THE FATE OF LEPROSY BACILLI IN FIBROBLASTS CULTIVATED FROM MACULAR AND TUBERCULOID LESIONS

by

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A preceding communication (1) reported the maintenance of fibroblasts from lepromata in serum media for periods of 14 to 34 days. The heavily bacillated macrophages died *in situ* and deposited their bacilli within the original explants. A relatively low proportion of smaller round cells which sometimes migrated into the plasma, were not sustained by serum media, as had been hoped, and disappeared early in the history of the cultures. The fibroblasts which persisted throughout the history of the cultures contained relatively few bacilli. Quantitative rating of the bacillary content of the original explants failed to disclose any increase in the total mass of the bacilli during tissue cultivation, a result in agreement with earlier microscopic counts in lepromatous tissue fragments maintained under bacteriological conditions (2). Since any bacilli which might be growing in these cells could not be distinguished from those acquired by phagocytosis it was not possible at that time to settle the question of bacillary multiplication in the growing cells.

This work was next extended to a study of the fate of the bacilli in fibroblasts from macular and tuberculoid lesions. The low bacillary content of these tissues appeared unlikely to interfere with learning how to maintain cells for long periods of time and to minimize the danger of reaching unsound conclusions because of excessive numbers of bacilli such as occur in lepromatous lesions. Being derived from persons with appreciable resistance to the disease, the reaction of such cells to leprosy bacilli was also of interest.

A crucial test of bacillary multiplication was based on cutting the new cell growth away from the original bacillated explants in order to learn whether the bacilli persisted or increased in successive sub-cultures. The serum media were supplemented with embryo juice to support continuous cell growth and to duplicate (in so far as it was consistent with the maintenance of cells) the con-

ditions used by earlier workers.* A partially synthetic supplement was used in a few instances in order to learn the results obtainable during extremely slow cell growth.

METHODS

The biopsied skin was first cut to the size and shape of the active margin or area of the lesion. It was then laid face down on a sterile slide and split by passing a very sharp knife parallel to the lower surface at a level which just exposed the stippling due to the papillae. This layer, including the epithelium, was then cut into explants (1-2 mm. surface dimensions) which will be designated as "papillary." A second series of explants was prepared from the deeper layer, and will be designated as "reticular."

The tissue cultures were maintained in 13 mm. serological tubes by methods already described (1). The components used in the preparation of the media were: placental serum (PL), chick embryo juice (EM), and the serum ultrafiltrate (SF) ** of Simms and Stillman (3). The composition of the various media will be given as final concentrations of each ingredient, based on 100 per cent serum, 100 per cent embryo juice (though prepared as 30 per cent), or serum ultrafiltrate. A medium composed, for example, of 25 per cent serum and 15 per cent embryo juice will be designated as: $PL_{25}EM_{15}$. The placental serum was collected as available and stored after cryochem dehydration; sodium bicarbonate, phenol red, and glucose were added from very concentrated solutions when the serum was dissolved for use. Chick embryo juice was prepared by adding two volumes of the salt solution lacking $NaHCO_3$ to each volume of pulp obtained from 11-day embryos with a Latapie grinder. This suspension was run for 30 minutes in the angle centrifuge, and the supernatant treated again in the same way. By the time the pH had been adjusted to neutrality and $NaHCO_3$ added to a concentration of 0.125 per cent, the concentration of this extract became approximately 30 per cent.

The supplementing solution (SS), modified after formulae by Vogelaar & Erlichman (4) and by Baker (5, 6) was composed to provide in the serum media: Baker's blood digest (30 mg. per cent N), Witte's peptone (100 mg. per cent N), thyroxin 0.001 mg. per cent, cysteine 5 mg. per cent and insulin 0.05 units per cent. This solution was used in the absence of any embryonic juice except that derived from hardening or patching the plasma.

An incubation temperature of 34°C. was chosen for several reasons: (a) it approximates skin temperatures more closely than does 37°C., (b) it permitted maintenance of cell populations for longer periods in the same tubes and offered greater possibility that a slow growth rate of the bacilli might keep pace with the rate of cell proliferation, and (c) it appeared

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* The studies of Timofejewsky and of Suwo and Kin were reviewed and discussed in a preceding paper (1). High concentrations of embryo juice, though the best source of the growth factors which must be added to serum media, cause the deposition of calcium phosphates in and around the explants. This precipitate is growth-inhibitory and usually fatal to the cultures. A special study, which will be reported elsewhere, was required to circumvent this difficulty.

** Purchased from Sterisol Division, Schering and Glatz, subsidiary of W. R. Warner Co., 113 West 18th Street, New York City.

possible that the lowered temperature might modify cell physiology in a manner favorable to the bacilli.

Cox (7) found that lowering the temperature from 39° to 35°C, favored maximum yields of Rickettsiae by the yolk sac method. Pinkerton (8) obtained similar results with Rickettsiae in tissue cultures. Sanders and Molloy found 23° superior to 37° for the propagation of the viruses of lymphogranuloma (9) and St. Louis encephalitis (10) in embryonic guinea pig brain cultures and of equine encephalomyelitis in embryonic chick tissue cultures (11). In order to be satisfied that 34°C. was not likely to be a temperature too low for leprosy bacilli, small numbers of human tubercle bacilli (H-37) were injected into the plasma near the centers of four fibroblast cultures. Obvious growth, chiefly extracellular, occurred within two weeks and overwhelmed the cells by the end of one month.

During the course of the work it was learned that media containing embryo juice could usually be left on cultures at 34°C. for twenty-one days without disadvantage, and that fourteen days was a safe routine interval for renewals. Large cultures required replacement of the media with supplementing solution every ten or seven days. It was unnecessary to renew at all the medium on explants which failed to grow within two or three weeks, and impossible to stimulate poor cultures by more frequent replacement of the media. The plasma was patched with one small drop of 50 per cent chicken plasma and of embryo juice each two or four weeks as required.

The culture sizes to be given, based on a scale of 1 to 4 in the case of early cultures, refer to the degree to which the margins of the explants were occupied by new growth, e.g., F1 means that outgrowth occupied 25 per cent of the margin and F4 that the explant was entirely surrounded by fibroblasts. An F1 rating required the presence of at least 20 cells while F2 and F3 were approximate multiples of 20 in early cultures. This simple method of description, though it does not take into account the increasing length of the fibroblast chains after the early period, is well suited to the relations to be emphasized in this and the following paper. Larger cultures are to be described as F5 and F6. The average areas of a series of cultures (of different ages and in different experiments) so designated and also measured with an ocular micrometer were: F4-5 sq. mm., F5-13 sq. mm., and F6-28 sq. mm.

The cultures were prepared for routine staining and examination as follows. The medium was replaced for two hours with the balanced salt solution (lacking $NaHCO_3$) to extract the serum; otherwise the plasma stained too intensively.* The cultures were then carefully removed from the tubes and the intact plasma was allowed to spread out on glass slides. As soon as the plasma was adherent (dry) at the margins, the slides were placed for five minutes in 60 per cent and later in 95 per cent alcohol for fixation and removal of salts and water. These preparations, which dried quickly and without distortion, were placed in Ziehl-Neelsen's carbolfuchsin for five minutes, rinsed, and then decolorized and counterstained simultaneously in Gabbet's 1:5 for 5 minutes. This method possessed the advantage of presenting each culture in its entirety and of being adapted to the study of a sufficient number of cultures to afford reliable comparisons.

* The presence of even small concentrations of formaldehyde in the extracting fluid or in the alcohols makes the plasma stain too deeply.

When large cultures were to be sampled and also cut for sub-cultures, a fan-shaped portion of plasma was left attached to the original explant so as to include all the cells from the center to the periphery of each sector sampled.

In the case of simple macular lesions the total numbers of bacilli in each culture were counted. The micro-organisms in the cells of early outgrowths from tuberculoid lesions were too numerous to permit counting the bacilli per cell. Hence, results were based on the percentages of cells which were found to contain bacilli. Since different proportions of positive cells tended to be arranged concentrically with respect to the explants, the counts were always made as though along the radii of circles. When several cultures of ordinary size were to form a pooled sample, it was the general practice to count 200 cells in each. In small cultures a record was kept for all cells. If 8 or more cultures were to form a single sample, only 100 cells were observed in each. This system worked out in such a way that the results in most instances were based on observations in 400 to 1200 cells. The percentages of cells containing bacilli was related to the concentrations per cell, and low percentages may be understood to indicate small numbers of bacilli in the positive cells.

EXPERIMENTAL RESULTS

Observations relating to the tissue cultures themselves will first be presented separately in order to simplify the summary of the individual experiments.

Tissue Cultures

The tissues explanted from the reticular layer of skin produced outgrowth within three to ten days. The cultures showed a predominance of spindle-shaped fibroblasts, and were more difficult than papillary cultures to maintain in a given series of cultures for prolonged periods. They differed from those of the papillary layer, or from lepromata, in that sheets or masses of epithelial cells from hair follicles and sweat glands were often prominent during the first generation. These epithelial structures occasionally grew in tube or rod-like forms directly out into the plasma. Since the epithelial cells were not observed to contain bacilli, no attempt was made to carry them forward in successive generations, but they sometimes persisted into the second or even third series of subcultures. In short, these cultures from the reticular layer were more complex, more inclined to rapid growth, and less easily maintained than the outgrowths of the papillary layer.

The outgrowth from explants of the papillary layer was more delayed, usually appearing after seven to twenty days. Explants destined to become positive were characterized within the first two weeks by the occurrence of pigmented epithelial or melanoblastic cells in the area immediately surrounding the explants. These cells often showed sprouting or slight elongation and persisted for sev-

eral weeks before being obscured by more rapidly growing fibroblasts. Some fibroblasts contained pigment when first seen and others acquired it later. Since many of the early cells contained both bacilli and pigment granules, the persistence of the inert particles after prolonged cultivation was eventually recognized to indicate that the bacilli seen in the same cells were likely also to have been derived from the original explants. Since the fibroblasts from the papillary layer were hardier than those from the reticulum, more prone to branch and ramify, possessed automatically of control particles, and their cultures were not complicated by persisting epithelium, they came to be the preferred type with which to work. Furthermore, Wade's description of tuberculoid lesions in African adults reveals that the epithelioid foci were found earlier and distributed more uniformly in this layer than they were around the nerves, vessels, follicles, and glands of the reticular layer (12). The earliest lesions in Philippine children born of leprous parents were found by Nolasco and Lara (13) likewise to involve the papillary layer more frequently than the structures of the reticular layer.

Infected round cells, or histiocytes with expanded cytoplasm, were not seen, and their ability to migrate from the tissues is doubted. Smaller cells of the monocytic or lymphocytic series were observed in a moderate proportion of early cultures, occurring more frequently than from lepromatous tissues. Monocytes, when present, wandered out into the plasma and matured into macrophages, but soon disappeared among the encroaching fibroblasts. They were not found in subsequent subcultures, and are not believed to have played any role in the fate of the bacilli.

As in the case of the lepromata cultivated earlier (1), serum media were capable of maintaining the fibroblasts from tuberculoid lesions for approximately one month. Frequent renewals hastened the removal of cell extractives from the explants and the plasma, and proved incapable of improving cultures or prolonging growth. $PL_{33}SF_{33}$ was superior to the serum media and maintained initial cultures for six (or eight) weeks. $PL_{25}EM_{15}$ markedly enhanced growth during one or two applications of the medium, after which the cultures became calcified and were usually lost. Higher concentrations of embryo juice were intolerable. $PL_{40}EM_{5}$ and $PL_{25}SF_{25}EM_{5}$ proved to be suitable for a long-term maintenance, since they provided sufficient growth-essential substance for fourteen days between renewals and did not induce calcification.

Serum medium with the supplementing solution ($PL_{33}SS$) proved of great interest because it provided for maintenance with approximately one-half the growth rate in the embryo juice media.

The restriction of cell division usually did not interfere with considerable increases in cell size. In one instance these two processes were so markedly distinguished that many cells attained lengths in excess of 1000 microns (1 mm.) and could actually be seen with the naked eye. The oxidation-reduction potential of this medium is apparently higher than that of media with embryo juice and the original explants remain populated with some cells for a longer time.

Several abnormal states of fibroblasts must be noted as not attributable to the presence of bacilli. Enormous cells were produced also in embryo juice cultures kept at room temperature (approx. 30°C.) to restrict the growth rate, but this temperature was employed in only a few observations since it did not permit continuous cultivation of fibroblasts. Under critically unfavorable conditions the fibroblasts expired by lysis, but if for one reason or another the growth became simply "stalled" their senescence was characterized by a gradual "balling up" until they presented the superficial aspects of large macrophages.

Low power examination of growing fibroblasts revealed no peculiarities characteristic of the presence or digestion of bacilli. In stained preparations from cultures which were stalled or growing slowly, cells containing 15 or more bacilli often appeared to be damaged; smaller numbers of bacilli occurred dispersely and were apparently well tolerated. In active cultures, on the other hand, the cells did not tolerate more than 15 or so bacilli without isolating the micro-organisms into segregation vacuoles near the nucleus and rapidly digesting them. Smaller number of bacilli were likely to remain dispersed and to excite no obvious cell response. Even in the absence of noticeable cell reaction, both intra- and extra-cellular bacilli began to show evidence of autolysis or extraction after six to twelve weeks. The single bacilli became difficult to find, while those in clumps were pale and fused.

Summary of Experiments

Experiment 1: Explants were prepared from the reticular layer of skin from the slightly reddened and elevated border of a macule in a child of 4½ years. This lesion presented tuberculoid characteristics and a moderate number of bacilli. The group of cultures in PL_{25} underwent lysis after intervals of 23-33 days, while those in $PL_{25}SF_{25}$ remained viable for 32 to 45 days and terminated as balled fibroblasts. Only those in $PL_{25}EM_{15}$ were suitable for preparing a second series of cultures after 32 days and for a third after 61 days. Due to difficulties with calcification of the plasma, only 3 cultures remained by the end of the third series and a total period of 107 days. Cultures sampled between the 20th and 32nd days exhibited from zero to moderate numbers of cells containing bacilli. The numbers of such cells varied greatly from culture to culture and from place

to place in a given culture. Inspection of all the cells remaining after 107 days revealed one cell with one bacillus.

Experiment 2: Explants were prepared from both layers of skin from an anesthetic macule in a child of 4 years. Sections revealed that this tissue contained a very small number of bacilli, several hours being required to find them.

Reticular cultures in PL_{25} failed after the usual interval, while those in $PL_{25}SF_{25}$ were cut for a second series at 18 days, and for a third after 30 days. These cultures were failing before the end of 60 days and were not carried further. Cultures in $PL_{25}EM_{15}$ were cut for a second series after 18 days, a third after 30 days, a fourth after 60 days, and a fifth after 79 days. These short intervals of transfer were always necessitated by a threatening degree of calcification. The cultures of the fifth series were kept in the same tubes from the 79th to the 116th days and became too calcified to be carried forward.

The greatest concentration of bacilli encountered in this experiment were present in circumscribed vacuoles in the cells of 12 cultures studied after 18 days, but were so obscured by acid-fast debris that their numbers could not be determined. The bacilli were found only after further decolorization of some of the preparations. Since this procedure was not standard and may have decolorized some of the bacilli, total counts for bacilli were not made. In 3 cultures counted for proportions, 5.4 per cent of the cells contained this debris. Forty-four bacilli were observed in the cells of 7 of 12 cultures after 30 days, and 17 bacilli in 4 of 16 cultures after 60 days. No bacilli were found in 8 cultures after 79 days, or in 10 cultures after 116 days. Since the original explants, with attached outgrowth, were divided in preparing the second and the third series of cultures, this experiment should be regarded as comprised partly of outgrowth from explants for the first 60 days and of outgrowth exclusively thereafter.

The papillary explants exhibited pigmented epithelial cells within 72 hours, but the general outgrowth of fibroblasts occurred between the 18th and the 40th days. The first series was maintained in the original tubes for 63 days, the second for 25 days (total 88), the third for 56 days (total 144), the fourth for 35 days (total 179), and the fifth series for 35 days (total 214, or 7 months). The short interval in the second series was necessitated by threatening calcification in $PL_{25}EM_{15}$, while maintenance for 56 days in the third may be attributed to the substitution of heated for fresh embryo juice. The fourth series was maintained in embryo juice of three experimental modifications. It was necessary to terminate the fifth series after 35 days, while some good cells remained, because of poor growth in juice from 9-day embryo.

The examination of 9 cultures after 63 days and of only 1 after 88 days failed to disclose bacilli. Eleven bacilli were found in 7 of 18 cultures after 144 days. Bacilli were not found in 12 cultures after 179 days,* or in 7 cultures after 214 days when the experiment was terminated. Small portions of explants were present in these cultures for 144 days.

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* Two of 4 cultures which were too small to be divided at 179 days revealed 16 bacilli after a total of 70 days in the fourth series. Both of these were derived from a culture known to contain bacilli in the third series.

Experiment 3: Explants were prepared from both the reticular and the papillary layers of skin from a macule in a child of 4 years. Bacilli were extremely difficult to find in the control material.

Reticular explants were maintained in the first series for 23 days, in the second for 19 days (total 42), in the third for 16 days (total 58), in the fourth for 14 days (total 72), and in the fifth for 56 days (total 128), which proved to be too long for these very active cultures, and they were lost. All series after the first were maintained in PL_{25} and EM_{15} of different sources and experimental modifications. The growth results demonstrated that the extracts of 9-day embryos avoided calcification but were incapable of maintaining cultures, and that heating of 11-day embryo juice at 65°C. for 30 minutes was the only successful modification. This heated solution in 15 per cent concentration did not cause calcification and maintained continuous cultures.

Two of 10 cultures at 18 days revealed 15 bacilli in 2 positive cells, 2 of 5 cultures at 42 days permitted finding 12 bacilli in 2 positive cells, while none of 4 cultures at 58 days, of 6 cultures at 72 days, or of 10 cultures after 128 days were found to contain bacilli.

The papillary cultures exhibited the first fibroblasts between 16 and 37 days. A second series was prepared after 48 days, a third after 44 days (total 92), and a fourth after 50 days (total 142). After a further 42 days (total 182, or six months) only 3 cultures remained in condition for sampling, and the experiment was terminated. These cultures were maintained in the second and third generations with remarkable success in $PL_{43}EM_5$, and were lost in the fourth due to over-optimism concerning the longevity of an entire series of culture groups which were not renewed for an interval of 28 days.*

Four bacilli were found in 3 of 10 cultures examined after 48 days, 2 bacilli in 2 of 4 cultures after 92 days, 2 bacilli in 2 of 12 cultures after 142 days, and 1 in the 3 cultures after 182 days.

The results of experiments 2 and 3 are summarized in table 1, in terms of the average number of bacilli found per culture. The time intervals are rounded off in weeks, while serial subcultures are indicated by small Roman numerals. The fibroblasts from reticular explants, which gave prompt outgrowth, revealed the greatest numbers of bacilli during the first 6 weeks of their history. They were found to be devoid of bacilli after subcultures were prepared exclusively from outgrowth. The fibroblasts from the papillary explants, which were characterized by delayed outgrowth, were found to exhibit the greatest numbers of bacilli at later intervals. They revealed some bacilli in the fourth series, after cultures were made exclusively from outgrowths, but the positive cells usually contained also pigment granules. These cells passed through serial subcultivation more

* It had previously been learned that intervals of 3, and sometimes of 4, weeks between renewals of $PL_{25}EM_{15}H$ (heated) were tolerated without stoppage of growth. Since results in the foregoing experiments had demonstrated that the bacilli disappeared quickly from the cultures with normal or maximum growth rates, this and other attempts were constantly being made to obtain a "stalling" of the growth rates and to maintain fairly stable cell population as *in vivo*. It was hoped that the original bacillary inoculum might be retained for long periods without dilution by cell growth. Unfortunately, if growth ceased entirely it could not be induced again, though the cells (as in this instance) might remain in good condition for extended periods of time.

TABLE 1. *Average numbers of intracellular bacilli per entire fibroblast culture from macular lesions.*

Experiment number	Cultures from reticular layer (weeks) 3	4	6	8	9	10	11	17	18
2	—(I)†	3.7(II)*			1.1(III)*		0.0(IV)	0.0(V)	
3	1.5(I)*		2.4(II)*	0.0(III)*		0.0(IV)			0.0(V)

	Cultures from papillary layer (weeks) 7	9	13	20	26	30
2		0.0(I)*	(II)*	0.6(III)*	0.0(IV)	0.0(V)
3	0.4(I)*		0.5(II)*	0.17(III)*	0.3(IV)	

† Approximately 5% of cells contained acid-fast debris, and also the greatest numbers of bacilli seen in the experiment.
* Indicates the period during which portions of original tissue explants were present in some of the cultures.
Roman numerals indicate the culture series as produced by transferring cells to new tubes.

slowly, and a separate time scale is required to show the results. Equal, or sometimes greater, numbers of bacilli were observed free in the plasma of sampled cultures, but since they correspond more or less with the intracellular bacilli as recorded, details are not included.

Experiment 4: Explants were prepared from the papillary layer of tuberculoid skin from a girl of 17 years, who had neural deformities of the hands and was badly scarred from lepra reaction three years previously. The lesion contained a moderate number of bacilli. The basic experimental control, however, now consisted of a group of cultures sampled after 14 days. The combination of serum with the supplementing solution ($PL_{33}SS$) permitted cultivation for approximately 6 months with only 2 series of subcultures.

Examination of 7 representative cultures after 2 weeks revealed bacilli in 2.5 per cent of the cells (see Table 2 and Figure 1). The remaining cultures were maintained for 9 weeks in the original tubes, when 6 which were sampled showed 8.3 per cent of cells with bacilli.

In the second series one group of subcultures was found contaminated after 3 weeks (total 12). Counts were made in 6 of those only slightly in-

TABLE 2. *Percentages of fibroblasts containing bacilli during the propagation of slow growing cultures from a tuberculoid lesion, Experiment 4.*

Series	Cultivation periods (weeks)	Cultures sampled Number	Average size	Per cent cells with bacilli Range	Average per cent
I	2	7	F 3.9	0.0 — 4.9	2.5
	9	6	F 5.0	2.0 — 16.0	8.3
II	12	6(1 ex)*	F 2.0	2.8 — 26.6	13.7**
	17	7(3 ex)	F 4.7	0.0 — 19.0	7.0
	22	2(1 ex)	F 3.5	0.0 — 3.0	1.5
III	26	10(2 ex)	F 4.1	0.0 — 3.0	0.7

*ex indicates cultures which contained one-half of an original explant. The average percentage at 12 weeks was increased slightly by the explant culture included; the average for 17 weeks was decreased slightly, since the three cultures containing explants were large; the figure for 22 weeks was due to a small explant culture; while the figure for 26 weeks was decreased by the inclusion of explant cultures.
**Bacilli were paler and showed the first signs of disintegration.
Practically every cell with bacilli contained also pigment granules.

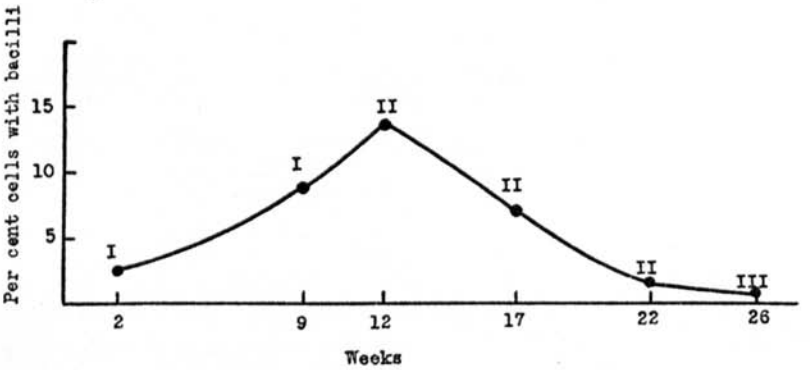

Figure 1. Delayed bacillation of fibroblasts from a tuberculoid lesion (See Table 2).

volved, and revealed 13.7 per cent of cells with bacilli. These micro-organisms already looked paler and more extracted than those at the outset and were usually associated with pigment granules. Seven cultures after 8 weeks in this series (total 17) averaged 7 per cent of cells with bacilli. Two cultures after 13 weeks in the second series (total 22) contained bacilli in 1.5 per cent of the cells.

A third series of cultures prepared after 8 weeks in the second series were maintained for 9 weeks (total 26 weeks, or 6 months). Ten cultures sampled after this interval revealed bacilli in only 0.7 per cent of the cells. Almost every cell with bacilli at this late date contained also remnants of pigment granules from the epidermis and the bacilli were very indistinct.

In order not to create the impression, from the average percentages shown, that results from individual tissue cultures were uniform or readily interpreted, the histories of 4 cultures of experiment 4 are set forth diagrammatically in figure 2. The circular areas, or their fractions, indicate the size of each culture when sampled, while darkened squares within the circle denote subcultures which received any portion of an original explant from the lesion. Attention is called to the disparity among the percentages of bacillated cells in the individual cultures of the first series after 9 weeks. Irregular distribution of bacilli within individual explants was revealed in one culture by proportions as high as 40 per cent of bacillated cells in one region and by an absence of bacilli in other areas. This illustrates the likelihood of unequal concentrations of bacilli in the four original explants themselves. This factor, however, was not the only determinant of the results, as may be seen be references to table 3, which indicates the rapidity with which the primary cultures attained their ultimate sizes. It may be seen that the sequence of sizes after 2, 4, and 6 weeks was related inversely to the proportion of cells later found to contain bacilli.*

* It is believed that unfaorable conditions of pH, oxidation, or nutrition occur in the centers of the larger cultures and inhibit continuous transport of the available bacilli. These factors doubtless determine an optimal zone of growth, lying concentrically around the center of each culture and at an increasing radial distance as the culture expands. In cultures which were growing slowly, a steady acquisition of pigment granules could be observed. In rapidly growing cultures the concentric optimal zone moves outward, while continuous subdivision of the bacilli or pigment granules among the expanding cell population soon decreases their incidence.

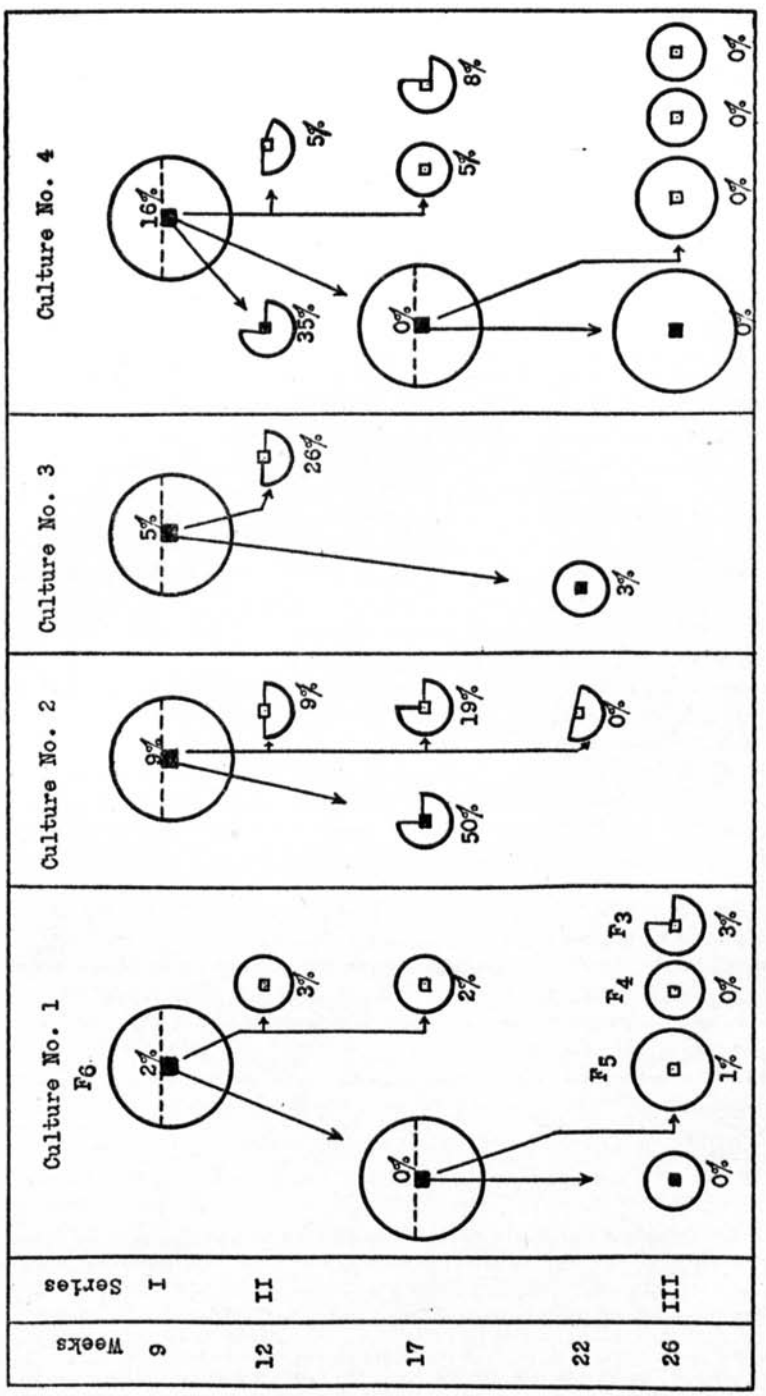

Figure 2. The variability of results in individual explants and their subcultures, Experiment 4.

The primary cultures are arranged according to their rate of growth in the first series (See Table 3). Percentages within circles and above the broken lines show that one-half of the culture was studied and the other half used for subcultures. Replanted portions of original explants are indicated by solid squares, and replanted outgrowth by open squares at the right side of each diagram. The sizes of circles (or fractions) denote the sizes of the individual cultures.

TABLE 3. *Inverse relationship between rate of culture growth and average percentages of fibroblasts with bacilli, Experiment 4, Series I, Figure 2.*

Culture number	Size of cultures (weeks) 2	4	6	8	Per cent cells with bacilli after 9 weeks
1	F 4	F 5	100*	110	2.0
2	F 1	F 4	90th	100	9.0
3	F 1	F 3	90th	100	5.0
4	F 1	F 2	80th	95	16.0

*Sizes at 6 and 8 weeks are diameters in ocular spaces; they correspond to F 6. th indicates thin cultures, cells not crowded to standard density.

The 4 small cultures in the second series, which were prepared from primary outgrowth and sampled after 12 weeks, contained a higher proportion of cells with bacilli (10 per cent) than was recorded in the primary outgrowth (8 per cent). It should be pointed out that the results in the primary series express the average degree of bacillation from the center to the periphery of each culture, while the new explants for the second series (as usual) were cut from outgrowth immediately adjacent to the primary explants, and contained cells richer in bacilli than the average. It happened that another 4 of these cultures from primary outgrowth remained practically stationary in size until 17 weeks, and still contained about 8 per cent of cells with bacilli. This result does not suggest bacillary multiplication in the second generation, and leaves the explanation on a mechanical basis as the only useful interpretation.

The replanting of the primary explants into the second series produced 2 large cultures (200 cells observed in each) in which bacilli were not found, and 2 small cultures in which 35 per cent and 50 per cent of the cells contained bacilli. These observations substantiate what has been said about the effect of rapid growth and of large size on the proportion of cells with bacilli. A third small culture was maintained in this series for 90 days, and revealed only 3 per cent of cells with pale and disintegrating bacilli.

In the third generation all cultures derived from the source with the highest incidence of bacilli in the first series (culture No. 4) were found without bacilli, while cultures descended from the source of lowest incidence (culture No. 1) still contained bacilli. It appears that the culture which transported the greatest number of bacilli from the primary explant during the first series left fewer bacilli to be carried out later, while the primary explant from which transportation was inhibited was able later to supply bacilli again.

Experiment 5: A second biopsy was obtained from the same patient with the hope of making further study of the increasing percentages of cells with bacilli during the early course of the experiment, but the results resembled those to be described in connection with lepromata. Three cultures were sampled at 2 weeks for control purposes and revealed 13 per cent of the cells to contain bacilli. Four groups of 8 cultures each were maintained in $PL_{33}SS$ in the original tubes from the 2nd to the 8th week under the oxygen and carbon dioxide pressures indicated in table 4. An accident, which destroyed all the cultures in all experiments occurred just after the preparation of a second series of cultures at 8 weeks, and terminated the experiment.

TABLE 4. *Percentages of fibroblasts containing bacilli during the first series of more rapidly growing cultures (from the patient who furnished the preceding lesion), Experiment 5.*

Growth period (weeks)	Number of cultures sampled	Conditions of incubation			Average size of cultures	Per cent cells with bacilli
		Temperature (°C.)	Oxygen (%)	CO_2 mm.		
2	3	34	8	30	F 3.5	13.0
	7	34	20	30	F 3.5	4.8
8	5	34	8	30	F 4.0	2.9
	5	34	8	60	F 5.0	2.5*
	7	37	8	30	F 5.8	1.2

*Bacilli paler than in other culture groups.

Seven cultures maintained for 8 weeks at 34°C. and in the usual atmosphere averaged F 3.5 in size and 4.8 per cent for cells with bacilli. Five cultures under 8 per cent oxygen and 30 mm. CO_2 averaged F 4.0 in size and 2.9 per cent for cells with bacilli. Five cultures under 8 per cent oxygen and 60 mm. CO_2 averaged F 5.0 in size and 2.5 per cent of the cells contained bacilli. Seven cultures at 37°C. and under 8 per cent oxygen averaged F 5.8 in size and revealed 1.2 per cent of cells with bacilli. The group of cultures under 60 mm. CO_2 was distinguished from the others by the presence of very pale bacilli and the fact that pigment granules were rare. After 8 weeks the bacilli in all cultures were found in circumscribed vacuoles and the corresponding cells usually contained also pigment granules. As shown in figure 3, the percentages of cells containing bacilli correlated inversely with the average sizes attained by the different groups of cultures, a relationship which will be strongly emphasized in the following paper.

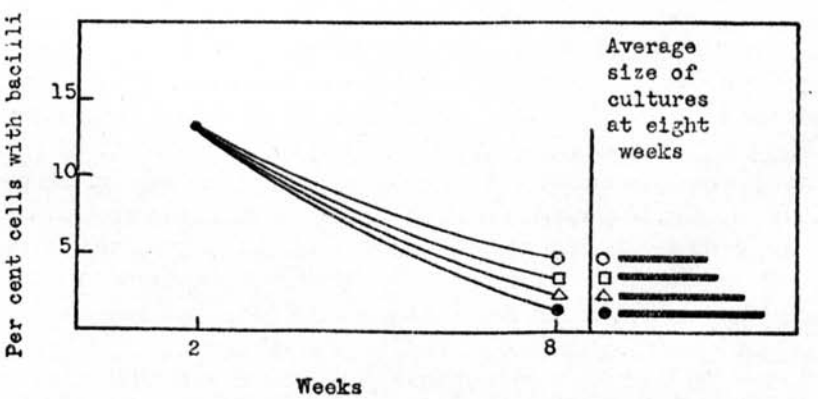

Figure 3. Proportion of cells containing bacilli, as related to culture size (See Table 4).

The last two experiments, though carried out with lesions from the same individual, gave strikingly different results in the same type of medium. In experiment 4 the primary outgrowth was delayed, developed slowly, and was composed of only a moderate number of enormous cells. These cells, which exhibited for a time an increasing bacillary content, divided more slowly than in any cultures studied before or since. They retained active relations with the explants for an unusual period of time, as was

evident from the impressive accumulation in the cells of pigment granules from the explants during the first nine weeks. In experiment 5, the cultures showed more rapid growth and division of the cells. The explants sampled after 2 weeks were found to be populated with cells, while those in the cultures after 8 weeks were not. There was never any visible increase in the pigment content of cells during the first generation. Thus, in one experiment the fibroblasts developed along the lines already described as favorable for the phagocytosis and transport of bacilli or pigment, while in the other the factors limiting transport and increasing the subdivision of the particles among the new cells were predominant.

DISCUSSION

These attempts to determine with some degree of accuracy the fate of leprosy bacilli in a constantly changing population of fibroblasts has revealed three methods by which results may be determined and expressed. The method of counting the total bacilli per culture was most tedious, but the only means of disclosing the bacilli in cultures from macular lesions. It revealed in two instances that the maximum number of intracellular bacilli occurred in the early samples of cells. In three instances, the maximum number of bacilli was observed after intervals of 6, 13, and 20 weeks, but still within the period when portions of original explants were present in some of the cultures. For reasons already discussed, this phenomenon was finally attributed to delayed transport of the bacilli from the primary explants. The method based on the proportion of cells containing bacilli, which in reality aims at comparing the growth rate of the bacilli with the growth rate of the cells, showed a complete disparity between the two rates. This method, like the first, was found in one experiment to reveal for a time a rising proportion of cells with bacilli, but this trend reversed after 12 weeks and the bacilli had disappeared almost entirely by the end of six months. Comparisons of the bacilli against the growth rate of the cells required a careful analysis of the way in which cell growth acquired or subdivided its bacillary load, and more must be added to this subject in the next paper. These first two methods are incapable of settling the question of bacillary multiplication on a limited scale or for a limited time, since the remarkable transport of bacilli may be delayed or discontinuous and must always be reckoned with. The eventual fading, disintegration, and disappearance of the bacilli could be ascertained much more conveniently by simply comparing the bacilli in cultures sampled early with those to be found after cultivation intervals of three to six months. A third method, which was appreciated too late to be used in the routine counts, is to rely upon non-proliferating control particles. In spite of any discrepancies between bacillary and cell growth, and regardless of the relative importance of the phagocyting and the

subdividing action of the growing fibroblasts, particles such as pigment granules could be relied upon to indicate that cells containing bacilli after the longest intervals were likely also to contain material from the original explants.

The advancing border of the tuberculoid lesion creates a stronger impression of the presence of viable leprosy bacilli than may, it seems to me, be gained by observing any other form of leprosy. The study of these tissues has furnished more interesting intimations of an occasional or limited activity on the part of the bacilli than have been seen in lepromatous materials. On one occasion a group of five acid-fast bacilli inside a cell was arranged as though it had arisen by a series of divisions, like the branches of a liverwort or of coral, with the larger ends of the tapering bacilli all pointing in the same direction. In another instance, groups of bacilli within several cells of a single culture appeared to have arisen by cross-branching or lateral sprouting. Each sprout had its base located exactly upon a granule in the presumably original bacillus. No sprouts or other bacilli occurred haphazardly at other points. Many other suggestive arrangements, of course, were observed, but their interpretation seems inadvisable, since the presence of any bacilli aside from a single compact group provides a possibility of chance arrangements. It appears doubtful that the groups mentioned could have been phagocyted without disturbing the original arrangement of the bacilli, and quite possible that they arose from limited activity within the cells. Such observations were confined to primary cultures from the papillary layer of skin which remained almost stationary in size.

Two types of bacilli found free in the plasma of the macular cultures, which were searched completely, also remain of undetermined significance. The plasma of some of the cultures contained an occasional acid-fast bacillus of unusual length, sometimes as long as three or four ordinary bacilli, but always devoid of branching or other distinctive features of multiplying acid-fast bacteria. In a few cases paired bacilli lying side by side, with their granules in precisely similar locations, were observed to have red granules in one end of the rods and blue ones at the other. In four instances when one-half of a culture had been used to prepare subcultures, the sampled portion revealed from 10 to 50 blue micro-organisms of morphology resembling leprosy bacilli. But in each case there followed no contamination among the subcultures, and no especial content of acid-fast bacteria. It seems doubtful that these blue forms can be dismissed as contaminants.

The morphological forms of bacilli suggestive of activity within fibroblasts have been noted as occurring in inactive cultures

which were terminated by sampling, i.e., in those which did not develop sufficiently high growth rates to be continued by subcultivation. Enhanced destruction of bacilli, on the other hand, was observed under circumstances which caused, or were associated with, greater degrees of cell activity. Fibroblasts from the reticular layer of skin have been characterized as growing more vigorously than those from the papillary layer. In Experiment 2 the bacilli in the primary outgrowth from reticular explants were found to be largely disintegrated into acid-fast debris by the 18th day. Fibroblasts from the corresponding skin layer in Experiment 3, being devoid of bacilli in the third series of cultures, were infected experimentally with bacilli from a nodule. Once the set-up permitted phagocytosis of sufficient numbers of bacilli the latter were promptly segregated in vacuoles and destroyed, leaving abundant acid-fast debris. This artificially increased bacillary content made it possible to confirm the first observations in naturally infected reticular fibroblasts. The inherently less active fibroblasts from the papillary layer of a macular lesion did not produce acid-fast debris when transporting moderate numbers of bacilli from tuberculoid lesions in Experiments 4 and 5. The cultures in these two experiments, though from the same patient, showed unmistakable differences in their metabolic activity and in their action on the bacilli. In Experiment 4, growth was restrained with unusual success, and the bacilli disappeared by fading during the third and subsequent months of cultivation. The fibroblasts in Experiment 5 contained more bacilli at the outset, were more active than desired, and by the end of two months had isolated the fragmenting bacilli in circumscribed vacuoles.

SUMMARY AND CONCLUSIONS

1. Cultivation and maintenance of the fibroblasts from macular or tuberculoid lesions for periods of two to seven months did not provide for multiplication of leprosy bacilli within these cells.

2. The total numbers of intracellular bacilli, or the proportion of cells with bacilli, sometimes increased during intervals as long as three months, but this rise was always followed by a decline in bacilli and by signs of their disintegration.

3. Evidence is presented that the concentration of bacilli in the outgrowing cells was influenced by early and transient or delayed or discontinuous transportation of micro-organisms from the primary explants of the lesions, and also by the degree to which the bacilli were divided among the growing cells.

4. Pigment granules, as well as bacilli, occurred in the fibroblasts from the papillary layer of skin. Their incidence was con-

trolled by the factors which determine the occurrence of bacilli, and they were usually to be found in the cells which contained bacilli for the longest intervals. These inert particles served to identify such cells as containing material from the original explants.

5. The fibroblasts from these clinically more resistant forms of leprosy were found capable of rapidly destroying the microorganisms. This capacity varied in accordance with the numbers of bacilli phagocyted and with the physiological activity of the cells.

REFERENCES

(1) HANKS, J. H. A study of the bacilli in tissue cultures of lepromata in serum media. Internat. J. Leprosy. **15** (1947) 21-30.

(2) HANKS, J. H. Fate of the bacilli in incubated lepromatous tissues and the question of microscopic growth. Internat. J. Leprosy. **13** (1945) 9-24.

(3) SIMMS, H. S. and STILLMAN, N. P. Substances affecting adult tissues *in vitro*. III. A stimulant (the "A" factor) in serum ultrafiltrate involved in overcoming adult tissue dormancy. J. Gen. Physiol. **20** (1937) 649-662.

(4) VOGELAAR, J. M. and ERLICHMAN, E. A feeding solution for cultures of human fibroblasts. Am. J. Cancer. **18** (1933) 28-38.

(5) BAKER, L. E. Artificial media for the cultivation of fibroblasts, epithelial cells, and monocytes. Science. **83** (1936) 605.

(6) BAKER, L. E. and EBELING, A. H. Artificial maintenance media for cell and organ cultivation. I. Cultivation of fibroblasts in artificial and serumless media. J. Exper. Med. **69** (1939) 365-378.

(7) COX, R. H. Use of yolk sac of developing chick embryos as medium for growing rickettsiae of Rocky Mountain spotted fever and typhus groups. Pub. Health Rep. **53** (1938) 2241-2247.

(8) PINKERTON, H. and HASS, G. M. The effect of temperature on the multiplication of *Rickettsia prowazeki* in tissue culture. J. Exper. Med. **56** (1932) 145-150.

(9) SANDERS, M. Studies on the cultivation of the virus of lymphogranuloma venereum. J. Exper. Med. **71** (1940) 113-128.

(10) MOLLOY, E. Cultivation of the St. Louis encephalitic virus. Proc. Soc. Exper. Biol. & Med. **44** (1940) 563-565.

(11) SANDERS, M. and MOLLOY, E. Cultivation of the virus of equine encephalomyelitis in serum ultrafiltrate and buffered salt solution (Simms). Proc. Soc. Exper. Biol. & Med. **45** (1940) 327-332.

(12) WADE, H. W. Tuberculoid changes in leprosy. I. The pathology of tuberculoid leprosy in Africa. Internat. J. Leprosy. **2** (1934) 7-38.

(13) NOLASCO, J. O. and LARA. C. B. Histopathology of early lesions in fourteen children of lepers. I. Analysis of previous skin blemishes in relation to sites of biopsies and other positive and probable lesions. Philippine J. Science. **71** (1940) 321-358.