GROWTH CHARACTERISTICS OF AN ACID-FAST MYCOBACTERIUM ISOLATED FROM HUMAN LEPROMATOUS LEPROSY

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Tissue culture experiments designed to elucidate the affinity of the leprosy bacillus to nerve elements were first reported in October 1958 ([14]). A preliminary note on continuous cultivation of an acid-fast microorganism isolated from lepromatous leprosy was published in the same year ([1]). The present paper reports *in vitro* studies on the growth pattern of the organism, and also its biology and bacteriology under culture conditions. The results of animal experiments will be reported separately.

MATERIAL AND METHODS

Dorsal root ganglia from human fetuses were dissected out, trypsinized, and planted on the glass surface of 4-ounce bottles in Eagle's synthetic medium supplemented with 20 per cent human serum and enriched with cystine ([4]). The line of stromal fibrocytes thus established was inoculated in its 10th passage with 0.5 cc. of fresh lepromatous tissue suspension prepared in Simm's balanced salt solution. The cultures were incubated at 36 ± 1°C. Weekly examination of cultures was carried out to mark the degree of turbidity, and smears were prepared every fourth week and stained by the Ziehl-Neelsen method. Increase in turbidity, numerous microorganisms in the smear, and a drop in pH of the medium were taken to indicate growth and multiplication of the organism.

The cells on the glass remained healthy and continued to grow simultaneously with increase in the turbidity of the fluid medium. The condition of the cells and of the organisms indicated symbiotic existence, so that the modified fluid of the stock cultures was presumed to contain intermediate metabolites useful for growth and multiplication of the bacillus. The culture was therefore transferred to a conditioned fluid of stock cell cultures, hereafter referred to as "modified fluid," in which it has been maintained since July 11, 1957.

The organism, designated the "ICRC bacillus," [1] is a strongly acid-fast, gram-positive rod varying in length from 1 μ to 5 μ, sometimes presenting the appearance of a beaded chain. A detailed history of subcultures from two biopsies of Case 1, and an explanation of material from newer cases during the year 1957-58, has been described and discussed previously ([1]).

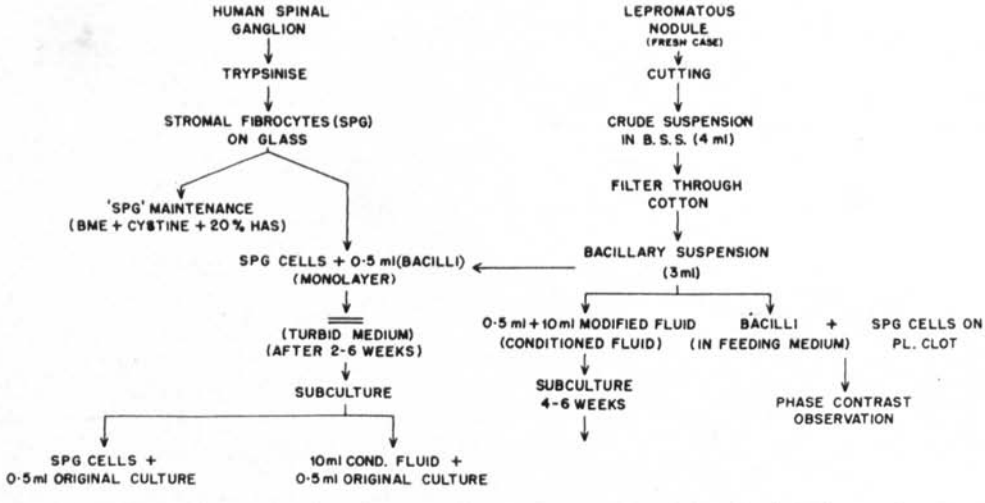
The general procedure adopted for the isolation and cultivation of the organism is outlined in Text-fig. 1.

During the last three years it has been possible to isolate morphologically similar strains of acid-fast microorganisms from three more cases of lepromatous leprosy. The organisms from all the four cases, after 6 to 8 months' cultivation in the modified fluid, have now become adapted to grow on solid bacteriologic media like Loewenstein-Jensen's, Herrold's egg, and Dubos'. Materials from three more recent cases harvested directly on the modified fluid in September 1959 are showing growth of microorganisms which have not yet been adapted to bacteriologic media.

[1]Signifying "Indian Cancer Research Center" bacillus.

GROWTH STUDIES

Growth rate.—Forty cc. of a bacillus suspension was prepared by adding 4 cc. of inoculum to 36 cc. of modified fluid. After thorough mixing, the suspension was distributed in 6 pyrex test tubes, 6 cc. in each tube, and the tubes were stoppered with rubber corks. The remaining suspension in the flask was used for hemocytometer counts, as well as for making standard smears. The culture tubes were incubated at 36 ± 0.5°C. The tubes with modified fluid alone served as controls. Tube cultures were thus set up for growth studies on material from all the four cases.



TEXT-FIG. 1. Chart showing the general procedure employed for the isolation and cultivation of the ICRC bacillus from lepromatous nodule tissue.

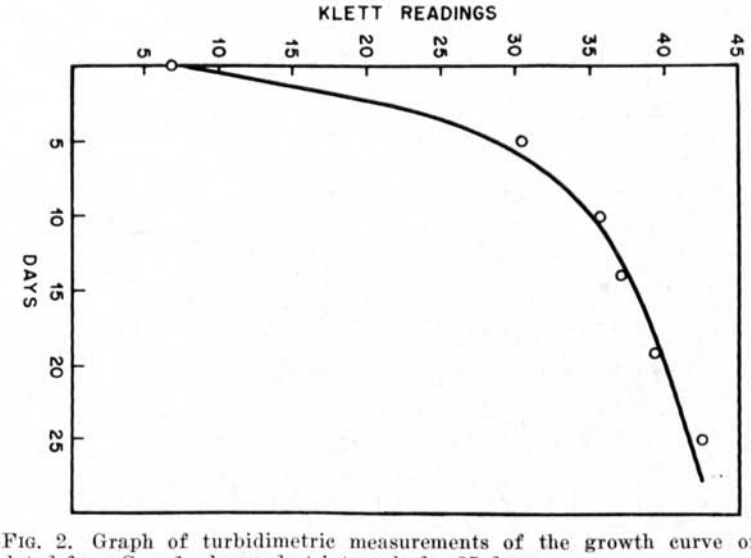
Enumeration of bacilli.—(*a*) Standard smear technique: Smears were prepared every 5th day for 20-25 days, according to a modified Breed technique described by Hobby *et al.* ([10]). Uniform 8 mm.-diameter circular smears were prepared with a platinum loop 2 mm. in diameter. Four smears per sample were made on each slide. Smears were heat-fixed at 100°C and coated with aqueous gelatin solution (0.5% in 0.5% phenol) and stained by the Ziehl-Neelsen technique for acid-fast organisms.

In counting, smears were examined under the high-dry objective (40X), with an eyepiece fitted with a graticule which served as a standard reference area. Three of the 4 smears on the slide were selected for counting. Nine or 10 fields per circular smear, spaced at 2 mm. distance, were examined so that the bacilli were counted in 27 to 30 fields per slide.

(*b*) Hemocytometer counts: Since the medium was free from debris, it was possible to count bacilli under the phase contrast microscope. A

preliminary examination indicated that the bacilli could be counted without further dilution of the suspension. Some difficulty was encountered in the enumeration of the bacilli from Case 2, because of their tendency to clumping.

(*c*) Turbidimetric measurement: Simultaneously with the enumeration of bacilli by direct counting methods, turbidimetric measurements were also made to explore the possibility of evolving a quick method for experimental work in growth requirements and other studies. A Klett-Summerson colorimeter and a Coleman spectrophotometer were used for the purpose. The graph in Text-fig. 2 represents the turbidimetric measurements giving the growth curve.



TEXT-FIG. 2. Graph of turbidimetric measurements of the growth curve of the ICRC bacillus isolated from Case 1, observed at intervals for 25 days.

Growth characteristics.—The results of standard smear counts and hemocytometer counts, done in October 1958, are tabulated in Table 1. The last column in the table shows proliferation ratios on the 15th day. The values obtained by both methods recorded in this column are comparable in all the cases except Case 1. These values show that bacilli multiply to a limited extent in the medium, the increase in population on 15th day being 2.5, 2.5, 2.8 and 4.5 fold in Cases 1, 2, 3 and 4, respectively, according to hemocytometer counting. According to the standard smear method, proliferation ratios on 15th day are 5, 2.8, 3.4 and 4. The turbidimetric curve for Case 1 shows a gradual levelling off after 15th day.

Table 2 shows standard smear counts for all the four cases according to the growth study carried out once again in October 1959. Proliferation ratios obtained were: Case 1, 5.9; Case 2, 8.0; Case 3, 5.4; and Case 4, 6.0 on 20th day.

TABLE 1.—*Growth rate of ICRC bacilli. Average number of bacilli per field (Method A) and per cc. (Method B). (October 1958.)*

Case No.[a]	Method	Days of growth						Maximum proliferation	Proliferation at 15 days
		0	5	10	15	20	25		
1	A	10.2	25.0	44.6	50.0	53.3	63.0	6.0	5.0
	B	16.5	37.6	42.9	41.3	41.8	45.3	2.7	2.5
2	A	10.6	18.6	20.0	28.3	48.0	59.0	5.5	2.8
	B	8.1	10.65	8.6	19.4	25.2	28.45	3.5	2.5
3	A	16.9	30.9	49.2	59.9	60.1	59.5	3.5	3.4
	B	9.4	16.5	24.25	25.9	32.55	35.6	3.7	2.8
4	A	12.1	27.4	39.4	49.2	57.4	67.9	5.6	4.0
	B	8.15	16.75	27.5	38.35	54.65	62.85	7.7	4.5

[a]Case 1: O.P.D. No. 25,275 (5/7/57)
Case 2: O.P.D. No. 27,736 (3/10/58)
Case 3: O.P.D. No. 27,773 (3/31/58)
Case 4: O.P.D. No. 20,860 (6/23/58)

TABLE 2.—*Growth rate of ICRC bacilli. Average number of bacilli per field, standard smear method (A). (October 1959)*

Case No.	Days of growth				
	0	5	10	15	20
1	52.8	145	205	215	308
2	30.7	97	153	184	240
3	51.1	195	273	280	280
4	46.3	130	213	214	271

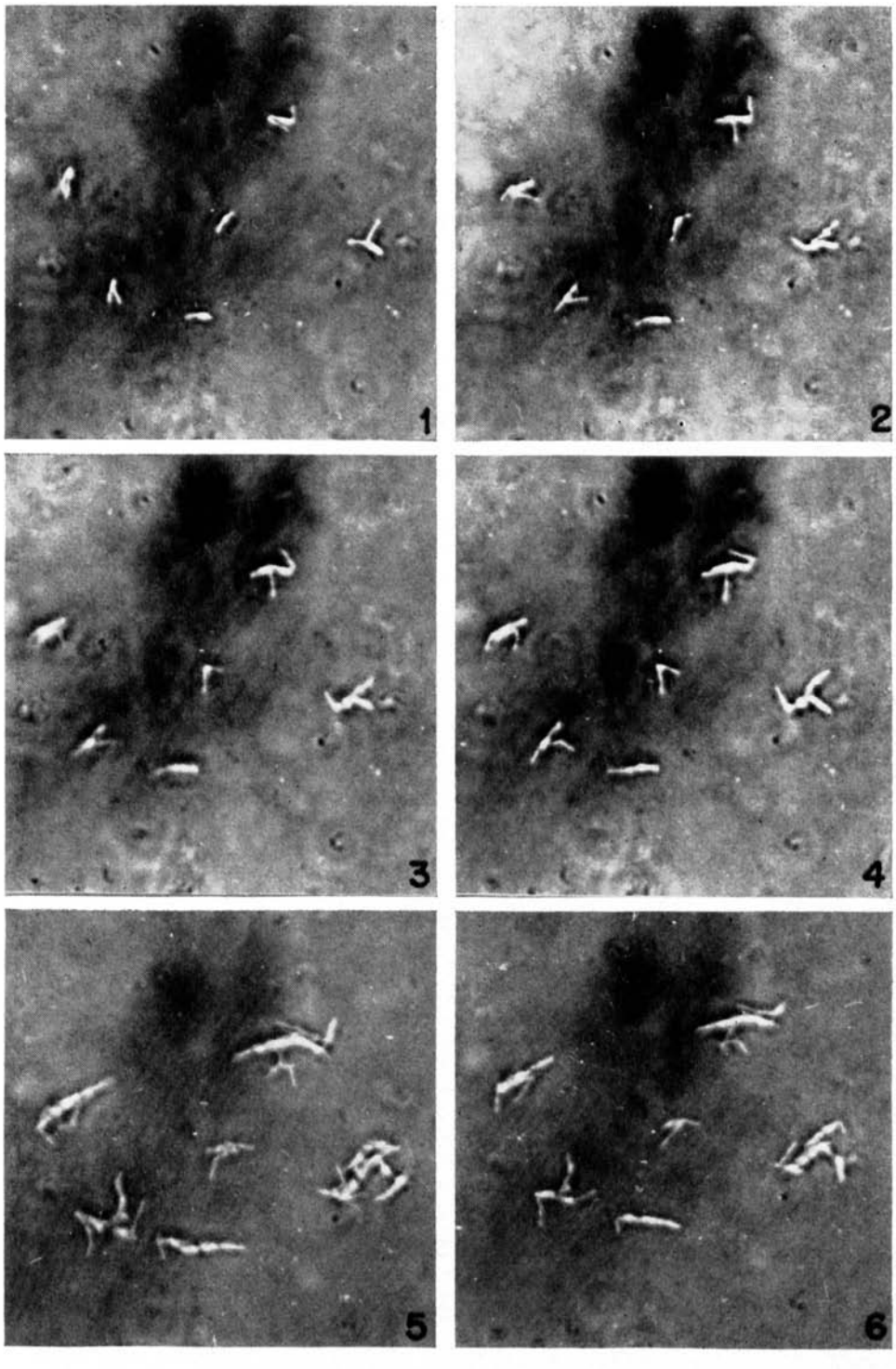
Growth pattern; phase contrast microscopy.—One drop of the bacillus suspension in modified fluid from a stock bottle of Case 1 was added to a mixture of one drop of chick plasma and one drop of chick embryo extract, forming a thin plasma clot. The hanging-drop preparation was then placed on a warm stage and observed under a high-dry, phase-contrast objective. Observations were recorded photographically every few hours for 124 hours on 35 mm. Plux-X film.

Six isolated groups of bacilli were located in one plane on the thin plasma-film coverslip culture. The growth patterns of the bacillus groups were recorded photographically as well as diagramatically at "0" hour and sequence pictures were taken every few hours, some of which are shown in Figs. 1 to 6.

DESCRIPTION OF FIGS. 1-6

FIGS. 1 to 6. Growth pattern of ICRC bacilli *in vitro* in thin plasma clot, bright contrast phase microscopy.

FIG. 1. 43 hours; Fig. 2, 56 hours; Fig. 3, 60 hours; Fig. 4, 64 hours; Fig. 5, 76 hours; Fig. 6, 88 hours. × 500.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The growth pattern of the organism may be described as a rod that first increases in length. Elongation is followed by a little thickening at the tip of the rod, which continues to grow in length until it attains double the length of the original bacillus, thus giving it the appearance of a beaded rod. A break occurs at the beaded point and the rod rotates around the bead to acquire a "V" shape. Another branch starts developing from the same beaded thickening, and the branching gradually increases. By 60 to 64 hours a few newly-formed rods are seen bending around the bead and forming doublets. Further multiplication occurs by 76 to 88 hours from doublets and triplets that are aligned to form closely-packed bunches of beaded rods. Further branching forms a network of colonies. In some cultures the bacilli finally form colonies having the appearance of cords or pellicles.

BIOLOGICAL STUDIES

Cytopathogenic effects.—An attempt was made to compare the cytopathogenic effects of the ICRC bacillus on cells *in vitro* with those of fresh *M. leprae*. Stromal fibrocytes grown from human fetal spinal ganglia (the "SPG" cell line) were scraped from the glass surface of stock bottles and planted on thin plasma clots of double coverslip cultures. In one set of cultures fresh pieces of lepromatous nodules, swarming with bacilli, were explanted in the thin plasma clot as in the previous experiment. Another set of cultures was inoculated with the ICRC bacillus suspension. These two sets of experimental cultures of SPG cells, one exposed to the fresh *M. leprae* material and the other to the ICRC bacilli, were carefully studied under the phase contrast microscope for 8 to 10 days, and observations were recorded with sequence photomicrography.

A careful study of the SPG fibrocytes on the surface of the plasma clot grown with fresh lepromatous tissue revealed phagocytosis of a few organisms. Frequently an isolated organism or a small group were found neatly lodged inside the cell cytoplasm with a peculiar "halo" around the body of the microorganism. This condition has been easily discovered in many stained preparations as well (Figs. 13 and 14).

Larger groups of organisms observed in the cytoplasm, either as irregular clumps or typical globi, sometimes displayed an appearance

DESCRIPTION OF FIGS 7-12
(Phase contrast pictures; magnification × 430.)

FIG. 7. SPG cells exposed to fresh lepra bacillus suspension. Note radiating cluster of bacilli in one cell (arrow).

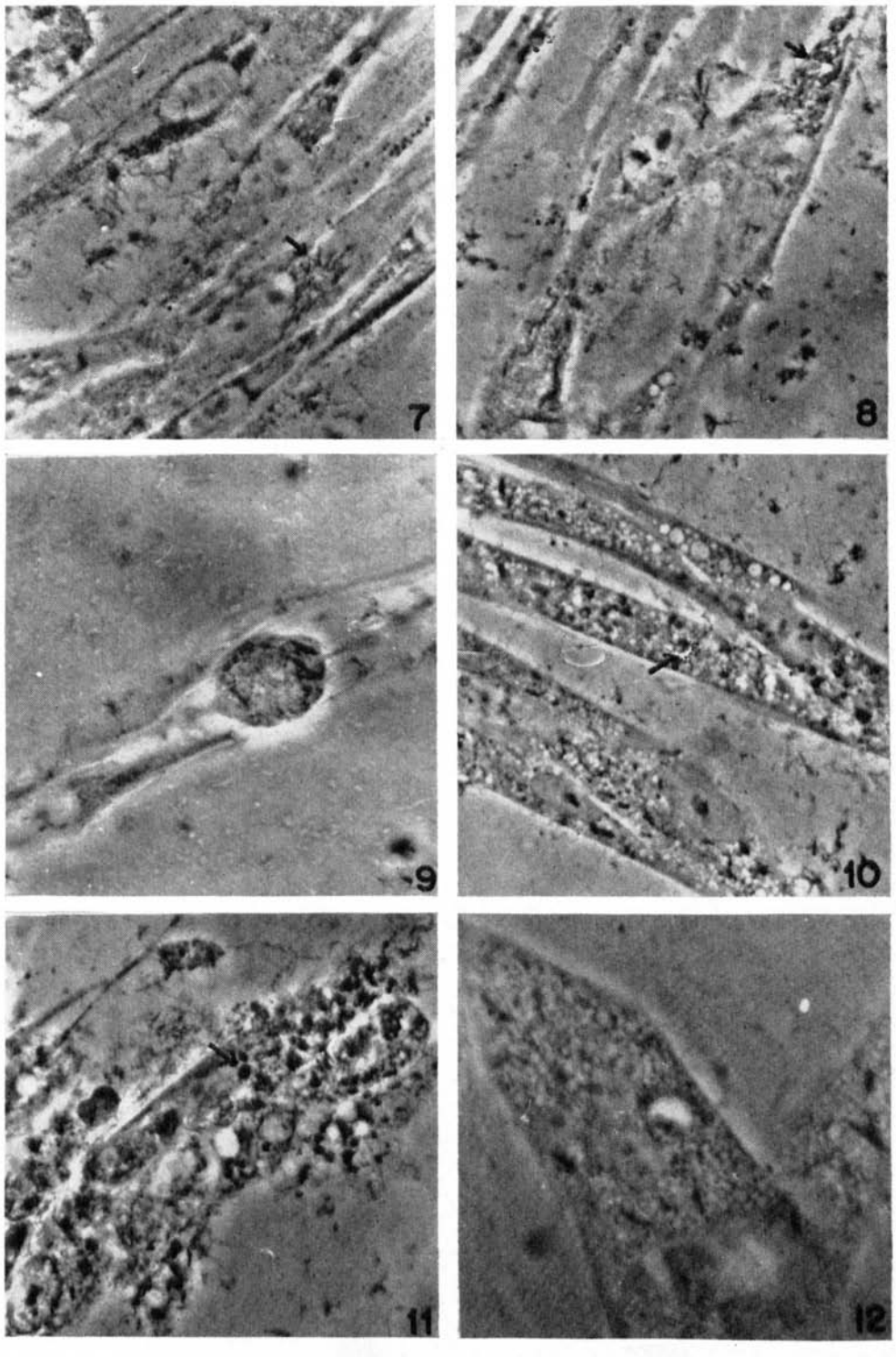
FIG. 8. Another field, the same explant as in Fig. 7. Varying numbers of bacilli in cytoplasm (large group at arrow). Few vacuoles seen.

FIG. 9. A dividing fibrocyte with bacilli in the cytoplasm.

FIG. 10. Same culture as in Fig. 7 to 9, after 6 days. Note fully vacuolated cytoplasm with tiny globi in it (as at arrow).

FIG. 11. Degenerating cells. Note dark round globi (as at arrow) and large vacuoles.

FIG. 12. Fully vacuolated or foamy SPG cell inoculated with ICRC bacilli.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of growth columns radiating from a common center (Fig. 7). A larger number of cells on the surface of the clot would phagocytose the bacilli in varying numbers, depending upon the location of the organisms and the contacts made. They formed bundles or radiating clusters of rods, or spread along the length of cytoplasmic processes (Figs. 7, 8). The bacilli thus lodged in the cytoplasm did not affect the cell shape and form, and not even the cell division (Fig. 9).

On the fifth day of explantation, vacuoles began to develop. First, one or two tiny vacuoles appeared which increased in number and size, finally filling the cytoplasm with vacuoles giving it a little foamy appearance. Complete vacuolation of cytoplasm occurred in about 18-24 hours (Fig. 10).

Closely-packed groups of bacilli were soon circumscribed by the boundary of tiny vacuoles (Fig. 10). Many of the groups had the typical appearance of globi, which can be clearly discerned in a stained preparation.

The SPG cells thereafter showed progressive degeneration. The cells got rounded, showing cytoplasmic bubbling; the nuclei became indistinct; and finally vacuoles packed with bacilli burst open, so also the cell wall was ruptured, spreading bacilli all over the surface of the clot (Fig. 11).

A set of Ziehl-Neelsen-stained preparations confirmed that the organisms observed under the phase-contrast microscope were strongly acid-fast. Auramine staining exhibited strong fluorescence under ultraviolet light. The length of the organisms varied from 1 μ to 5 μ. The morphology of the organisms clearly resembled that of the bacillus in the original tissue smear.

SPG cells exposed to ICRC bacilli followed the same sequence of cytopathogenic changes, and at the end of the week the cell with foamy cytoplasm showed similar vacuolation as seen in the other set of cultures (Fig. 12). The behavior of fresh *M. leprae* and of the ICRC bacillus inside the SPG cells was closely comparable, under controlled experimental conditions.

Cytopathogenic effects on HeLa cells.—Leighton tube cultures were set up with HeLa cells and incubated at 37°C in a stationary position. A

DESCRIPTION OF FIGS. 13-18

(Photomicrographs of stained preparations, Ziehl-Neelsen and hematoxylin.)

FIG. 13. SPG line cells exposed to fresh lepra bacillus suspension. × 400.

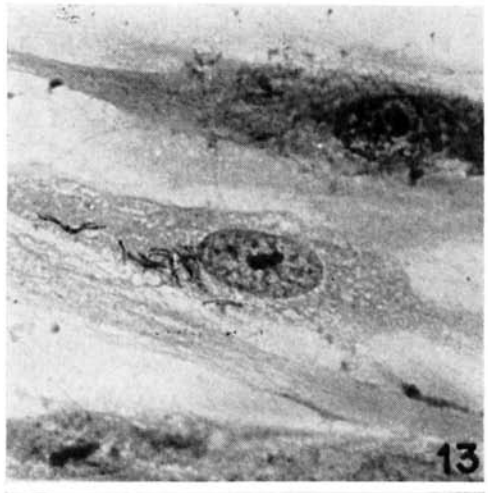
FIG. 14. SPG cell with neatly arranged bacilli in the cytoplasm. × 400.

FIG. 15. HeLa cell with fresh lepra bacilli; 24-hour culture. Note the typical globus. × 637.

FIG. 16. HeLa cell with fresh lepra bacilli, showing typical network; 48-hour culture. × 637.

FIG. 17. HeLa cell exposed to a suspension of the ICRC bacilli; varying numbers of bacilli in cells; 72-hour culture. × 637.

FIG. 18. HeLa cells with ICRC bacilli; 72-hour culture. Note globus-like formation. × 637.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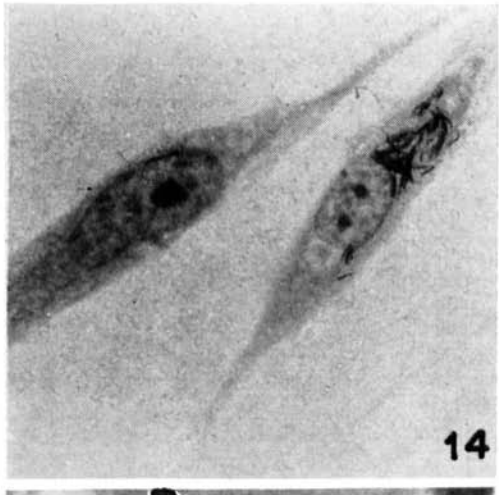
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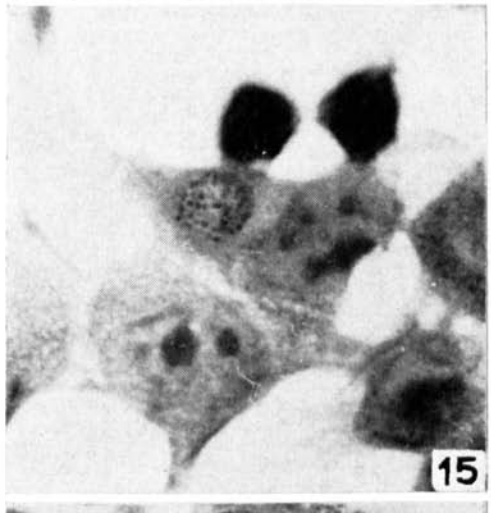
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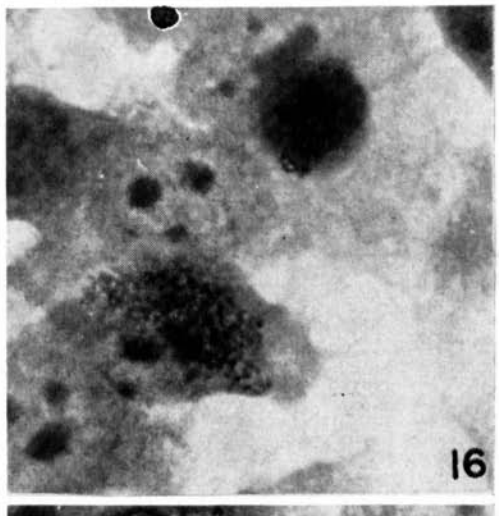
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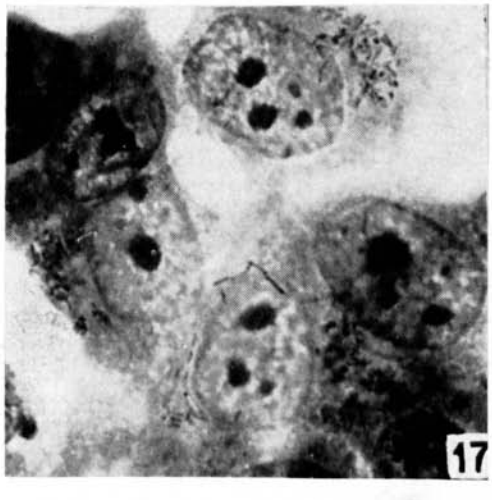
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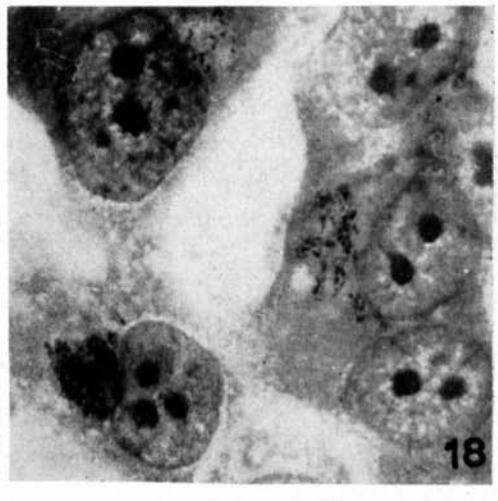
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few drops of the fresh lepra-bacillus suspension was added. The cells on the tubeslip were thus directly exposed to the organisms in the fluid medium. Sixteen tubes of HeLa cells were set up at a time, of which 4 were kept as controls and 12 were inoculated with the lepra bacillus suspension. Three slips, 1 control and 2 inoculated, were fixed every 24 hours and stained with carbol-fuchsin. Careful study of these slips indicated increase in the number of organisms taken in by the cells after an interval of 24 hours. Groups of bacilli inside the cells presented various arrangements, some showing typical globi (Figs. 15 and 16).

HeLa cells cultures were also inoculated with ICRC bacilli in the same way. The HeLa cells phagocytosed the bacilli, and the pattern of arrangement of the bacilli in the crytoplasm was closely comparable to that of fresh lepra material as described (Figs. 17 and 18).

Bacteriologic characteristics.—Extensive bacteriologic studies have been carried out on the 4 strains of organisms isolated from 4 cases of lepromatous leprosy. This work was undertaken at the Armed Forces Medical College, Poona, and a full report of it will be published separately. Organisms from all the 4 cases are now adapted to bacteriologic media like Loewenstein-Jensen's, Herrold's egg, etc. The organisms were strongly acid-fast short rods, varying in length from 1.5 μ to 5.0 μ. Longer forms up to 7 μ in length have been found occasionally. They produce smooth, slightly creamy, thin growths on the solid media. A heavy inoculum initiates growth in 4-7 days, and reaches a maximum in 18-21 days. On exposure to light there is no pigment formation, nor any change in color. The bacilli grow only on media containing either egg yolk or a serum component. Tests for catalase activity were negative. On storage the organism retains viability at 4°C, 25°C, and 37°C up to 5 months.

Pathogenicity to animals.—A heavy inoculum of the bacillus suspension (2×10^9 organisms per animal) given intraperitoneally to mice produced many pin-point nodules on the visceral organs, such as the liver, spleen, pancreas and testis. Typical isolated granulomas were formed around portals in the liver, and good-sized miliary leproma-like lesions were also developed at the edge. Four strains of mice—C_3H-(Jax), dba(-MTI), dba(Bar) and a hybrid of Paris strain XVII x C_{57}-(B1) have been used for the experiment. The dba mouse appeared to be more susceptible than the other strains. Variable doses of the inoculum have been tried, and progressive lesions have been observed up to 12 months. Lesions of varying size and pathology were found in the liver, spleen, pancreas and in the adventitial tissues of the testis. The skin near the site of injection occasionally developed nodules which were full of acid-fast bacilli. A detailed report on the animal experiments and the histopathologic study of the lesions will be published elsewhere.

DISCUSSION

A great many reports have been published on attempts to isolate and cultivate acid-fast organisms from human leprosy ([12]). Some of these organisms either lost their acid-fast character, developed pigment, or failed to grow indefinitely. The method used for isolating the ICRC bacillus from lepromatous leprosy and its continuous cultivation is different from any previously used. This bacillus was first isolated on a cellular substrate of fetal spinal ganglion origin, and then adapted to the cell-free conditioned fluid. Isolation from 3 cases was done directly in conditioned fluid, and the strains have now been adapted to standard bacteriologic media for over three years. Multiplication at first was roughly judged by increase in turbidity, but later was investigated quantitatively by standard smear technique as shown in Tables 1 and 2. This technique was introduced by Hanks ([10]) and later used by Rees ([15]) and others ([7, 8, 18]) to study limited multiplication of the murine leprosy bacillus in a strain of rat fibroblasts *in vitro*. The method of enumeration of bacilli is a modification of the Breed technique and gives only comparative figures. However, it can be modified to give absolute counts by using the Agla syringe to prepare the standard smear ([9]). From the tables it appears that the logarithmic growth phase lasts for 15-20 days, after which increase in number is very slow. Unlike other fast-growing organisms, the number of organisms does not increase but remains stationary. The hemocytometer count gives absolute numbers of bacilli in a unit volume, but the method is not adaptable to regular use because of errors involved due to clumping. The comparative count, once obtained, is useful to estimate the number of organisms in a given sample by the standard smear method.

The turbidity measurement technique has its limitations, but it might provide a useful and quick method for screening antileprosy drugs or studying growth-inhibiting effects of agents like blood serum, etc.

As regards growth pattern and arrangement of organisms inside the cell, interesting data have been collected on the ICRC bacillus. Human leprosy bacilli have a characteristic arrangement described as "packets of cigars." Such a pattern would essentially result from close, parallel alignment of bacilli. Elongation, breaking and turning, as well as gradual formation of doublets and triplets, was observed in the ICRC bacillus under the phase microscope. This process continued until a palisade arrangement or packet of cigar-like arrangement resulted. *M. marianum*, a chromogenic acid-fast organism, has been reported to exhibit a similar arrangement of bacilli ([13]).

M. tuberculosis and *M. leprae* are supposed to have an intimate association with cells. Tissue-culture techniques have been useful to study this relationship and the formation of the tubercle since 1924. Recently Shepard ([16, 17]) and Fjelde ([6]) have described the behavior of various

mycobacteria, including *M. tuberculosis*, in HeLa cell cultures. The reaction to lepra bacilli has so far been investigated only in histologic sections of lepromatous tissue ([5, 11]). Benewolenskaya ([2]) had observed that bacilli had apparently no harmful effect on cells from cultures of normal and leukemic blood, liver and spleen. Macrophages actively phagocytosing bacilli became "lepra" cells after 5 to 7 days and finally degenerated.

The phase microscopy record of cytopathogenic changes occurring in SPG cells in plasma clot, exposed to both fresh and cultivated bacillus material, is very interesting. Development of vacuoles in cytoplasm of SPG cells was so intense that the nucleus was pushed aside or indented by vacuoles. The exact cause of vacuolization has not yet been determined ([3]). It may be noted, however, that the foamy cytoplasm of the vacuolated cell infected with bacillary material *in vitro* compared closely with the histiocytic lepra cell described by pathologists ([5]). Other cell types, like HeLa and HLS_2 ([11]), on the tubeslips exposed to bacilli in the fluid medium also showed characteristic globus-like aggregates of the organism in the cytoplasm (Figs. 17 and 18).

Shepard and Fjelde have used a similar system to study the behavior of different mycobacteria inside the cells. In such a system there are two drawbacks: (*a*) the large volume of the medium, and (*b*) the rapid multiplication of cells in tissue culture. The former could reduce the effect due to dilution of any cytopathogenic agent introduced in the system, while the latter would obliterate the final result either by crowding a very large population or by toxic substances released by the cells themselves. The plasma-clot cultures of SPG cells offered better conditions in this respect, the result of which has already been discussed.

It is important to note that the *in vitro* behavior of the ICRC bacillus inside the cell cytoplasm presented striking similarities to that of the fresh *M. leprae* suspension inoculated on cells *in vitro*. These findings, together with the bacteriologic, immunologic and pathogenic properties of the bacillus, would perhaps facilitate confirmation of identity of the organism isolated from lepromatous leprosy.

SUMMARY

An acid-fast microorganism has been isolated from four cases of human lepromatous leprosy in a tissue culture system consisting of the SPG fibrocytic cell line derived from human fetal spinal ganglia. The organisms, designated the "ICRC bacillus," can be grown and maintained in the conditioned fluid of the stock cells. It can become adapted to solid bacteriologic media after about six months maintenance in the modified fluid.

The *in vitro* behavior of this organism has been studied in detail. Growth characteristics, growth pattern, growth rate and bacteriologic studies are reported and discussed in the light of relevant literature.

RESUMEN

En 4 casos de lepra lepromatosa humana, se ha aislado un microbio ácidorresistente en un sistema de histocultivos compuestos de raza fibrocítica SPG derivada de ganglios raquídeos de fetos humanos. El microbio, denominado "bacilo ICRC," puede cultivarse y mantenerse en el líquido condicionado de las células stock. También puede adaptarse a medios bacteriológicos sólidos después de unos seis meses de mantenimiento en el líquido modificado.

Se ha estudiado a fondo el comportamiento *in vitro* de este microbio. Se presentan y discuten a la luz de la literatura pertinente las características de proliferación, el patrón del desarrollo, la velocidad del desarrollo y los estudios bacteriológicos.

RESUMÉ

Un micro-organisme acido-résistant a été isolé de quatre cas de lèpre lépromateuse humaine sur une culture de tissus consistant en une lignée cellulaire des fibroeytes SPG dérivés de ganglions spinaux de foetus humain. Ces organismes, désignés par le terme de "bacilles ICRC," peuvent être dévelopées et maintenus dans le liquide de la culture tissulaire. Ils peuvent s'adapter à des milieus bactériologiques solides après environ 6 mois dans le liquide modifié.

Le comportement in vitro de ces organismes a été étudié de façon détailée. Les charactéristiques de leur croissance, l'aspect et la vitesse de cette croissance, ainsi que diverses études bactériologiques, sont rapportées et discutées en tenant compte de la littérature à ce sujet.

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