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In Vitro Cultivation of Leprosy Bacilli in Hyaluronic Acid-Based Medium 2. Progress and Developing Concept of the Role of Hyaluronic Acid Suggested by Culture and Armadillo Infection Studies [1, 2]

O. K. Skinsnes, B. Andersson Kuba, P. H. C. Chang and T. Kuwahara [3]

Pathologic evidence accumulating over past decades indicates that *M. leprae* is a successful intracellular parasite only in those forms of leprosy characterized by near total or relative absence of cell-mediated immunity. Multiple studies have indicated that these hosts produce high levels of humoral antibodies to this pathogen. These antigens are agglutinative and precipitative of *M. leprae* and it is not improbable that the presumed "obligate intracellular" nature of *M. leprae* results from highly effective phagocytosis due to an opsonic effect of the antibodies. The pathogen, then, would not necessarily be "intracellular" because of any "obligativeness" in its nature but would be adaptively "obligate"—or more fundamentally would essentially be a "facultative" intracellular pathogen. Accumulated evidence further indicates that *M. leprae* itself has remarkably little acute inimical effect on host cells or tissues per se, apart from induced immunologic reactions such as those of *erythema nodosum leprosum* or delayed-type hypersensitivity and, of course, the slow inflammatory effects so well seen in the characteristic nerve involvement. Intracellular parasitism is not necessarily an inherent characteristic of leprosy since relatively severe and effective host response occurs with tuberculoid type leprosy where the bacilli are rapidly suppressed or destroyed. Were it not for the fortuitous existence of an immunologically incompetent segment in the parasite's host, e.g., had the spectrum of host response been as it is in tuberculosis, the concept of "obligate" intracellular parasitism relative to leprosy might not have arisen.

Once the concept was accepted and it was concomittantly found that *M. leprae* isolated from host tissues resisted many attempts at *in vitro* cultivation, there followed quite reasonably the concept that if cultivation were to be achieved it probably would necessitate the determination of some unusual metabolic requirement or combination of required substances.

Our initial preliminary report of the cultivation of mycobacteria isolated from leprous tissues and alleged to be *M. leprae* accordingly assumed, with some hesitancy, that hyaluronic acid (HA) in the LA-3 medium presented an unusual energy source for the cultivated bacilli. The hesitancy arose from some difficulty in understanding biochemically how and why this might be so, though the concept was not totally untenable. Subsequent extended collaborative studies by Kato and Ishaque [8, 9] on adequate quantities of cultured and armadillo derived bacilli have shown that bacilli from both sources are metabolically similar, are highly competent and adaptive metabolically and apparently do not utilize hyaluronic acid as an energy source.

These formulations and determinations

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[3] O. K. Skinsnes, M.D., Ph.D.; B. Andersson Kuba, EM Technologist; P. H. C. Chang, R.M.T.; and T. Kuwahara, M.D., ALM Leprosy Atelier, Department of Pathology, John A. Burns School of Medicine, University of Hawaii, 3675 Kilauea Avenue, Honolulu, Hawaii 96816.

present the background for reassessment of the possible role of hyaluronic acid or related substances in *in vitro* cultivation attempts. The observations and formulations herein presented are so directed.

MATERIALS AND METHODS

Culture maintenance. Cultured isolates are consistently maintained through successive subcultures on LA-3 medium as a base line for adaptation studies. From this stream, inocula may be placed in other media such as Dubos, Sabouraud liquid broth, etc. for comparative study, after the cultures have adapted adequately to permit such growth. Thus, HI-75 is currently in its 54th, MO-75 in its 44th, and AR-H6 in its 15th subculture in LA-3. These successive subcultures have never been interrupted by sojourns in other media though derivative inocula from them have been so utilized.

Cultivation technic. Basic technics of cultivation, as originally described ([37]), remain essentially the same and no attempted alterations of the basic medium have improved its performance.

Kato ([14, 15]) has reported similar success with a modified (KI-1) medium which appears to work equally well and perhaps better. It has the advantage of being less costly and easier to prepare. However, if it is to be used in the study of early culture adaptation it must be filtered in order to remove confusing yeast forms.

The inocula while in the medium undergo a long period of adaptation before developing acid-fast rods and full replicative growth. The ideal conditions for this are not clear. However, it currently appears that maintenance of the pH of the culture medium as originally described is important. It also seems that early aeration of the medium may be inimical, i.e., during the first weeks before the culture is prepared to elongate into acid-fast rods. The inoculum should be heavy, on the order of 10^7 or more.

Isolation of inocula. Optimum bacillary isolation is achieved by aseptic biopsy and preparatory conditions. Our currently preferred approach is to check this by growing the completed inoculum in thioglycolate medium. If no growth occurs in 24-48 hours the culture medium is inoculated and its penicillin content seems adequate to prevent growth of possible gram positive contaminants. Higher concentrations (2,000 units/ml) of penicillin than detailed for the LA-3 medium have an inhibitory effect on the growth of the leprosy isolates.

A series of TEM and general mycobacterial studies in this laboratory have demonstrated that the use of NaOH is inimical to the bacillary wall and inhibitive of growth. Though mindful of the 1934 proscription by Duval and Holt ([5]) of the use of any iodine compound in isolation technics, our same studies indicate that periodate or sodium periodate may be preferable to NaOH if decontamination is indicated by positive thioglycolate culture from the inoculum. Periodate in preliminary trials seems not to have an inhibitory effect and is effective in preventing contaminant growth, but its practical value in present culture attempts is not yet clear.

Lepromin preparation. Mitsuda type lepromin was prepared by standard methods from seven day old and eight week old HI-75 cultures. Obvious problems were evident regarding standardization which is customarily related to 160 million bacilli/ml in lepromin prepared from human tissue-derived bacilli. Cultured bacilli on TEM droplet preparations show considerable variation in size from tissue-derived bacilli. Also, the general size of the cultivated bacilli may change as they increasingly adapt to cultivation, as may the proportion of their constituents. There is also the problem of what age of cultivation should be used since there is no information available on the average age of bacilli in tissue in terms of relating age to logarithmic or other phases of multiplication.

Since the work of Dharmendra ([4]), delayed-type hypersensitivity and the lepromin reaction have generally been related to protein fractions, though this concept may presently need refinement. However, on this basis a suspension of armadillo-derived bacilli was prepared in saline, without phenol, having a count of 160 million bacilli/ml. Its protein concentration was determined by the Folin-Ciocalteau method and found to be 100 μg/ml. Culture lepromins were adjusted by their protein concentration against this standard. Such lepromin from a seven day old HI-75 culture had a CHO content of 0.87 mg/ml, and an eight week old culture 1.54 mg/ml, as compared with commercial cane sugar's content of 68 mg/ml as determined by the carbazole reaction.

In view of the probable role of polysaccharides in generating the Arthus reaction in lepromatous leprosy, a similar lepromin from an eight week old culture was prepared, the preparation including incubation with 10 mg% lysozyme in phosphate buffer at 37°C for 60 minutes. Its CHO content was 0.35 mg/ml and also presented the possibility of alteration in antigen structure.

Bacillary acetone powder. Acetone powder, rather than bacillary culture, is preferred for adsorption of shared mycobacterial antigens from serum since the material can be prepared in quantity, is easily stored, ready for use and is less hazardous.

M. tuberculosis was grown in 750 ml of Dubos liquid medium for seven weeks from a heavy inoculum. After preparation this yielded 4 gm of dried TB acetone powder.

Unsonicated TB bacilli were removed from the medium by centrifugation and washed in PBS. They were then treated with 100% acetone several times in a ratio of two volumes to one volume of original culture, dehydrated and the bacillary powder was ground with a covered mortar and pestle. It was then washed in PBS containing 1:10,000 thymersol and separated by centrifugation (5,000 rpm, 10 minutes). For adsorption the powder is used in a ratio of 200 mg/ml serum.

Peroxidase labeled antibody preparation.

1. Pooled LL serum, usually 30-40 ml, containing 1:10,000 thymersol preservative, is adsorbed against *M. tuberculosis* acetone powder for three hours at 37°C and then left overnight in the refrigerator. The TB powder is used in a ratio of 100 mg powder/25 mg protein/ml serum. Just before use ten volumes PBS are added to one volume acetone powder, mixing carefully with a glass rod; allowing the mixture to stand for 5-10 minutes, then centrifuging for 15 minutes at 10,000 rpm with discard of the supernatant.

2. The centrifuged precipitate is mixed uniformly with the LL serum by means of a glass rod, allowed to stand for three hours at 37°C with occasional shaking. The mixture is then stored overnight in a refrigerator, centrifuged at 12,000 rpm for 30 minutes and the adsorbed supernatant serum separated.

3. To this is added 0.1 ml L-ratio phospholipid per 1.9 ml supernatant serum, mixing carefully and then refrigerating overnight. L-ratio phospholipid ([24,25]) consists of a 0.4% solution of 1:1 cardiolipin-lecithin in absolute ethanol.

4. Salt fractionation of the serum is carried out as previously described ([24,25]). The resultant antibody containing immunoglobulin fraction is dialyzed against PBS once and the protein concentration determined by the Folin-Ciocalteau technic.

5. Fifty milligrams of the gamma globulin preparation is mixed in 2 ml 0.5 M cold carbonate buffer at pH 10. To this there is added 50 mg horse radish peroxidase (type VI, Sigma) and 0.25 ml 0.5% FNPS (p,p'-difluoro-m,m'-dinitrophenyl sulfone) in acetone and the mixture agitated gently for 30 minutes at room temperature. This is followed by dialysis overnight in PBS. Then the precipitates are removed by centrifugation at 10,000 rpm for 15 minutes.

6. Before final fractionation, the unreacted peroxidase may be removed from conjugated and unconjugated gamma globulins by precipitating the globulins in a solution of 50% saturated ammonium sulfate. The globulin precipitate is then redissolved in PBS and the ammonium sulfate removed by dialysis against PBS.

7. The gamma globulin solution is fractionated in a Bio-Gel P300 column with each fraction collected being about 2.9 ml. The first 21 fractions are discarded, leaving the final 22-30 pooled fractions to be eluted by PBS and to yield the final preparation.

Direct immunoperoxidase TEM identification technic.

1. Tissue isolated or cultured bacilli are rinsed several times in sodium cacodylate buffer at pH 7.2 by centrifugation (10,000 rpm, 30 minutes).

2. The bacillary suspension is incubated for 20-30 minutes in the peroxidase conjugated antibody preparation, diluted 1:5 with 0.15 M Na-cacodylate buffer, pH 7.2.

3. The bacillary suspension is rinsed several times in 0.15 M Na-cacodylate buffer by centrifugation (10,000 rpm, 30 minutes).

4. The final bacillary centrifugate is then incubated in the 0.15 M Na-cacodylate buffer containing 0.05% 3,3-diaminobenzene and 0.01% hydrogen peroxide ([3]) for 45 minutes with occasional gentle agitation.

5. Similarly rinsed several times by centrifugation in 0.15 M Na-cacodylate buffer.

6. The final pellet is fixed in 1% OsO_4 in veronal acetate buffer at pH 7.2 for 1.5 to 2

hours. The pellet is then dehydrated through the usual ethanol series and embedded in Araldite.

7. Ultrathin sections are cut at 600 Å on a LKB Ultrotome III for TEM examination.

TEM bacillary drop. A small droplet of medium or other solution containing concentrated numbers of bacilli is placed on a Formvar supported grid so as to form a "bead" extending to the edges of the grid. The droplet is touched with the edge of a Whatman No. 1 filter paper to remove most of the liquid and the grid dried at room temperature. The grid is then held over 1% OsO_4 for one to two minutes and rinsed by dipping in glass distilled water. Ideally the water is sterilized after distillation so as to avoid possible surface contamination by fungal forms growing in water. After the rinse the grid is again dried and is then ready for electron microscopy.

Use of this technic makes monitoring cultures by TEM virtually as simple as by light microscopy and the visualization obtained is far greater. It does not replace ultrathin sectioning for detailed study of mycobacterial structure.

SEM specimen preparation. Bacillary cultures are centrifuged at 10,000 rpm for 20 minutes, the pellet is resuspended in PBS, pH 7.2., and again similarly centrifuged and the pellet is resuspended in 0.1 M phosphate buffer.

A few drops of the PBS bacillary suspension are filtered through a 0.2 μ GE-Nuclepore membrane filter (13 mm diameter), utilizing a Swinny filter adapter (0.5 inch diameter) having a supporting 25 Seitz filter disc (Scientific Products). The adapter is attached to a 3 ml disposable plastic syringe. This results in the deposition of culture particles greater than 0.2 μ diameter on the surface of the Nuclepore filter and this deposit constitutes the SEM specimen. The pores in the membrane provide a useful approximate gauge to the size of particles viewed. The membrane specimen is not permitted to dry during ensuing processing up to the point of critical point drying.

Fixation is accomplished by carefully squeezing a few drops of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, through the membrane filter utilizing the same filter apparatus. The same technic is employed for dehydration through a 40-50-70-80-90 and 100% ethanol series.

The filter membrane specimen is then transferred to the critical point dryer container which is immediately immersed in the intermediate fluid, Freon 113, and transferred to the precooled critical point dryer chamber in a Bomar SPC-900/Ex instrument where Freon 13 is used as the transition fluid.

Following critical point drying the membrane is mounted, specimen holding surface up, on an aluminum stub by means of a small amount of silver paint. The specimen, on the stub, is then gold coated in an argon gas atmosphere within a Denton Vacuum Evaporator (model DV-515) equipped with a Denton DSM-5 cold sputter module. The specimen, on the stub, may then be stored or viewed. In this laboratory viewing is with a Coates & Welter Field Emission Cwikscan 104A SEM.

It is evident that in this processing any particulate material in processing fluids that does not pass through the 0.2 μ filter will be deposited on the membrane with the original specimen. Mold forms and particles growing in distilled water are particularly troublesome and for work on cocco-bacillary culture forms or possible protoplast forms it is necessary to use fresh solutions prepared in sterilized glass distilled water from which such contaminating forms are excluded.

Armadillo infection. Nine, nine-banded armadillos captured in the neighborhood of Fort Sam Houston, Texas, were all Mitsuda lepromin negative. On 7/3/74 each animal received 5×10^6 pooled human leproma (two biopsies) isolated acid-fast bacilli (Saigon patients) in each of three subcutaneous sites (total 1.5×10^7). These had been refrigerated for 23 days. On August 23 they each received an additional 8×10^7 i.p. similar bacillary isolate from pooled (five biopsies) Hong Kong patient biopsies which had been refrigerated for 45-52 days. Total number of bacilli received by each animal was thus 9.5×10^7. The sole initial purpose of these infection attempts was to obtain large amounts of leprosy bacilli for studies contemplated, recognizing that at that time the reported success rate was 30-40%. The animals were followed by repeated nasal scrapings and by heart puncture search for bacilli. At 22 months six animals remained, three having succumbed, two to severe parasite infestation and one to attempted splenectomy. None gave any evi-

dence of infection.

By this stage the cultivation of leprosy isolated bacilli had been reported ([37]) utilizing hyaluronic acid and the question of the role of hyaluronic acid assumed greater significance. Accordingly, all six armadillos were subjected to a regimen of 0.25 gm HA (Sigma, Grade III-S, human umbilical cord) in 10 ml PBS given each week intraperitoneally beginning on May 25, 1976.

HA, like heparin, is known to have hemorrhage supportive effect and in the concentration used was viscous and difficult to handle so the schedule was changed to 0.1 gm/10 ml PBS given intraperitoneally every week. This treatment was continued in each animal until either spontaneous death or sacrifice or till HA administration was terminated in surviving animals after a total of 371 days. HA was discontinued because of the associated hazard of intraperitoneal hemorrhage.

RESULTS

Armadillo, HA supplemented infection. Because of the small number of armadillos available, dividing them into experimental versus control groups relative to the use of HA would probably have rendered their numbers too small to have significance. The information available at that time was that successful infection occurred in about 30-40% of inoculated animals. Accordingly, resort is made to utilizing published reports of others for control comparison.

The record of the six armadillos is presented in Table 1. All animals at death or sacrifice presented disseminated mycobacteriosis especially involving dermis, lymph nodes, liver and spleen. There was extensive intraneural bacillary invasion. The gross and histopathologic findings in these animals were compatible with those reported as characteristic for *M. leprae* infection of armadillos save that infestation was heavier ([19, 20]). By comparison, Kirchheimer ([18]), utilizing 15 armadillos infected with 10^7 human leproma-derived bacilli, reported 33% dissemination in 1,095 days, and 66.6% in 1,676 days. Comparison with later reports apparently utilizing armadillo bacilli would be invalid since isolates from this source seem to have a much higher percentage of viable pathogens.

The inoculum load of a total of 9.5×10^7 bacilli per animal in these subjects was higher than that used by Kirchheimer at 10^7. However, our initial three sites/animal intradermal inoculation of a total of 1.5×10^7 bacilli utilized biopsies which, due to problems of logistics, had been refrigerated (4°C or wet ice) for 23 days and the intraperitoneal inocula of 8×10^7 bacilli/animal were derived from five pooled human biopsies which had been similarly refrigerated, also without any preservative, for from 45-52 days.

These were, perhaps, marginally infective isolates since significant change in infectivity at similar storage for 29 days and total loss of viability at 54 days has been reported ([32]) on the basis of mouse foot pad studies. If

TABLE 1. *100% armadillo* M. leprae *infection with hyaluronic acid.*

	Armadillo (AR)					
	H-1[a]	H-2	H-5[b]	H-6	H-7	H-8
Total duration infection (days)	1,217	791	1,369	973	1,004	913
Duration HA (days)	371[c]	105	371[c]	287	301	231
Total intraperitoneal HA (gms)	7.55	3.75	7.55	6.35	6.55	5.55

[a]Gave anatomical evidence of "healed," fibrosed inoculation site leproma and draining lymph node leproma ("primary complex") with reactivation at both sites.

[b]Failed to show elevated inoculation site nodules.

[c]HA stopped 146 and 296 days before demise without apparent effect on course of infection.

Note: There were no gross signs, e.g., fibrosis, adhesions, etc., intraperitoneally of any HA effect.

the same were true of the present inocula, then the larger intraperitoneal inoculation may have been virtually or totally ineffective leaving the lesser, partially vitiated intracutaneous inoculations of 1.5×10^7 bacilli per animal as the source of 100% rapid dissemination in these animals. The probability of a significant role on the part of HA is enhanced by these experimental conditions.

Comparison of tissue bacillary content also speaks strongly to a significant role of HA. Storrs *et al* ([39,40]) reported that 854 gm of leproma from five armadillos and 988 gm leproma from eight armadillos yielded an estimated 17 gm and 15-20 gm wet weight bacilli, respectively. Taking the latter figure as two separate determinations this works out to 1.99 gm/100 gm, 1.52 gm/100 gm, and 2.02 gm/100 tissue, respectively.

In the present experiment, 152 gm of dermal and adjacent lymph node lepromas yielded 4 gm/100 gm wet weight bacilli. Pooled leproma, liver and spleen yielded 500 mg/100 gm bacilli. One hundred and fifty grams of pooled leproma, liver and spleen from AR-H1 yielded 450-500 mg/100 gm. These determinations were made on bulk material shared with Dr. Kato. On additional separate lots of 4 gm, 12 gm, 10 gm, and 217 gm armadillo leproma received from Gulf South Research Institute (GSRI), Kato's determination was 52 mg/100 gm, 280 mg/100 gm, 35 mg/100 gm, and 92 mg/100 gm, respectively.

The tissue bacterial load in armadillo No. 8 (the first reported infection), as calculated from bacterial counts rather than by actual weight determinations, was reported per 100 gm tissue as being 1.78 gm in the lymph node, 1.32 gm in the spleen, 220 mg in uninoculated skin, 78 mg in the lung, and 44 mg in the liver.

It seems clear that administration of hyaluronic acid had an infection enhancement effect in armadillo supportive of similar effects previously reported ([26]) in mice infected with human leproma derived bacilli. A further consideration suggests that this effect is on bacilli rather than on the host.

In mice, the presence of numerous extracellular bacilli was noted ([26]). In armadillos, numerous microcolonies of acid-fast bacilli were found extracellularly by light microscopy (Fig. 1); transmission electron microscopy (TEM), Figure 2; and scanning electron microscopy (SEM), Figure 3. These with diameters of 30-60 μ were too large to be contained in macrophages or to be re-engulfed by macrophages but give morphologic evidence of being growing colonies. Thus, *M. leprae* under suitable conditions would appear to be a facultative rather than an obligate intracellular pathogen, a finding consonant with its *in vitro* cultivation.

Serum from armadillo AR-H6 was processed for FITC LL-Ab immunofluorescence by the procedure previously described ([24]). Mycobacterial isolates from this same ar-

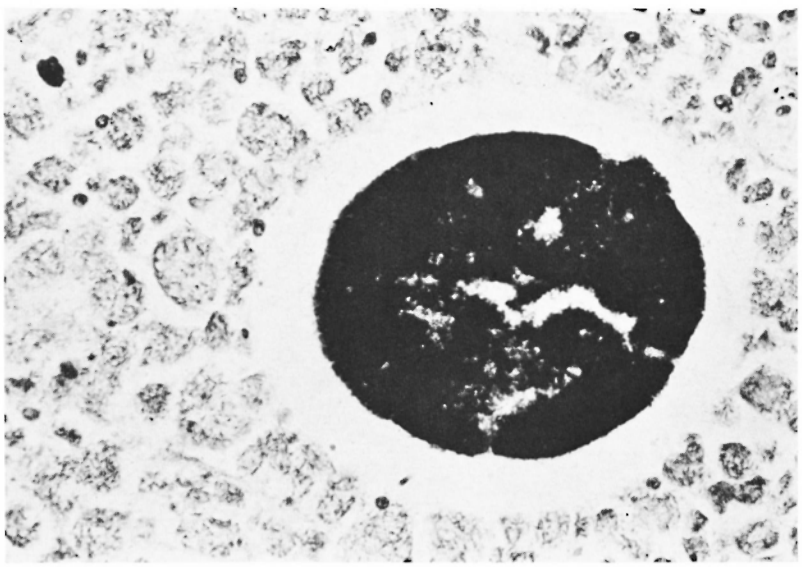

FIG. 1. Extracellular microcolony, armadillo AR-H1 skin. Acid-fast stain. X 1,000 original.

madillo were cultivated in LA-3 by procedures described ([37]). This isolate did not grow on conventional mycobacterial media such as Lowenstein-Jensen medium. The armadillo antibody preparation reacted with the culture from the armadillo, as expected, and also with our other strains of mycobacteria cultivated from human lepromas. The armadillo culture also showed positive immunofluorescence with antibody preparation from pooled human lepromatous serum, as did the human isolate cultures which were derived from patients other than those providing the pooled serum (Fig. 4).

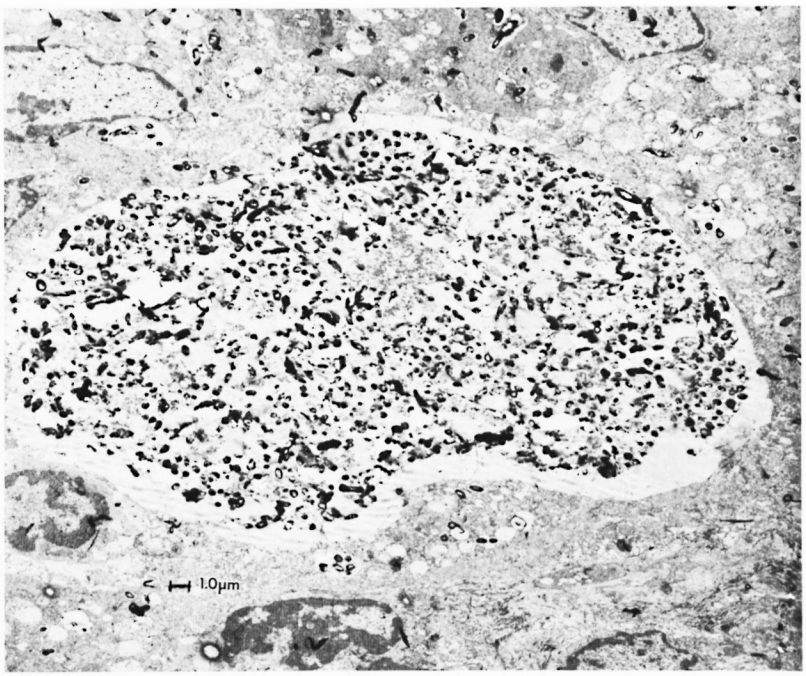

FIG. 2. Extracellular microcolony, AR-H1, TEM ultrathin section. ×6,500 original.

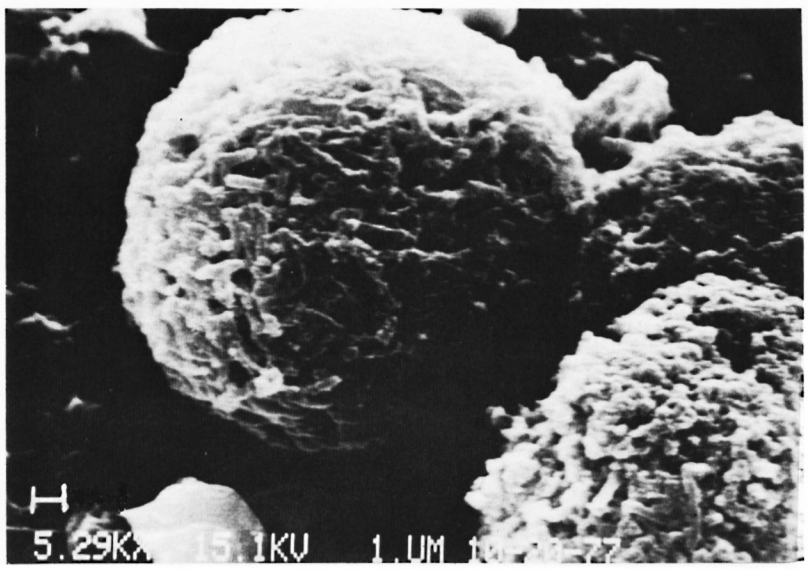

FIG. 3. Extracellular microcolony, AR-H1, SEM.

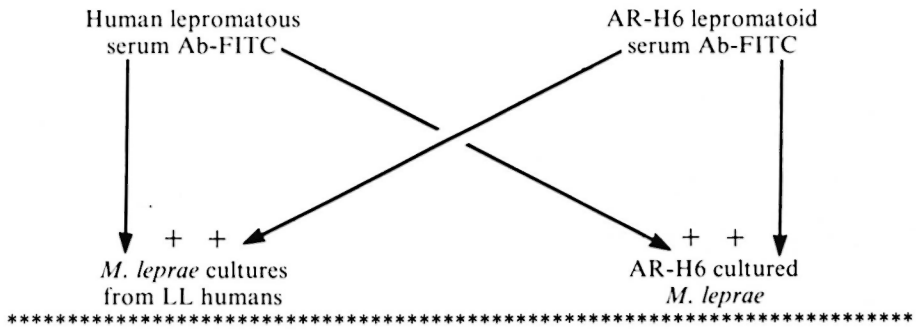

FIG. 4. Immunologic cross reactivity. Armadillo and human cultures and sera.

Other mycobacteria, including strains of *M. scrofulaceum*, did not give positive fluorescence with either the human or the armadillo serum preparations.

Guinea pig, HA supplemented inoculation. Human leproma-derived mycobacterial isolate inoculated into five adult guinea pigs, which were similarly treated with weekly injections of 20 mg HA per week for 67 weeks, did not yield any bacillary growth.

Early adaptation in cultures. Twenty-three human leproma culture attempts have been made in this laboratory. Seven yielded positive cultures, of which two died out after two subcultures. Of the others, six were lost to contaminants and in eight no growth was achieved. Of five culture attempts from HA treated armadillos three have been positive, one lost to contaminant and one resulted in growth that aborted. Kato has achieved five cultures from human lepromas on KI-1 medium and in three of three attempts from infected armadillos, two from our AR-H6 and AR-H1 and one from an armadillo with feral infection ([12]).

In these and other attempts in progress by others one is reminded of the statement by Wolbach and Honeij ([41]) in their 1914 comprehensive review of leprosy cultivation attempts:

> Whatever their significance may be, the nature of the organisms, their free growth at ordinary temperatures and upon ordinary media, do not accord with our ideas of a parasite so highly specialized as the leprosy bacillus must be. It is difficult to understand why these cultures are so difficult to obtain in the first generation and so easy to maintain afterwards.

The statement is remarkably prescient of present experience. A number of the attempts they reviewed involved placenta based media—media which therefore contained quantities of hyaluronic acid.

The initial leprous isolates, directly from tissues or stored in LA-1 do not grow on Lowenstein-Jensen or other standard mycobacterial media but yield growth in LA-3 and in KI-1 ([14, 15, 37]) media. A striking and regular characteristic of this growth is that it requires a variable but long period of adaptation, measured in weeks or even months, before acid-fast bacillary growth is achieved. This long adaptation period witnesses against an energy giving role of HA. If it were a needed unique energy providing nutrient it would be reasonable to expect the cultures to grow reasonably quickly since in host tissues the bacillus is associated with the presence of HA or other acid mucopolysaccharides ([35]). If enzyme adaptation or development were crucial, the bacillus should therefore already have this and bacilli in direct isolates from host tissues have been shown by light and TEM histochemistry to possess β-glucuronidase ([23, 25]).

During the period of slow adaptation virtually all of the original acid-fast inoculum disappears, i.e., no acid-fast bacilli remain. Instead there are present amorphous (light microscopy) non-acid-fast materials in which minute non-acid-fast spheroid particles appear which yield immunofluorescence with LL-Ab preparation (Fig. 5). They vary in size with common diameters of about 1.5 μ often seen. This size is too large to pass

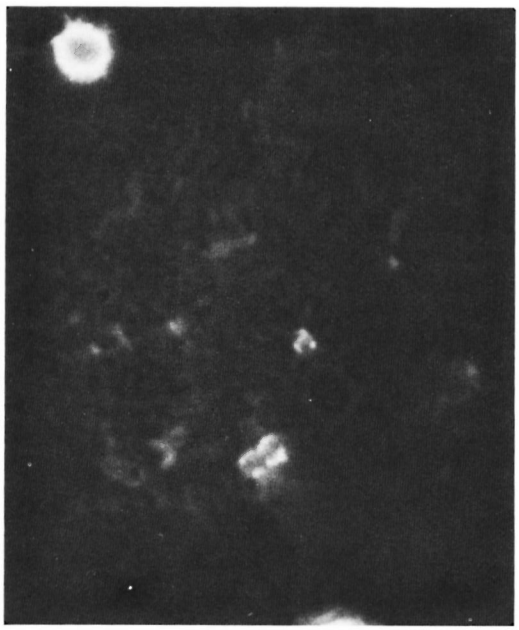

FIG. 5. FITC, LL-Ab immunofluorescent response of early adaptive culture forms. ×1,000 original.

through the 0.2 μ Seitz filter through which the LA-3 (containing yeast extract) is passed for final sterilization, thus eliminating the likelihood of possible confusion with yeast forms present in the yeast extract.

In due course there is increase in a light, "fluffy" deposit, always on the bottom of the culture container, which lightly spirals upward when the container bottom is gently agitated.

With electron microscopic visualization two distinct variant morphologic forms are seen; one, for lack of a better term, designated "protoplast" (Figs. 6, 7) and the other tentatively termed "spheroblast" (Figs. 8-10). On oil immersion light microscopy these two variants can usually not be clearly differentiated. Both forms are, at this time, non-acid-fast by "differential acid-fast staining," i.e., utilizing methylene blue at pH 10 in the acid-fast stain.

Since the "protoplast" form does not present an enveloping membrane or capsule while the spheroblast does, it seems reasonable that the latter may be derived from the former by the development of such an envelope. The "protoplasts" morphologically, as suggested by some available TEM and SEM evidence, may be derived from bacillary mesosomes as, for example, those illustrated by Hirata recently ([7]) but this has not been determined as a fact. The "protoplasts" appear to have a lower density than the spheroblasts

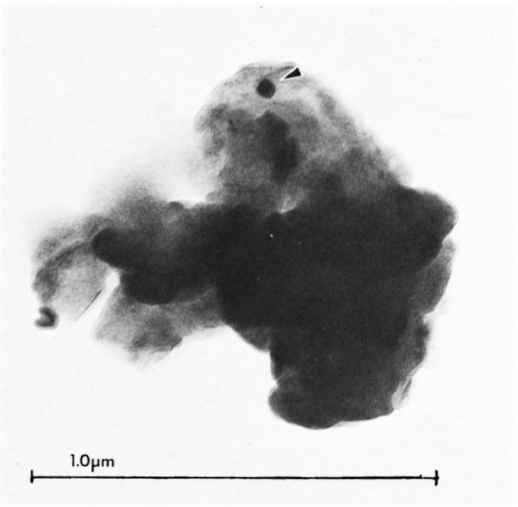

FIG. 6. TEM, drop preparation, one adaptive form in 1° cultured isolate. Same type as SEM, Figure 7. Apparent metachromatic granule (arrow). ×87,500 original.

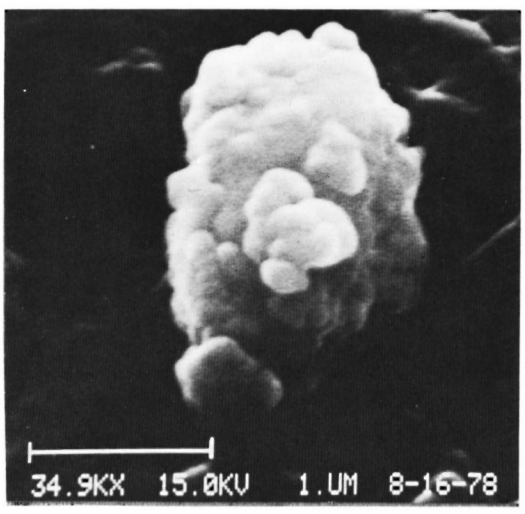

FIG. 7. SEM, early adaptive form in 1° culture isolate, equivalent to Figure 6.

and have been difficult to obtain in adequate quantities from early cultures to permit "catching" them for TEM ultrathin sectioning for the purpose of studying possible internal mesosome-like membranes.

The spheroblasts have walls of differing thickness (Fig. 10), suggesting that there is progressive build-up of these walls. Preliminary attempts are in process directed at developing ^{14}C hyaluronic acid and studying, by autoradiography, its possible incorporation into these wall structures. Within the spheroblasts, cell structures develop, including lipid accumulations.

Spheroblasts increase by cell division (Fig. 11). This also militates against the possibility that they might be yeast forms from the sterile yeast extract utilized in LA-3. Spheroblast forms are found, though in relatively lesser numbers, in subcultures on LA-3 (Fig. 12) to the present 54th transfer over a three year period. By this time the cultures are well adapted to the medium as evidenced by more uniform morphology and relatively few elongated rods.

These processes do not occur in sharply defined "waves" save for the initial adaptation period when no acid-fast rods are found. Thereafter there is intermingling of forms and it is evident that the described "protoplast" and "spheroblast" forms are part of the ongoing mycobacterial reproductive process. Indeed, "beaded" bacilli are found by both TEM and SEM, suggesting that the cap-

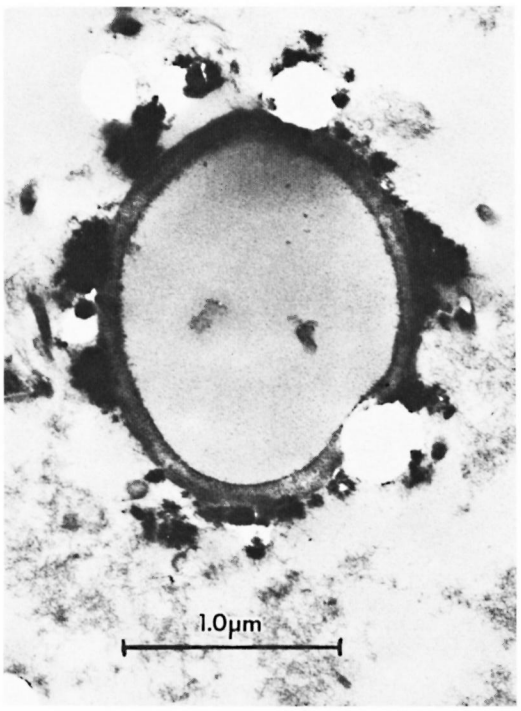

FIG. 9. TEM drop preparation, "spheroblasts" in 1° culture isolate. × 33,566 original.

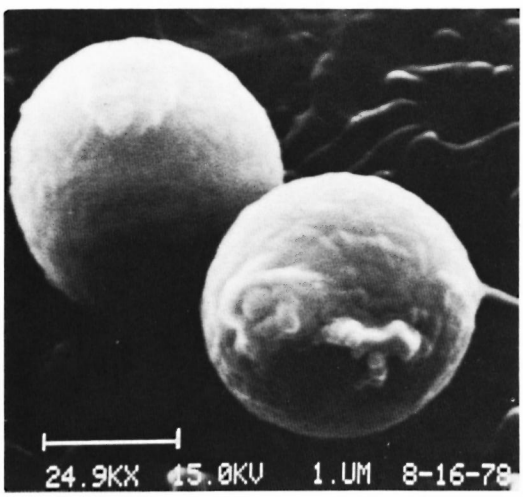

FIG. 8. SEM, "spheroblasts" in 1° culture isolate.

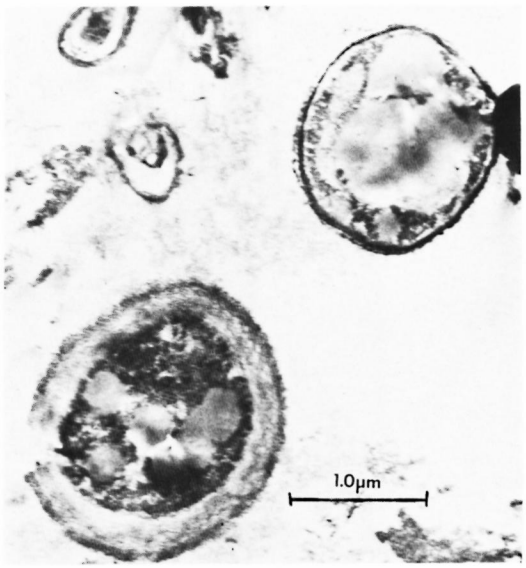

FIG. 10. TEM drop preparation, "spheroblasts" in 1° culture isolate. × 28,500 original.

sules of acid-fast rods may disrupt to give rise to the reproductive forms (Fig. 13).

The question of the possible presence of the described forms in human lepromas arises from these considerations as well as from occasional reports in the literature, perhaps the most recent being that of Chatterjee ([2]). TEM search for these is being made utilizing ultrathin sections of human and armadillo lepromas. In the meantime, TEM photos of such sections in our files have been reviewed and in a number of instances forms are found similar to those seen in culture (Fig. 14). This does not specifically prove that the cultures are those of *M. leprae* but it adds incremental witness to the possibility and, even if the cultures were not *M. leprae*, suggests the probable importance of such forms in leprosy.

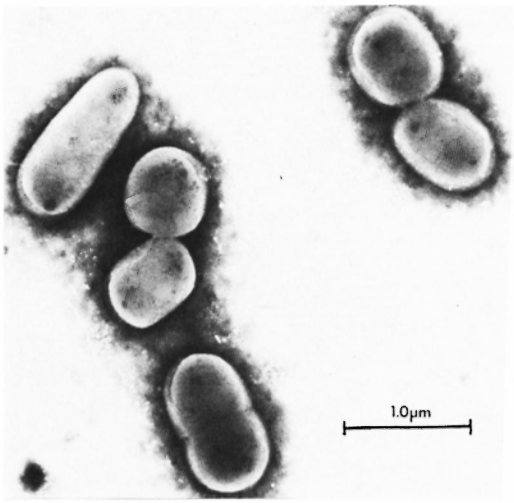

FIG. 11. TEM drop preparation, reproductive cocco-bacillary forms in LA-3 culture, strain HI-75, subculture 3. × 29,850 original.

Culture identification. *Immunofluorescence and immunoperoxidase.* Cultures isolated in this laboratory have all given positive direct immunofluorescent reactions with the technic previously described ([24, 25]). The intensity of the immunofluorescent response is variable with the age of the cultures as illustrated by Figure 15. In order to achieve more precise visualization of specific immunologic response, the direct immunoperoxidase technic described above has been similarly applied to these cultures. Figures 16 and 17 illustrate the characteristic positive response as illustrated by strain HI-75. Figure 18 presents the negative response presented by *M. scrofulaceum*. Other mycobacteria, negative to direct immunofluorescence, have also been negative to this technic but the survey is not yet complete.

The indirect immunofluorescence reaction in general accord with the technic developed by Abe and Yoshino ([1]) is being pursued and the technic adapted to indirect immunoperoxidase.

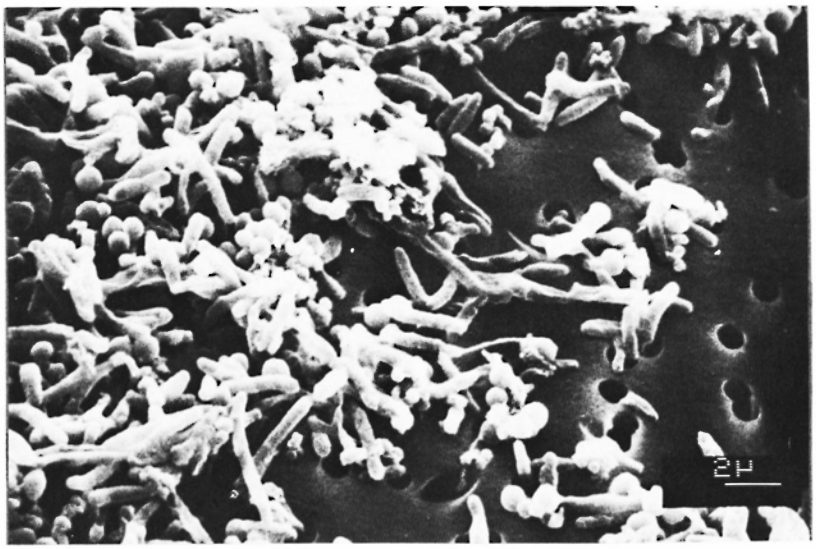

FIG. 12. SEM, culture HI-75, subculture 12 at about 8 weeks. Shows bacillary and cocco-bacillary forms.

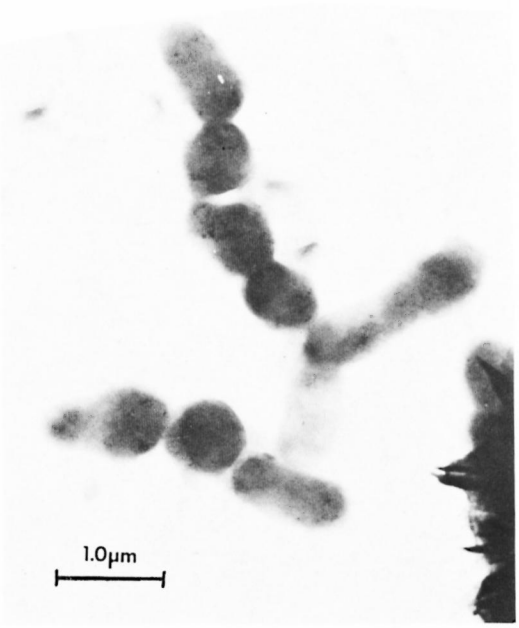

FIG. 13. TEM drop preparation, HI-75, subculture 3 at 18 days. Bacilli apparently reproducing by shedding "capsular wall" and giving rise to spheroid forms. X 19,000 original.

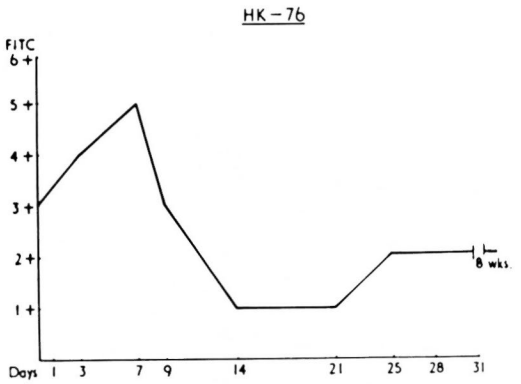

FIG. 15. Intensity immunofluorescent reaction in strain HK-76, subculture 2, over a period of 8 weeks.

Present experience is that the direct immunologic technics provide higher percentage of cultured bacillary visualization whereas the indirect technics provide better visualization with bacilli directly derived from tissues. This may be related to the presence of accretions of host antibodies on the tissue-derived bacilli as previously discussed ([33]). Similar problems with indirect immunofluo-

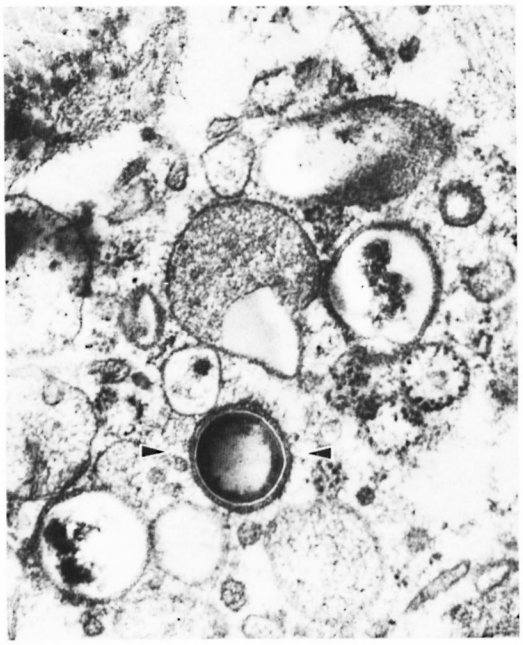

FIG. 14. TEM, ultrathin section untreated lepromatous leproma containing apparent "spheroblast" form. X 39,000 original.

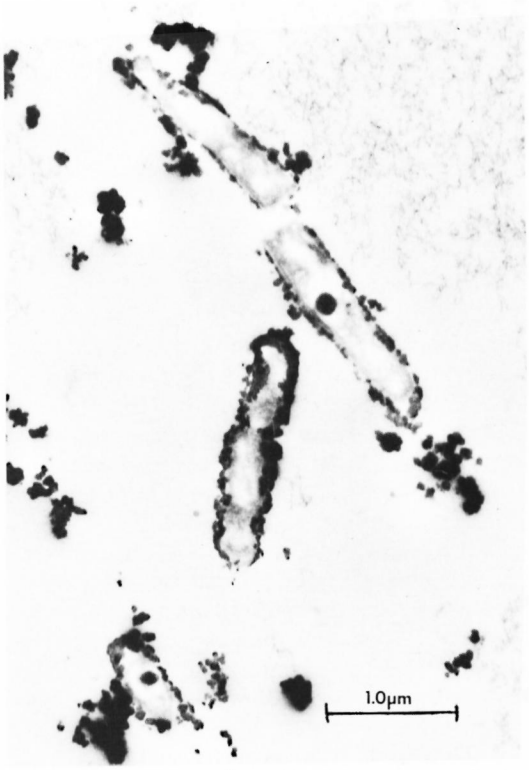

FIG. 16. TEM ultrathin section, positive LL-Ab immunoperoxidase response, strain HI-75, subculture 49. X 28,500 original.

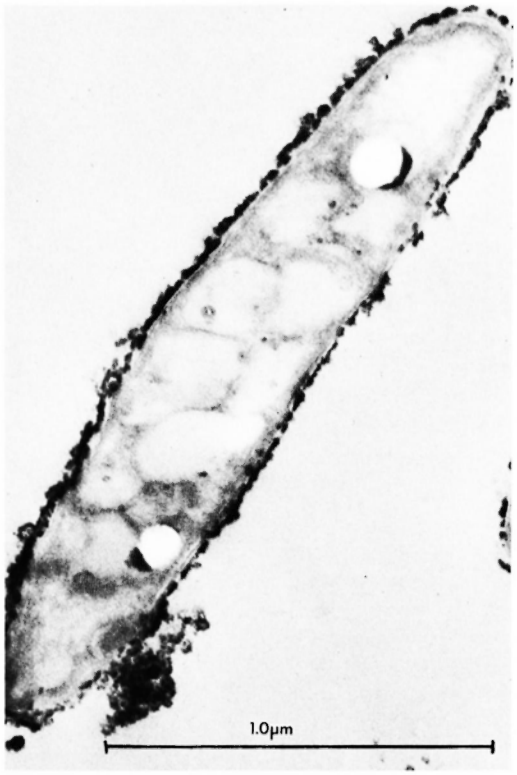

FIG. 17. TEM ultrathin section, strain MO-75, subculture 39. × 86,000 original.

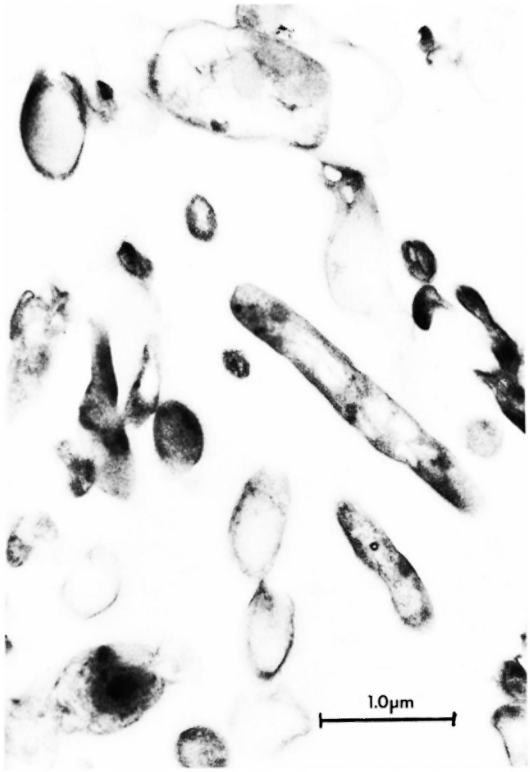

FIG. 18. TEM ultrathin section, negative LL-Ab immunoperoxidase response, *M. scrofulaceum* (CDC) in LA-3. × 28,500 original.

rescence studies of streptococci were reported by Roberts and Sherris ([31]) who found blocking antibodies on tissue-derived bacteria. Kanai ([10]) found similar accretion of host substances on tissue-derived tubercle bacilli. Roberts and Sherris found that if the streptococci were exposed to NaOH the cells did not react to the indirect immunofluorescent technic but responded to the specific direct technic. When this and other variations are employed with Abe's indirect technic, better results are obtained with cultured bacilli. These studies are still evolving.

Culture lepromin response. Cultures continue to give evidence of continuing growth adaptation for many subcultures and this relates also to their immunologic characteristics which seem related to the adaptation of their cell walls, particularly as related to their polysaccharide content. Mitsuda type lepromin from a ninth subculture of HI-75 gave positive Mitsuda responses in five BT and TT leprosy patients at four weeks—slightly stronger than with control standard human derived lepromin. Ten LL patients gave negative response to both. In ten LL patients the culture preparation evoked a reaction at 24-48 hours which was judged to be Arthus in nature though this is difficult to differentiate from a Fernandez reaction. Mitsuda type lepromin from the 41st subculture of the same strain but treated with lysozyme resulted in elimination of the 24-48 hour reaction in LL patients while the 48 hour reaction (Fernandez) was positive in TT/BT patients. However, at four weeks both TT/BT and LL patients presented severe Mitsuda type reactions, though stronger in TT/BT. In the LL patients this subsided and scarred. In the TT/BT patients, healing and scarring also occurred save that in two TT cases reactions persisted till the sixth month. Thus, quantitative antigenicity, through adaptation, is possibly adequately enhanced to stimulate the deficient LL response.

Animal inoculation. *Korean chipmunk.* Lew made a comparative study of HI-75, Kato's Dakar culture and *M. scrofulaceum*

(ATCC 19981) in chipmunk infection and reported:

> The Korean chipmunk *(Eutamias sibiricus asiaticus)* is highly susceptible to infection with *M. scrofulaceum* (IJL **44** [1976] 539-540). Three groups of four animals each were inoculated intraperitoneally with bacillary suspensions of 1.3 × 10^7 bacilli of *M. scrofulaceum* (ATCC 19981), HI-75 and Dakar leprosy isolates, respectively. Animals were sacrificed at two, six and ten weeks after inoculation. The spleens of all animals inoculated with *M. scrofulaceum* showed II to VI + bacilli (Gaffky scale). Those inoculated with the HI-75 and the Dakar leprosy isolates did not present any bacilli or signs of infection.
>
> It was concluded that neither HI-75 nor the Dakar strains are *M. scrofulaceum* akin to ATCC 19981 type ([21]).

Mouse foot pad. No feedback has yet been received from a laboratory expert in the mouse foot pad technic which volunteered to make a study and was provided with a culture for that purpose in May of 1976. Our own limited attempts, thought initially to be possibly consonant with findings for tissue isolated *M. leprae,* were not in the long run indicative of similar response in that very limited infection occurred and in many instances disappeared. Not being enamored of or impressed with the accuracy of bacillary counting we utilized only histopathologic sectioning. We recognized that most laboratories expert in this technic and critical of procedures related thereto would not readily accept our efforts in this line since our laboratory is not highly experienced in the technic. However, for theoretical reasons ([34]) we do not expect this technic to be of identification value for cultured bacilli.

Armadillo infection. This approach would seem to be one that should be utilized. Laboratories in possession of adequate numbers of these animals have their own extensive programs and one such laboratory which was approached was, understandably, not receptive to a trial with cultures.

Repeated efforts have been made to obtain adequate numbers of armadillos for inoculation trials over a period of more than 20 months, both commercially and otherwise. There have been major difficulties illustrated by the last commercial shipment of 11 animals, all of which died within a week of arrival without having been subject to any experimental procedure. Two major sources of promised supply failed to deliver. A few animals are being maintained and others sought. A considerable number are required since they should be separately infected with early spheroblast forms, as well as smooth colony and rough colony variants.

In vitro culture versus in vivo characteristics. The characteristics of *in vitro* mycobacterial isolates compared with perception of *in vitro M. leprae* characteristics as presently perceived are summarized in Table 2.

Suggestions have been made that the cultures discussed are *M. scrofulaceum* ([29, 38]). A comparative summary is presented in Table 3 of characteristics of LA-3 cultured leprosy isolates and standard strains of scrofulaceum. While agreeing that the leprosy isolates have many characteristics in common with scrofulaceum—so much so that if only standard, commonly determined characteristics are considered, it can be called "scrofulaceum"—it is still evident that by other parameters one of which may well be the disease leprosy, the leprosy derived isolates are not a simple contaminant and are not biologically identical with bacillary strains commonly designated as scrofulaceum. Some uncommon, open-minded, and more sophisticated taxonomic studies are indicated before rushing to judgement.

The report by Stanford *et al* ([38]) also noted drug sensitivity studies on HI-75 and, concluding that this culture was not sensitive to DDS, doubted that it is *M. leprae.* However, a subsequent study by Kato *et al* ([17]) originating in three separate laboratories but reported in a joint publication as producing identical results, found to the contrary. The HI-75 and Dakar strains were found to differ from *M. scrofulaceum* (four strains) but to have drug sensitivities identical to those of *M. leprae* as found in the human patient. The authors propose that the *in vitro* cultured bacilli be used in the search for antileprosy drugs on the basis that this model is more accurate, less expensive and far less time consuming that utilization of the mouse foot pad.

DISCUSSION

It is evident that the identification of *M. leprae* in *in vitro* cultures, be the reported cultures true *M. leprae* or not, is a far more complicated problem ([34]) than was envisaged when criteria for such identification were laid down by the VIII and IX International Leprosy Congresses in 1963 and 1968

([28,42]). Despite long-held supposition that *M. leprae* might have unusual or fastidious energy requirements, it is becoming evident from studies of quantities of armadillo-derived bacilli that *M. leprae* is actually highly competent metabolically. Comparative studies of armadillo-derived bacilli and cultures of mycobacterial isolates from human and armadillo-derived bacilli and cultures of mycobacterial isolates from human and armadillo lepromas have revealed no unique, identifying metabolic characteristic ([8,9]).

Pyridine extractability of acid-fastness has been mooted as an identifying characteristic. However, this factor seems to be a function of the age of the bacilli ([36]) and is, therefore, not specific.

Thus, by far the most valuable means of

TABLE 2. M. leprae *particularities.*

IN VIVO		*IN VITRO*
Conceived	Perceived	Perceived
Slow growth	Slow, but occasionally rapid	Slow, adaptive primary culture; rapid in subculture
Uniform morphology except for degenerative changes	Spheroblast + non-acid-fast + acid-fast bacilli	Spheroblast → non-acid-fast → acid-fast bacilli
Only solid, acid-fast forms viable	"Granular" rods nonviable	Some granular forms may give rise to spheroblasts which are viable
Non-solid forms degenerative and nonviable		
Deficient metabolic competence	Highly competent metabolically by Warburg analysis	Highly competent metabolically by Warburg analysis
DOPA reaction unique	DOPA reaction invalid	DOPA reaction by culture hyaluronic acid
	Specific indentification by pyridine acid-fast extraction	Acid-fast extractability a characteristic of aged bacilli—not diagnostic
Obligate intracellular	Facultative intracellular	Obviously not obligate intracellular
		Same drug sensitivity spectrums as *in vivo*
	Specific response to LL antibody	Specific response to LL antibody
	—Direct & indirect immunofluorescence	—Direct & indirect immunofluorescence
	—Indirect immunoperoxidase	—Direct & indirect immunoperoxidase
	No Arthus reaction in LL patients to lepromin	Arthus reaction in LL to "lepromin" from some media and some culture ages
		LL Arthus reaction vitiated by lysozyme treatment—culture lepromin + Fernandez and Mitsuda reactions in TT. Mitsuda reaction may be + in LL with late, adapted subcultures
No primary growth on conventional mycobacterial media		Adapts to conventional media several subcultures

TABLE 3. *Comparative characteristics of an* in vitro *cultivated bacillary strain from human leproma and commonly designated* M. scrofulaceum *strains.*

HI-75	*M. scrofulaceum*
1. Positive reaction with FITC-LL-Ab immunofluorescence.	1. No reaction with FITC-LL-Ab immunofluorescence.
2. Positive immunoperoxidase reaction with LL-Ab.	2. No immunoperoxidase LL-Ab reaction.
3. Positive Mitsuda type reaction in tuberculoid patients at 4 weeks comparable to standard lepromin.	3. No comparable Mitsuda reaction.
4. Not agglutinated by available scotochromogenic sera ([29]).	
5. Same spectrum of drug sensitivity as *M. Leprae* in humans ([17]).	5. Different spectrum of drug sensitivity than *M. leprae* ([17]).
6. No infection of Korean chipmunk within 10 weeks ([21]).	6. Disseminated infection in Korean chipmunk in 2-10 weeks ([21]).
7. No growth of initial isolate on common mycobacterial media.	7. Direct growth on common mycobacterial media.
8. Prolonged adaptive period *in vitro* before cultivation is achieved and gradual subculture adaptation to common mycobacterial media.	8. No prolonged adaptation period before growth after isolation from tissues.

identification have been direct and indirect immunofluorescence and immunoperoxidase technics. Relating to percentage of bacilli positive in the preparations of specimens examined, the direct method yields higher results with cultivated organisms and the indirect with bacilli directly isolated from tissues. The difference appears to lie in factors related to host-derived accretions, e.g., antibodies, on bacilli thence derived as has been previously discussed ([33]). Some modifications in the indirect immunofluorescent technic as applied to leprosy, according to work in progress, seems to considerably enhance the percentage of positively reacting bacilli from cultures.

The lack of evidence that *M. leprae* and cultivated isolates from lepromas utilize hyaluronic acid for energy derivation, the evidence that both isolate types are highly competent metabolically and the long adaptive incubation period required for growth to occur in the culture media suggests that the problem of cultivation lies elsewhere than with some exotic energy pathway requirement.

On these bases the remarkable effect of hyaluronic acid on mycobacteriosis initiated in the armadillo by mycobacterial isolates from human lepromas suggest that HA facilitates extracellular existence on the part of the pathogen and permits more rapid increase in the numbers of bacilli present than is the case with similar infection not reinforced by HA supplementation. *M. leprae* is thus revealed as a facultative rather than obligate intracellular parasite.

Illustrations and descriptions in published accounts ([19, 20]) of the histopathology of armadillo infection by mycobacterial isolates from human lepromas indicate that in these, non-HA supplemented, hosts there also occur large extracellular microcolonies. Armadillos are far more susceptible to rapid, widespread and heavy *M. leprae* infection than human subjects. It may well be that their macrophages are less debilitative of bacillary cell walls than human macrophages, thus permitting some extracellular growth in the presence of intercellular constituents—a trend enhanced by supplemented HA.

Thus far cultures are easier to achieve from armadillo than from human untreated lepromas.

The concept of "leaky" pathogen cell walls ([6, 27]) occasioned by continuous exposure to macrophage enzymes during intracellular parasitism coupled with the knowledge that polysaccharides are important components of the bacillary walls suggests

that the presence of excess quantities in the extracellular environment enables the bacilli to repair the cell wall defect and maintain the integrity of the bacilli. The large extracellular colonies in these armadillos appear to be derived from the intracellular microcolonies ("globi" in human LL) on dissolution of their host macrophages. They are then too large to be rephagocytosed and continue to grow extracellularly. It may be that the prominent acid mucopolysaccharide (including HA) in lepromatous macrophages ([35]) enables *M. leprae* to maintain its cell walls, despite effects of cell enzymes on it, in a fashion adequate for survival and reproduction as it lives in the intracellular nutrient bath until accumulation of its lipid metabolic products vitiates the macrophage which is now a "foam" cell and can no longer support the life of the bacilli.

Of course, polysaccharides are not the only components of capsules that may be vitiated by intracellular life and HA is probably only one approach, but probably an adequate one, to wall constitution. The recent reports of Kato and associates ([11, 16]) on the significance of cholesterol open new vistas.

Assuming that the witness of immunofluorescence and immunoperoxidase technics to the *M. leprae* identity of the *in vitro* cultivated isolates, supported by other observations such as their sameness in drug sensitivity ([17]), is true, then many of the unexpected *in vitro* findings can be related to the changes in cell walls occasioned by cultivation. Thus, increased mycobacterial antigenicity might well result in a change in the fragile balance between host defense and pathogen in the mouse foot pad. On the other hand, the Korean chipmunk differentiation from *M. scrofulaceum* is not nullified because this is based on the pathogenicity of *M. scrofulaceum* for this experimental animal. The failure of guinea pigs to demonstrate growth of *M. leprae* in the presence of HA supplementation is also not remarkable in view of the noted ability of this host to develop cell-mediated immunity. This would not be likely to be nullified by increased antigenicity of the pathogen.

Thus far the cultured leprosy isolates have been of smooth colony variety. Rough colony varieties can be anticipated either among direct isolates or by changed cultivation technics. Collaborating scientists already indicate that this has been achieved in as yet unpublished results. Since, generally, rough colony variants of mycobacteria are more pathogenic than the smooth, dissociation into the former would suggest that the first cultivation technics are not optimum but are an on-ramp to the highway of cultivation understanding.

It is becoming evident that there is nothing mythical about the double existence of *M. leprae,* imaginatively designated its "Janus Face" by Kato ([13]). This can now be hypothetically formulated as the saga of a pathogen having at some time in the dim past, and perhaps even now in an undetected state, the ability to survive in nature elsewhere than in the human host. Somehow, somewhere, perhaps repetitively and possibly even today under uncommon circumstances this pathogen, which was probably highly competent metabolically, attained disease-producing entrance to the human host. By virtue of its particularity it was able to survive distribution in a proportion of its host subjects probably because the pathogen did not possess significant tissue destructive potential and because the deficient host was unable to process this pathogen's constituents into an effective immunologic stimulant. Even the immunologically deficient host cell environment, however, is not uninimicable to the pathogen. Quite possibly its ability to produce resistant reproductive cocco-bacillary forms was significant to its survival and transmission. *In vitro* cultivation of the pathogen removes this inimical influence, permitting the pathogen to re-establish its genetically retained characteristics and reveal its previously unseen second face.

The intracellular existence of *M. leprae* is unique in that even on transfer from host to host it is virtually a macrophage to macrophage transfer. There is not known to be, and epidemiologic evidence speaks against, any intermediate host or environmental period of recuperation from the effects of intracellular habitation.

This interpretive formulation is clearly hypothetical but data suggesting the formulation have been presented. If there be in it a modicum of actuality, then the cultivation of this pathogen provides a unique model, which can be repeated again and again from human, armadillo and, perhaps, other sources for comparative study of the effect

of adapted intracellular life as against extracellular relicative existence.

SUMMARY

Progress is summarized relating to the verification, identification of *M. leprae* and understanding of the process of adaptation the pathogen passes through before *in vitro* growth takes place. It is recognized that hyaluronic acid apparently does not serve as a source of energy but the possibility is presented that it plays a role in the reconstruction of *M. leprae* cell walls made "leaky" by constant intracellular life. This apparently occurs, in culture, initially by the development of coccoid forms which after a period of weeks finally give rise to acid-fast bacilli.

If these understandings are correct and the bacillary cell walls are vitiated by enzyme and other action occasioned by intra-macrophage existence, then cell wall antigenicity may also be vitiated or altered by intracellular parasitism and restored by *in vitro* cultivation. The possible importance of this hypothesis in the understanding of immunologic responses in leprosy, and in the possibilities for therapeutic use and vaccine development are discussed.

RESUMEN

En este trabajo se resumen los resultados recientes sobre la verificación e identificación del *M. leprae,* y sobre lo que sabemos acerca del proceso de adaptación que sufre el patógeno antes de que ocurra su crecimiento *in vitro.* Reconocemos que el ácido hialurónico parece no servir como fuente de energía para el parásito, pero presentamos la posibilidad de que éste participe en la reconstrucción de las paredes celulares del *M. leprae,* las cuales pueden hacerse "demasiado permeables" debido a la constante vida intracelular. Esto parece ocurrir en los cultivos, en donde inicialmente se desarrollan formas cocoides que después de algunas semanas dan origen a los bacilos ácidoresistentes.

Si esta idea es correcta y si las paredes celulares del bacilo son alteradas por la actividad enzimática y por otras condiciones de la vida intra-macrofágica, entónces, la antigénicidad de las paredes celulares también podría resultar alterada por el parasitismo intracelular. Esta alteración podria ser restaurada por el cultivo *in vitro.*

Se discute la importancia potencial de esta hipótesis en relación con: el mejor entendimiento de la respuesta inmune en la lepra, la posibilidad de una nueva forma de terápia, y el posible desarrollo de una vacuna.

RÉSUMÉ

On a resumé le progrès relatif à la vérification, l'identification de *M. leprae,* et la compréhension de la méthode de l'adaptation de la pathogène avant qu'elle puisse pousser *in vitro.* On reconnait que l'acide hyaluronique apparemment ne sert pas comme source de l'énergie, mais on a présenté la possibilité qu'il joue un rôle dans la reconstruction de la paroi cellulaire de *M. leprae* rendue "leaky" (qui coule) par son existence constante intracellulaire. En culture, *M. leprae,* apparemment, se développe au commencement en formes coccoïdes et après quelques semaines les formes coccoïdes donnent naissance aux BAR.

Si ces explications sont applicables et les parois cellulaires des bacilles sont viciées par activité enzymatique ou d'autre action occasionée par l'existence dans les macrophages, on peut proposer que la qualité antigénique des parois cellulaire pourrait être changée par parasitisme intracellulaire et restituée par cultivation *in vitro.* L'importance de cette hypothèse dans la compréhension des réponses immunologiques dans la lèpre, les possibilités thérapeutiques, et le développement d'un vaccin sont mentionnés.

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