ATYPICAL ACID-ALCOHOL-FAST BACILLI CULTURED FROM HUMAN URINES

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During the past seven years at this hospital the urines of all patients with skeletal tuberculosis have been examined at regular intervals with a view to detecting early renal infection. Our practice is to collect the first specimens passed on three successive mornings in sterile jars. These specimens are centrifuged in sterile tubes and samples of the deposits examined for pus and red cells and for acid-alcohol-fast bacilli. The deposits are stored at -10° C. until three have been examined, then they are pooled, the pool treated by Petroff's method and portions of the pool inoculated on to media and into a guinea-pig.

In the course of this work acid-alcohol bacilli have appeared on culture media which do not appear to be *M. tuberculosis* or any of the well-known saprophytes. These mycobacteria (for we believe them to be so) were not studied in detail until they appeared in a sequence of urines from eight patients during January and February, 1954. This series all appeared to belong to the same species with the exception of two chromogens. In January and February, 1955, the urines of 21 patients, and in October and November, 1955, the urines of 16 patients, were found to contain a similar organism. The appearance and the disappearance of these mycobacteria could not be explained by any change in the method of collection, in procedure, or in personnel. Two patients whose urine contained them were out-patients and passed their urine into the sterile jars supplied by the laboratory without attending the wards.

A prolonged search has been made to track down the source of these mycobacteria without success. The Löwenstein media have been repeatedly examined for contamination at source. Cultures were made of washings of the glassware and of the reagents used from the time patients pass their urine to the time it is inoculated onto the Löwenstein media. The search has been fruitless. Pus, synovial fluid, and other liquid material sent from the wards for bacteriological examination has not been con-

taminated to the same extent, though a similar organism was responsible for a false interpretation of a sensitivity test of *M. tuberculosis*.

Incidence of Acid-alcohol-fast Mycobacteria

The incidence has been of the order of 0.5% of pooled urine deposits, the majority being in the three series already mentioned. The average incubation period before growth appeared on Löwenstein's medium on primary isolation differed slightly in these three groups:

Series	Period	No. of Cultures	Average Time	Range
1	Jan. Feb., 1954	8	2 weeks	
2	Jan. Feb., 1955	21	3 "	1-8 weeks
3	Oct. Nov., 1955	16	1 week	1-10 days

During this period eight strains of *M. tuberculosis* were isolated from urines, none of which was resistant to isoniazid or streptomycin.

Methods

After growth had been obtained from the primary inoculum on Löwenstein's medium at 37° C. the following tests were carried out:

- (1) Subculture on Löwenstein's medium and on glycerol agar at 37° C. to observe rate and type of growth, and on Löwenstein's medium at 22° C.
- (2) Subculture on nutrient agar at 22° C. and 37° C.
- (3) Subculture on Hirsch's charcoal agar medium, 10% horse blood agar, 10% horse blood+Glover's medium, Dubos Tween albumin medium and Loeffler's serum.
- (4) Survival at 60° C. when subcultures on glycerol agar slants were held at 60° C. for four hours, then cooled and stored at 37° C. for two weeks and examined for growth.
- (5) Microscopic examination of cultures and fermentation of rhamnose and arabinose were tested by the method described by Gordon and Smith (1953).
- (6) Catalase activity was measured by taking equal volumes of an aqueous solution of 20% Tween 80 and

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30 volumes H_2O_2 , adding one loopful of culture and observing the rate and amount of gas liberated (Middlebrook, 1954).

(7) **Neutral Red Test.**—Five millilitres 50% methyl alcohol was put in a centrifuge tube plus two loopfuls of a Dubos or Löwenstein culture rubbed in and incubated for one hour at 37° C. The supernatant is decanted and replaced by another 5 ml. methyl alcohol and incubated for one hour and decanted. Five millilitres alkaline buffer (1% sodium barbital in 5% NaCl solution not over two weeks old) plus 0.2 ml. of 0.05% aqueous solution of neutral red is added and the tube incubated at 37° C. and well shaken every 15 minutes. The colour is graded from – to ++++ according to the degree of red on the bacteria. The reading is made easier by adding 2 ml. petroleum ether, shaking for two minutes and putting in the refrigerator for a day or two, when the positives will develop a deeper red at the interface.

(8) **Methylene Blue Reduction Test.**—This was tested by the method described by Bloch (1950).

(9) **Sensitivity to Antimicrobials.**—Isoniazid and streptomycin were diluted in Glover's medium to which 10% horse blood and the test organisms were added. After two weeks the bottles were centrifuged and the deposit examined after staining by Ziehl Neelsen's method. Control cultures without antibiotics were always included. This method gave a sharp end-point of no growth. Sensitivity to other antibiotics was determined grossly by incorporating the drugs in 25 mm. filter paper discs, which were laid on 10% horse blood agar, and streaking up to the discs. The results were

read after 14 days. The plates were kept in tins to prevent desiccation.

(10) **Survival of Organisms in Mice.**—All strains were grown in Dubos Tween albumin medium for one week and brought to a concentration of 1 mg./ml., and 0.5 ml. of this suspension was inoculated intraperitoneally into groups of five mice per culture. One mouse from each group was killed by ether at the end of three, four, five, six, and seven weeks respectively and the spleen removed and homogenized. In addition to 0.02 ml. of the undiluted homogenate, similar volumes of 1/10, 1/100, and 1/1000 dilutions were inoculated on to the surface of glycerol-blood-agar. These were incubated and the colonies counted after two and four weeks.

(11) **Guinea-pig Inoculation.**—Guinea-pigs were inoculated into the thigh with 2 mg. wet weight of a 14-day culture. They were killed after six weeks and smears of glands and spleen examined for acid-fast bacilli. In the case of the chromogenic strain which produced manifest disease, six guinea-pigs were inoculated, the last three being with 1 loopful of a seven-day Löwenstein culture emulsified in 1 ml. of saline which was injected into the thigh. In these instances tissues were fixed and examined histologically, and other portions were homogenized and cultured.

Results

The results of the morphological and cultural studies are set out in Table I, and a characteristic growth pattern is illustrated in Fig. 1.

TABLE I
MORPHOLOGY AND CULTURAL CHARACTERISTICS

	45 Mycobacteria	Two Chromogens	Tap-water Strain	<i>M. smegmatis</i>	<i>M. phlei</i>	<i>M. fortuitum</i>
Subculture on Löwenstein medium at 37° C. at 22° C.	4–7 days Moist, smooth or granular, confluent buff Fairly good	7–10 days Moist, smooth yellow-orange No growth	4–7 days Moist, smooth yellow No growth	2–3 days Rough, spreading waxy, pale orange or smooth, glistening butyrous. Growth abundant at 22° C.	2 days Rough, spreading orange, or smooth butyrous Growth abundant at 22° C.	1–4 days Heaped, rough, creamy
Size and acid fastness on glycerol agar at 37° C. for 48 hours	1–3 μ Acid-alcohol fast	(1) 2–8 μ (2) 1–3 μ	1–10 μ Acid-alcohol fast	1–7 μ Acid-alcohol fast	1–3 μ Acid-alcohol fast	1–6 μ Acid-alcohol fast
Nutrient agar at 37° and 22° C. at 2 weeks	22° C. No growth 37° C. Poor growth	22° C. No growth 37° C. Poor growth	No growth	Profuse in 72 hr.	Profuse in 72 hr.	Profuse in 72 hr.
Survival of 60° C. for 4 hours	29 survived out of 42 tested	1 survived out of 2 tested	Survived	4 survived out of 5 strains tested	3 survived out of 3 strains tested	1 survived out of 2 strains tested
Charcoal agar medium at 37° C. at 2 weeks	Moist, smooth confluent, flat grey	Moist, smooth yellow-orange	Moist, smooth yellow	Dry, wrinkled spreading, heaped Pale orange in 4 days	Dry, wrinkled spreading, heaped light orange in 4 days	Non-spreading, salmon, raised, rough in 4 days
10% horse blood agar at 2 weeks	Growth in 1 week	Moist, smooth yellow	Moist, smooth yellow	Good spreading growth in 4 days	Good spreading growth in 4 days	Rough, profuse growth, non-spreading in 4 days
Dubos Tween albumin medium at 2 weeks	No pellicle—semi-granular	No pellicle Even turbidity	No pellicle “Chain formation”	Pellicle Heavy turbidity	Pellicle Heavy turbidity	No pellicle—granular
Loeffler's serum at 2 weeks	Growth in 4–7 days. No pigment. Amorphous masses	Growth in 4–7 days. Pigmented. Amorphous masses	Growth in 4–7 days. Pigmented. Amorphous masses	Good growth in 4 days. Pigmented. Clumps but no cording	Good growth in 4 days. Pigmented. Clumps but no cording	Good growth in 4 days. Short, thin rods
Growth pattern in Glover's + blood at 2 weeks	Amorphous masses	1. Filamentous 2. Amorphous masses	Filamentous	Amorphous masses	Amorphous masses	Rafts and slight cord pattern

TABLE II
BIOCHEMICAL TESTS

	45 Mycobacteria	2 Chromogens	Tap-water Strain	<i>M. smegmatis</i>	<i>M. phlei</i>	<i>M. fortuitum</i>
Fermentation of rhamnose and arabinose	Rhamnose all -ve Arabinose all -ve	R - A -	R - A -	R all - A all +	R all - A all +	R all - A all -
Catalase	Series 1. ++ 2. ++ 3. ++	1. ++ 2. +++	+++	++	+++	++
Neutral red test	19/43 +	1. ++ 2. -	-	-	-	-
Methylene blue reduction test	15-60 min.	1. 12 hr. 2. 13 min.	3 min.	3 min.	3 min.	14 min.

TABLE III
DRUG SENSITIVITY TESTS

	45 Mycobacteria	2 Chromogens	Tap-water Strain	<i>M. smegmatis</i>	<i>M. phlei</i>	<i>M. fortuitum</i>
I.N.A.H.	Resistant to 10 µg./ml.	Both resistant to 25 µg./ml.	Resistant to 10 µg./ml.	Sensitive	Sensitive	Sensitive
Streptomycin	Resistant to 25 µg./ml.	Both resistant to 25 µg./ml.	Resistant to 25 µg./ml.	"	"	"
50 units penicillin	Resistant	1. Sensitive 2. Resistant	Sensitive	Resistant	2/3 resistant	"
5% sulphamerazine	"	1. Sensitive 2. "	"	Sensitive	Sensitive	"
50 µg. chloromycetin	"	1. " 2. "	"	Resistant	Resistant	"
50 µg. aureomycin	"	1. " 2. "	"	"	"	"

The results of the biochemical and drug sensitivity tests are set out in Tables II and III.

The results of the tests for bacterial survival in mice for the "mycobacteria" strains are shown in Table IV. None were pathogenic for guinea-pigs.

TABLE IV
INFECTION OF MICE

Mycobacteria	50 or More Colonies from 0.02 ml. Spleen after				
	3 Weeks	4 Weeks	5 Weeks	6 Weeks	7 Weeks
	20/23	12/22	7/20	5/15	2/11
Chromogen 1	Confluent growth	No growth	Confluent growth	Not examined	Not examined
Chromogen 2	50 colonies	No growth	Confluent growth	10 colonies	No growth

FIG. 1

To sum up, an acid-alcohol-fast bacillus, 1-3 µ in length, in urines from 45 patients was found and has the following characteristics: In liquid media (Glover's blood) it grows in amorphous masses; on glycerol-containing solid media it forms a smooth, moist, buff-coloured colony and grows well at room temperature and survives 60° C. for four hours; it can survive for at least seven weeks in mice but is non-pathogenic for guinea-pigs. Its lack of pigment and its inability to ferment rhamnose and arabinose and other features distinguish it from *M. smegmatis* and *M. phlei*. Colonially it appears to be more like an avian bacillus or *M. fortuitum*, but its growth

at 22° C. distinguishes it from the former and it is shorter and more uniform in size than the latter. It is resistant to most antimicrobials and could be taken for a drug-resistant tubercle bacillus. It differs from isoniazid-resistant tubercle bacilli in that it combines catalase production with non-pathogenicity for guinea-pigs, but being able to grow at 22° C. on glycerol-containing media is the important characteristic that distinguishes it from drug-resistant variants of *M. tuberculosis*.

Chromogenic Bacilli.—The first of these was isolated from the urine of a girl, Betty C., who had had a tuberculous hip since 1935. Saprophytic bacilli had been recovered from her urine in January and February, 1954, and one is included in the above strains. When a chromogenic bacillus was cultured from her urine in December, 1954, no particular attention was paid to it until the autumn of 1955. It had been freeze-dried in the interim, and when re-cultured was still highly chromogenic. A sample, 0.5 mg., was inoculated into two guinea-pigs and produced macroscopic disease in six weeks, and acid-fast bacilli were found in the spleen, liver, and regional lymph nodes, but in two other pigs no disease was manifest, but the spleen sections showed evidence of an inflammatory process, not very suggestive of tuberculosis, and with no demonstrable acid-fast bacilli. Further pathogenicity tests are in progress, and at present it can only

be said that a chromogen has been isolated from the urine of a tuberculous patient and that this has many characteristics of a pathogenic strain.* It does not grow on Löwenstein medium at 22° C., it takes 12 hours to reduce methylene blue, and give ++ in the neutral red test. Attempts to demonstrate a mixture of chromogen and a pathogen have not been successful.

The other chromogen appears to be a true saprophyte, and warrants no further comment.

Conclusions

An acid-alcohol-fast bacillus has been recovered from the urines of 46 patients. It is non-pathogenic to guinea-pigs but is capable of surviving in mice for at least seven weeks. It does not appear to conform to any of the saprophytes.

A chromogenic acid-alcohol-fast bacillus has been recovered from the urine of one tuberculous patient. Its pathogenicity is in question.

REFERENCES

- Bloch, H. (1950). *Amer. Rev. Tuberc.*, **61**, 270.
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* These tests have now gone as far as we are prepared to take them. Eight more guinea-pigs have been inoculated with the culture of this strain, and no evidence of tuberculosis has been produced. This is a very unsatisfactory conclusion to offer, and there is no explanation. There is no evidence whatsoever that we were dealing with a mixture.