between 8 and 10 μ m to release cytoplasmic antigens. The resultant material was used as antigen in immunodiffusion studies. Control antigens from non-infected tissues were prepared by the same method from 20-g amounts of normal mouse livers and spleens and from similar amounts of normal rat livers and spleens.

The suspensions reserved for antiserum production were purified by a method based on the observations of Mudd (1925) that mycobacteria, unless coated with antibody, cross from the aqueous to the oily phase at oil-water interfaces. The suspensions were made up to 10 ml. with normal saline and shaken with 10 ml of an equal mixture of Whitemor oil and heptane (Stanford, 1973). The shaking was repeated several times over the course of a day and the emulsion was left at 4°C overnight to separate. The oily layer was removed and centrifuged for 20 min. at 10,000 r.p.m. to deposit the mycobacteria in the oil. The clear oil was discarded and the deposit washed once in heptane and twice in acetone, before being resuspended in 5 ml of normal saline. This last suspension of bacilli, with almost all the tissue antigen removed, was treated in the ultrasonic disintegrator as described above. Each of the extracts was used for immunisation of two rabbits as previously described.

Tests. The system of immunodiffusion analysis employed has been described by Stanford and Beck. Each of the extracts of the six strains of *M. lepraemurium* was tested with each of the antisera. A mouse-tissue antigen control was included in tests employing the antiserum to strain 1 and a rat tissue antigen was included in tests employing the antiserum to strain 3. Extracts of strains of *M. avium*, *M. chelonae*, *M. marianum*, *M. ranae*, *M. smegmatis*, *M. tuberculosis* and *M. ulcerans* were also tested with the antiserum to strain 1 and extracts of three strains of *M. lepraemurium* were tested with antisera to *M. avium*, *M. ranae* and *M. ulcerans*. The extracts of strains other than of *M. lepraemurium*, and the corresponding antisera, were available from previous studies.

RESULTS

All six extracts of *Mycobacterium lepraemurium* produced the same pattern of precipitates with both of the antisera prepared against members of the same species. The best serum (against strain 1) produced 17 precipitation lines, of which seven were also produced with the normal mouse-tissue control. With this antiserum, four antigens could be demonstrated in extracts of *M. chelonae*, *M. ranae* and *M. smegmatis*, and five in extracts of *M. marianum*, *M. tuberculosis* and *M. ulcerans*. Extracts of both serotypes, A and B, of *M. avium* (Stanford and Muser, 1969) possessed eight antigens that reacted with this antiserum. Five of these eight antigens were the same as those present in the other slow-growing species. Two of the antigens demonstrable with extracts of *M. lepraemurium* were not present in the normal tissue preparation or in extracts of the other mycobacteria.

Extracts of *M. lepraemurium* contained five antigens demonstrable with the antisera to *M. ranae*, *M. tuberculosis*, *M. ulcerans* and *M. avium*, two more with the last three antisera but not with that to *M. ranae*, and three more with the antiserum to *M. avium* alone. The collated results are shown in the histogram (the figure).

DISCUSSION

The results obtained with the extracts of the six strains of *Mycobacterium lepraemurium* and two homologous antisera show the species to be homogeneous. All strains produced the same ten precipitation lines, excluding those also formed with the normal tissue antigens. Further studies with antigens of other species and antisera prepared against them showed *M. lepraemurium* to possess five antigens common to most if not all mycobacteria, two antigens characteristic of slow-growing species, three antigens shared with *M. avium* alone and two antigens specific to itself. *M. lepraemurium* is therefore a slow-growing mycobacterium with especially close antigenic relationships with *M. avium*. The details of this relationship are shown in the histogram. Of the six antigens

FIGURE.—Antigenic relationships between *Mycobacterium lepraemurium*, *M. avium*, and other fast- and slow-growing mycobacteria. Each hatched or dotted area represents possession of an individual antigen, which is shared vertically. Hatched antigens were demonstrable with the two antisera prepared against *M. lepraemurium*; dotted antigens were demonstrable only with antisera prepared against other species.

previously considered specific to *M. avium*, four are present in both serotypes A and B, and two are present in serotype-A strains alone. *M. lepraemurium* shares three of the antigens present in both serotypes A and B. Thus if a system of purely serological classification is used, *M. lepraemurium* would be reduced to a serotype or subspecies of *M. avium*. Whether or not they should be considered serotypes, subspecies or separate species, there can be little doubt that they share a closer common origin with each other than with other mycobacteria.

M. avium is one of the least homogeneous of mycobacterial species and covers a wide spectrum of phenotypic, pathogenic and serological activity. Classical strains are highly pathogenic for birds and rabbits, grow well at temperatures up to 45°C, and are found in the wild as pathogens of birds and pigs, and rarely of cattle, sheep and man. Such strains are of immunodiffusion type A and of agglutination types 1, 2 and 3. Strains at the other end of the spectrum known as "battey" bacilli or *M. intracellulare* are non-pathogenic for birds and rabbits, fail to grow at 45°C, and are found in the wild as pathogens of pigs and man, and rarely of cattle. These strains belong to immunodiffusion type B and to any of 20 or more agglutination types. Strains of both types have been isolated from soil, and are therefore considered as opportunist pathogens. That a species showing such diversity should have a non-cultivable variant is perhaps hardly

surprising, although the nature of such a variant would make it an obligatory pathogen.

The basis for the differences between *M. lepraemurium* and the cultivable *M. avium* might be a major irreversible mutation, a very small change such as the deletion or repression of a single gene controlling some vital enzyme only otherwise available in living animal tissues, or even an effect of episome loss or bacteriophage-mediated conversion. It may be impossible to confirm or refute the first possibility, but the others are open to investigation. A final decision concerning the taxonomic status of *M. lepraemurium* must await the outcome of such investigations.

SUMMARY

Six strains of *Mycobacterium lepraemurium* Marchoux and Sorel have been subjected to immunodiffusion analysis. They were found to be identical with each other and to possess 12 demonstrable antigenic constituents. Five of these were antigens common to all mycobacteria, two were shared only with slow-growing mycobacterial species, three were amongst those antigens hitherto considered specific to *M. avium* and two were specific to *M. lepraemurium* alone. Thus it was concluded that *M. lepraemurium* is a member of the slow-growing group of mycobacteria with an exceptionally close serological relationship to *M. avium*.

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