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A Study of Alleged Leprosy Bacillus Strain HI-75[1]

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The recent report of the *in vitro* cultivation of the leprosy bacillus on media enriched with hyaluronic acid ([11]) has attracted considerable interest. In an attempt to establish the identity of the cultured organism, subcultures were sent out to a number of bacteriologists at their request. This paper gives an account of the studies performed on subcultures of strain HI-75. This organism was isolated from a relapsed lepromatous patient of Filipino origin living in Hawaii. The biopsy material, following decontamination with sodium hydroxide, was directly inoculated onto the hyaluronic acid-based medium LA-3 from which the strain was subsequently obtained.

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MATERIALS AND METHODS

Culture 1. Approximately 200 ml of a liquid culture consisting of a pool of four subcultures (S 5A/6A/7A/8A, aged 7–37 days at time of posting) was sent to London (J.L.S.) from Honolulu (by O.K.S.).[3] On arrival this material was subcultured onto slopes of Lowenstein-Jensen medium with and without mycobactin, Sautons medium, blood agar, nutrient agar, MacConkey agar, CLED agar, liquid blood culture medium and filtered aspirated joint fluid (thought to be rich in hyaluronic acid). Additionally, 10 ml of the original culture was sterilized by filtration and reinoculated with a loopful of the original culture.

A series of smears of the original culture were made and stained by the Ziehl-Neelsen and auramine methods and by Murohashi's modification of Lack's method. The organisms from a 5 ml sample of the original culture were deposited by centrifugation, negatively stained with 4% ammonium molybdate pH 6.8 and examined with the transmission electron microscope. Other small samples of the culture were sent to R.J.W.R. for mouse inoculation experiments and to S.R.P. for comparison with the culture received in Antwerp.

[3] The request was for at least 0.5 gm bacilli for antigenic analysis, hence the quantity and mixed subcultures.—OKS

The organisms in the remaining 180 ml of original culture were removed by centrifugation, thoroughly washed in phosphate buffer and broken in an ultrasonic disintegrator ([12]).

Part of the ultrasonicate was centrifuged for 30 minutes at 75,000 × g to remove remaining whole cells and much cell wall debris. This deposit was sent to PD for cell wall analysis.

The supernate was filtered to a particle size of less than 0.22 μ and used to prepare a skin test reagent ([8]). This reagent was dispatched to J.W.C. in Uganda for testing of leprosy patients. The remaining ultrasonicate was used to produce an antiserum in a rabbit and for immunodiffusion analysis with a range of antisera to various species of mycobacteria ([12]).

Part of the spent medium of the original culture was concentrated 20 times by pervaporation and examined for antigens specific to *M. leprae* in immunodiffusion analysis with a rabbit antiserum to leprosy bacilli extracted from armadillo tissues ([13]).

Culture 2. Approximately 15 ml of HI-75 subculture S10 (aged 27 days at posting) was sent to Antwerp (S.R.P.) where part of it was immediately used as inoculum for a set of identification tests for slow-growing mycobacteria ([7]). It was also inoculated onto a sample of LA-3 medium sent from Honolulu and onto a locally prepared batch containing half the recommended concentration of hyaluronic acid and solidified with agar. A part of culture 2 was exchanged with London for a part of culture 1 for comparative studies.

Following successful and sustained cultivation of both the above on routine microbiological media, without the addition of hyaluronic acid, the following studies were carried out on the isolated organisms:

a) Electron microscopic examination of negative stained and sectioned organisms.

b) Routine bacteriologic procedures for identification.

c) Drug sensitivity tests to isoniazide (1 μg/ml × 5 μg/ml), dapsone (1 μg/ml) and thiosemicarbazone (25 μg/ml).

d) Agglutination serotyping using Schaefers sera.

e) Preparation of ultrasonicates for comparison with the original culture sent to London, and species identification.

f) Lipid chromatography of hexane-ethanol (50:50) extracts of organisms harvested from Lowenstein-Jensen medium. These extracts were spotted onto thin layer silica gel plates and the run performed using the system of Tsukamura and Mizuno ([14]). Chromatograms were developed using 5% ethanolic molybdophosphotungstic acid ([4]). The extracts were run in parallel with others prepared from strains of Schaefer serotypes, 21, 41, 42 and 43 and with three strains isolated in Britain.

g) Inoculation of mice.

RESULTS

Microscopy. Ziehl-Neelsen stained smears of both original cultures showed some pleomorphism which was more marked in culture 1. When the Murohashi modification of Lack's stain for viability was applied to the latter culture by Dr. E. Nassau, it was found to contain mainly large green (presumed alive) bacilli and a lesser number of small red (presumed dead) bacilli. Ziehl-Neelsen staining of subcultures of both the London and Antwerp cultures on Lowenstein-Jensen medium showed them to be similar organisms with minimal pleomorphism.

Electron microscopic examination with negative staining showed culture 1 (S 5A/6A/7A/8A) to contain two types of organisms, a majority of large bacilli averaging 2.0 × 0.64 μ and a small number of much smaller organisms averaging about 1-2.0 × 0.21 μ. A differential count of organisms present in smears of culture 1 stained by carbol fuchsin showed that there were 95% of the large organisms and 5% of the small organisms.

Culture 2 (S10) contained almost exclusively the large type of mycobacterium and scanty organisms of much larger size and different morphology considered not to be mycobacteria and probably a contaminant which got in during the preparatory stages for electron microscopy. The organisms subcultured twice on Lowenstein-Jensen medium without hyaluronic acid, appeared to be identical with the predominating type in both the London and Antwerp cultures.

Culture. Subcultures of the organisms received in both London and Antwerp grew readily within two or three weeks on every kind of medium inoculated except MacConkey's medium and the solid LA-3 containing half the recommended amount of hyal-

TABLE 1. *Results of biochemical tests performed on organisms growing on routine media.*

	London subculture	Antwerp subculture
Gordon's sugars Gordon's organic acids	none utilized	not done
Amidase spectrum	5.6	3.5.6.
Nitrate reduction	–	–
Niacin reaction	–	–
Phosphatase reaction	not done	–
10 day Arylsulphatase	+	not done
10 day Tween 80 hydrolysis	–	not done
Catalase	+	+

uronic acid prepared in Antwerp. Good growth occurred at 32°C and 37°C, but growth was slow at room temperature and did not take place at 45°C. Second and subsequent subcultures produced good growth after 10-12 days at 32°C. All colonies produced were of a dull yellow color which did not increase in intensity following exposure to light.

Lipid chromatography of organisms grown on Lowenstein-Jensen medium showed them to have similarities with the control strains of *M. marianum,* but they could not be precisely allocated to any of the recognized types.

Drug sensitivity tests showed the organism to be resistant to isoniazide, dapsone and thiosemicarbazone. The results of other biochemical tests on the cultured organisms are shown in Table 1.

Immunodiffusion analysis. With the antiserum raised to the organisms harvested from the original culture sent to London, ultrasonicates of that organism and of the organisms subsequently cultured in both Antwerp and London produced the same pattern of precipitates. Only four of the precipitates formed when this serum was tested with ultrasonicates of leprosy bacilli extracted from armadillo tissues. Similarly, when tested with an antiserum to armadillo-derived *M. leprae,* only the common mycobacterial antigens could be demonstrated in ultrasonicates of the London and Antwerp organisms. This was also the case when these ultrasonicates were tested with antisera to *M. avium* and *M. gordonae.* However, there appeared to be a closer relationship with *M. marianum* (syn. *scrofulaceum*), antisera to which demonstrated the presence of some *M. marianum* specific antigens as well as the common mycobacterial antigens in the London and Antwerp organisms. When ultrasonic lysates of stock strains of *M. marianum* were tested with the antiserum to HI-75, more precipitates formed than was the case with lysates of other mycobacterial species. However, the known strains of *M. marianum* contained one less than the full complement of antigens demonstrable in the homologous reaction. The concentrated filtrate of culture 1 contained fewer mycobacterial antigens than the ultrasonicate.

Agglutination typing. The organism cultured in Antwerp was not agglutinated by antisera to the scotochromogenic strains tested, but it was agglutinated by antisera to Schaefer's types Howell and avium 7.

Skin testing of leprosy patients. Only 3 out of 69 patients tested produced positive (mean diameter of induration of 5 mm or more at 72 hours) reactions to the skin test reagent (Table 2).

Mouse foot pad infection. A sample of culture HI-75 as received in London was quantitated for acid-fast bacilli diluted in 1% albumin-saline and inoculated at a concentration of 1.0×10^4 into each of both hind foot pads of female CBA-strain mice, using the standard bacteriologic and histologic assessments for characterizing *M. leprae* by technics previously published [9, 15].

Mice were killed 1, 5.5 and 14 months after inoculation, one foot pad from each mouse was homogenized for assessing bacterial growth and the other fixed for histologic assessment. At one month there was

TABLE 2. *Skin test results.*

mm induration	Categories of leprosy patients tested			
	Lepromatous	Borderline (BB + BL)	Borderline (BT)	Tuberculoid
0	27	3	4	24
2	1	1		1
3	1			1
4	1	1		1
5	1		1	
6	1			
Nos. positive	2/32	0/5	1/5	0/27

already a fivefold increase in acid-fast bacilli, the organisms being then confined within macrophages and the morphology of all these bacteria was identical with the larger forms present in the original inoculum. At 5.5 months, when live inocula of *M. leprae* would have yielded approximately 1.0×10^6 acid-fast bacilli, culture HI-75 yielded only 2.7×10^5 of the same larger forms which by now were grossly degenerate. At 14 months the yield of AFB was similar or less than at 5.5 months. The histologic picture at 5.5 and 14 months showed the bacilli still confined to macrophages, none being present within endothelial lining cells of vessels, within striated muscle fibers or within or associated with neural tissues. Moreover, all the bacilli appeared degenerate and were of the larger form seen at one month. Therefore, none of the features produced in the foot pads of mice inoculated with culture HI-75 resembled those obtained with *M. leprae*.

This study is continuing and further mice will be killed every few months until two years after infection.

Cell wall chemistry. Bacterial cell walls prepared from culture 1 were separated from non-wall particles in a sucrose density gradient. Published technics for solvent extraction, hydrolysis and chromatography were used ([3]). Lipid extracted by chloroform-methanol ("unbound lipid") consisted mainly of triglyceride: no glycolipid or peptidoglycolipid was present. This makes it unlikely that the organisms belonged to the "avium-intracellulare" group of mycobacteria.

Three types of mycolic acid could be separated by thin-layer chromatography, using the technic of Minnikin, Patel and Goodfellow ([6]) as modified by Mr. L. A. Davidson (personal communication). The pattern of mobilities was similar to the mycolic acids of *M. avium* and *M. vaccae* and unlike that of *M. tuberculosis*. *M. leprae* from armadillos yields only two mycolic acids by this technic, one of which has a mobility different from any of the compounds from HI-75.

The walls contained arabinose and galactose in the ratio 2.6:1, with only small amounts of other sugars. Arabinogalactan is a characteristic component of the mycobacterium-nocardia-corynebacterium group of organisms ([1,5]).

Amino acid analysis showed that, unfortunately, the walls contained a large amount of non-cell wall amino acids, probably originating from unbroken bacteria. Diaminopimelic acid, muramic acid and glucosamine were present. There was much less glycine than either alanine or glutamic acid; whereas *M. leprae* from armadillos contains glycine as a major component of the wall ([2]).

These results show that the cell wall of HI-75 is unlike that of *M. leprae* extracted from armadillo tissues in every way.

DISCUSSION

We have been unable to find any evidence for the presence of *M. leprae* in culture HI-75 with the possible exception of the observation of numbers of an organism of a similar size to *M. leprae* in negatively stained smears of the culture received in London. However, these were not present in the later subculture received in Antwerp. Examination of organisms taken from the original

culture received in London failed to demonstrate any *M. leprae* specific antigens either by immunodiffusion analysis or by skin testing of leprosy patients. Mice inoculated with the material have failed to demonstrate any of the growth or histologic characteristics of *M. leprae* infection after 1, 5.5 or 14 months of observation. The cell wall of the cultured organism differed considerably from that of the leprosy bacillus.

The bacteriologic results indicate that the readily culturable organism present is *M. marianum* (syn. *scrofulaceum*), although it is not of the type commonly encountered in Britain or Belgium. It has a different lipid chromatography pattern and is of different immunodiffusion serotype from the collection of strains with which it was compared. It agglutinates with antisera to the avium/intracellulare types 7 and Howell, but not with antisera to Schaefer's types of *M. marianum*.

The 4% (3/69) of Ugandan leprosy patients responding positively to the skin test reagent prepared from culture HI-75 compares favorably with a small pilot study among normal Ugandan adults, 3% of whom produced positive reactions to Marianin ([10]).

There are several conceivable sources for the cultured organism; it might be a modified form of the leprosy bacillus itself as suggested by Kato (widely circulated communication), it might have been present with the leprosy bacillus in the tissues of the patient from whom the culture was made, or it might be a laboratory contaminant. It cannot be the first of these since the organism predominating in culture, and exclusively present in culture 2, differs from the leprosy bacillus in every way other than being a *Mycobacterium*. It might be the second since numerous species of culturable mycobacteria have been recovered from leprous and other tissues over the last 100 years, including *M. marianum*. A laboratory contaminant is, however, the most likely explanation since, in common with many mycobacterial species, *M. marianum* is ubiquitous in the environment. The isolation of the same unusual variant in both Antwerp and London indicates that the probable contamination occurred before the dispatch of the cultures from Honolulu.

Despite our present findings, the presence of numbers of an organism with dimensions similar to those of *M. leprae* in subcultures S 5A/6A/7A/8A which was not seen in S10 is interesting. It suggests either that a second mycobacterium (possibly *M. leprae*) present in larger numbers in earlier subcultures has been overgrown by *M. marianum* in the later subcultures, or that it is a remnant of a very heavy initial inoculum. The latter would seem unlikely since the earliest subculture studied was S5A-S8A mixed. Similar studies on different strains isolated by the Skinsnes system are urgently required to confirm or refute the successful cultivation of the leprosy bacillus.

SUMMARY

Subcultures of strain HI-75 of Skinsnes' leprosy bacillus received in Antwerp and London have been studied bacteriologically and compared. Both contained moderately large acid-fast bacilli readily subcultured and maintained on ordinary mycobacteriologic media. These organisms were found to be a variety of *Mycobacterium marianum* (syn. *scrofulaceum*) and were considered likely to be a laboratory contaminant. The earlier subculture studied also contained numbers of a much smaller mycobacterium (of a similar size to *M. leprae*) which appeared to be dead and which did not grow on the ordinary media. Skin tests and immunodiffusion analyses performed with extracts of the earlier subculture failed to demonstrate the presence of the specific antigens of leprosy bacilli. Similar studies on other cultures of Skinsnes' bacillus must be performed to confirm or refute its identity as *M. leprae*.

RESUMEN

Se realizaron estudios bacteriológicos comparativos con los subcultivos de la cepa HI-75 del bacilo de la lepra de Skinsnes recibidos en Antwerp y en Londres. Ambos subcultivos contuvieron bacilos resistentes al alcohol ácido de tamaño moderadamente grande que fueron faciles de subcultivar y mantener en medios micobacteriológicos ordinarios. Se encontró que estos organismos son una variedad del *Mycobacterium marianum* (syn. *Scrofulaceum*) y se consideraron como contaminantes de laboratorio. Los primeros subcultivos estudiados también contuvieron una micobacteria mucho más pequeña (del tamaño correcto para el *M. leprae*) la cual no pareció ser viable y no creció en los medios ordinarios. No

se logró demostrar la presencia de antígenos específicos del *M. leprae* cuando los extractos obtenidos de los cultivos más tempranos se probaron en pruebas intradérmicas y en reacciones de precipitación en gel. Deben hacerse más estudios de este tipo con otros cultivos del bacilo de Skinsnes para confirmar o negar su identidad con el *M. leprae.*

RÉSUMÉ

On a procédé à l'étude bactériologique et à la comparaison de souscultures de la souche HI-75 du bacille de la lèpre de Skinsnes, qui ont été reçues à Anvers et à Londres. Dans les deux cas, les échantillons contenaient des bacilles acido-résistants de dimension modérée, qui pouvaient facilement être mis en culture et maintenus sur des milieux ordinaires pour mycobactéries. On a constaté que ces organismes étaient une variété de *Mycobacterium marianum* (syn. *scrofulaceum*), et devaient être considérés comme des contaminants de laboratoire. La première sous-culture étudiée contenait également un certain de nombres de mycobactéries beaucoup plus petites, de la dimension qui répond à *M. leprae,* mais celles-ci apparaissaient mortes et n'ont pu être cultivées sur milieu ordinaire. Les épreuves cutanées, ainsi que des analyses d'immunodiffusion pratiquées sur des extraits de la première sous-culture, n'ont pas permis de mettre en évidence la présence d'antigènes spécifiques pour les bacilles de la lèpre. Des études similaires, menées sur d'autres cultures du bacille de Skinsnes, devraient être conduites afin de confirmer ou de réfuter leur identité avec *M. leprae.*

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