THE FATE OF LEPROSY BACILLI IN FIBROBLASTS CULTIVATED FROM LEPROMATOUS LESIONS

by

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Earlier work (1), involving the maintenance of fibroblast tissue cultures from lepromatous lesions in serum media for periods of approximately one month, failed to disclose any increase in the total mass of bacilli in the primary explants, but permitted no conclusion concerning the question of bacillary multiplication in the growing and surviving fibroblasts. A preceding paper (2), dealing with the fate of leprosy bacilli in the growth zone of fibroblast cultures from macular and tuberculoid lesions, presented evidence that, although the numbers of bacilli or the proportion of bacillated cells might rise for a time during cultivation, the micro-organisms were in reality being derived from the explants. After intervals of 60 to 90 days, and sometimes much earlier, the proportion of cells containing bacilli declined steadily. The occasional bacilli found in cultures after six months were usually associated with pigment granules from the explanted skin. These non-proliferative particles served to identify the bacillated cells as containing material from the original explants.

Such lesions, though ideal material with respect to their low bacillary content, were obtained from the forms of leprosy with considerable clinical resistance. The present communication reports similar studies in the fibroblastic outgrowth of lepromatous nodules.

METHODS

The nodules, with one exception, were obtained from patients less than 35 years of age. The lepromatous infection was believed to be advancing in each of these patients and they were free of ulcers or other complicating skin conditions.

In the present work the new cell growth was always cut away from the heavily bacillated explants in preparing the first subcultures. India ink, previously injected into the nodules, was employed as a source of inert particles with which the incidence of bacilli could be compared. Lacking information on the composition of India ink (Higgins), the preferred preparations for injection were obtained by sedimentation of 5 per cent ink in the Swedish angle centrifuge, suspending and washing the carbon once, and then resuspending as a 1:100 dilution in M/150 Na_2HPO_4 and 0.1 per cent sodium citrate as stabilizers. Washed carbon is flocculated by hydro-

gen or calcium ions. This suspension remained finely dispersed during autoclaving and prolonged storage. The ideal dose provided to be a few hundredths cubic centimeter of a 1:500 dilution.

The preparation of tissue cultures in 13 mm. tubes (1), the incubation temperature, designation of the ingredients in the media, intervals between renewal of media, description of culture sizes, procedure of sampling and staining cultures, and the method of counting to determine the proportion of cells with bacilli have already been described (2). In the presence of phagocyted carbon, a separate accounting was made for cells containing bacilli and carbon, bacilli and no carbon, carbon and no bacilli, and of cells lacking bacilli and carbon.

EXPERIMENTAL RESULTS

The information in a preceding paper (2) concerning the suitability or the shortcomings of various media was confirmed with fibroblasts from lepromata and will be repeated here only in so far as the media specifically influenced the results. The addition of embryo juice to serum media did not bring about the important migration of bacillated macrophages reported by Timofejewsky (3) and by Suwo and Kin (4). The low proportion of smaller round cells which appeared in a few of the cultures were apparently of blood origin, and disappeared without influencing the results to be described. The form of fibroblasts from lepromata varied from spindles to stellate or extremely ramifying types. Careful counts in two experiments failed to indicate that any one of the forms was more likely to contain bacilli than the others.

The observation that active fibroblasts from leprid lesions are stimulated by moderate numbers of bacilli and are capable of segregating and destroying them has been mentioned (2). In the present work this phenomenon was not observed. It was evident, nevertheless, that high bacillary numbers and/or physiological activation of the cells did modify the cell response to an appreciable degree. In all types of media the most heavily bacillated cells contained unusual amounts of carbon, which might be attributed to their having been stimulated by the bacilli or explained by assuming that such cells possessed extraordinary phagocytic capacity. In cultures stimulated by the presence of embryo juice such stuffed cells, and those only, flocculated the carbon so sharply that they were easily recognized by low power examination of the living cultures. Stained preparations revealed that the bacilli and carbon in other than "stuffed" cells were usually to be found together and in some particular portion of the cytoplasm, either at the proximal end (toward explant) of the nucleus, surrounding the nucleus, or at its distal end. Their location was apparently related to the division cycle of the cells. The normal position in resting cells was

near the proximal end of the nucleus, while dividing cells always exhibited particles around the nucleus. It was by this means that the bacilli and carbon were divided so equally between daughter cells along the course of rapidly growing cell chains and could reach the most distal cells of large cultures without growing.

Smith, Willis, and Lewis (5) earlier described an equal partition of avian tubercle bacilli between daughter cells in cultures of chick embryo fibroblasts. Motion pictures taken by Dr. Geo. O. Gey, Division of Cell Physiology, Johns Hopkins Hospital, reveal the remarkable mechanism involved. Just before cell division, the nucleus rotates actively, stirring up the mitochondria and cytoplasmic or foreign particles until they are uniformly distributed around the nuclear membrane. This explains why, as division proceeds, the cytoplasmic constituents are distributed so uniformly among the daughter cells.

In F2 or F3 cultures showing a slow growth it was at times possible to recognize new cells which were bringing ink and bacilli from the explants. It is to be doubted that this process occurs in large or rapidly growing cultures, since acquisition of particles from the explant is reduced as the optimal growth zone moves peripherally (2). This circumstance, combined with the distribution of the bacilli along cell chains, often resulted in cultures with fewer bacillated cells near the explant than there had been in the early cultures.

Two opposing sets or factors were again recognized to control the proportion of bacillated cells and the concentration of bacilli within the fibroblasts. These were: (a) factors which cause large numbers of bacilli to be brought out of the explants by growing cells and (b) factors which limited transport or resulted in a progressive decrease in the incidence of bacilli among cells. High bacillary content in original explants was usually associated with high cell content, with the result that large numbers of bacilli were carried into the growth zone by a rapid outburst of growth. These circumstances, if followed by slow subsequent growth, accounted for the older cultures with high bacillary content, both because the bacilli were transported from the explants for a longer interval during slow growth and because they were not rapidly diluted by cell division. Low bacillary content in early cultures was usually associated with indolent outgrowth from those explants which contained fewer cells and bacilli. Either type of early culture (luxuriant or sparse) revealed decreasing bacillary concentrations during continuous growth, especially if rapid, both because the bacilli were shared among the new cells being produced and also because explants in the midst of a dense zone of active cells more

quickly became necrotic due to unfavorable conditions of nutrition, pH, or oxidation.

The use of several media in initiating some of the experiments assisted in clarifying the influence of early luxuriant growth on the proportion of bacillated cells. The presence of embryo juice in serum media enhanced early cell growth remarkably, and almost all the cells in young cultures so prepared contained large numbers of bacilli. In the presence of carbon many cells were strikingly loaded with coarse black granules, always indicative of heavy bacillation. In plain serum media a lower proportion of early cells contained bacilli, and their carbon could not be perceived during the routine low power examination of living cultures. It must not be inferred that the influence of any medium entirely supersedes the initial growth energy of the explants themselves, since rapid outgrowths occur also, though less frequently, in the less stimulating media. The basic potentiality for early outgrowth is related to high cell content, which in turn accounts for the high bacillary and, as will be shown, carbon concentration in such explants.

TABLE 1. *Influence of early growth, and of subsequent growth rate, on the bacillary content of fibroblasts from lepromata.*

Characterization of growth	Growth period (days)	Size of cultures at 6 days	Size of cultures when sampled	Per cent cells with bacilli	Average per cent
Early growth luxuriant	6	F 5 F 4 F 5	F 5 F 4 F 5	90 96 94	93
Early growth good	14	F 4 F 3 F 4	F 4 F 3 F 4	87 73 80	80
Early growth poor	14	F 1 F 1	F 1 F 2	18 42	30
Early growth good, followed by stabilization*	30	F 3 F 4 F 4	F 3 F 3 F 3	67 83 74	75
Early growth good, followed by steady growth	30	F 4 F 3 F 4	F 5 F 6 F 6	39 29 31	33
Early growth poor, followed by stabilization	30	F 1 F 1 F 1	F 1 F 2 F 2**	19 25 37	21

* Growth inhibition or stabilization was due in some instances to calcification and in others to incubation at 30°C.

** This culture was somewhat intermediate. It became F 2 before 11 days, hence attained its full growth fairly early. Other intermediates are excluded.

The results of the microscopic studies in Experiment 1 (see Table 1 and Figure 1) illustrate this point. All of the cultures studied at each sampling date were arranged according to the percentage of cells containing bacilli, and their histories carefully reviewed. Irrespective of the medium, cultures showing good or lux-

uriant outgrowth within a few days contained the general order of 75-95 per cent of cells with bacilli at 6 and 14 days, while those with poor initial growth averaged only 30 per cent.

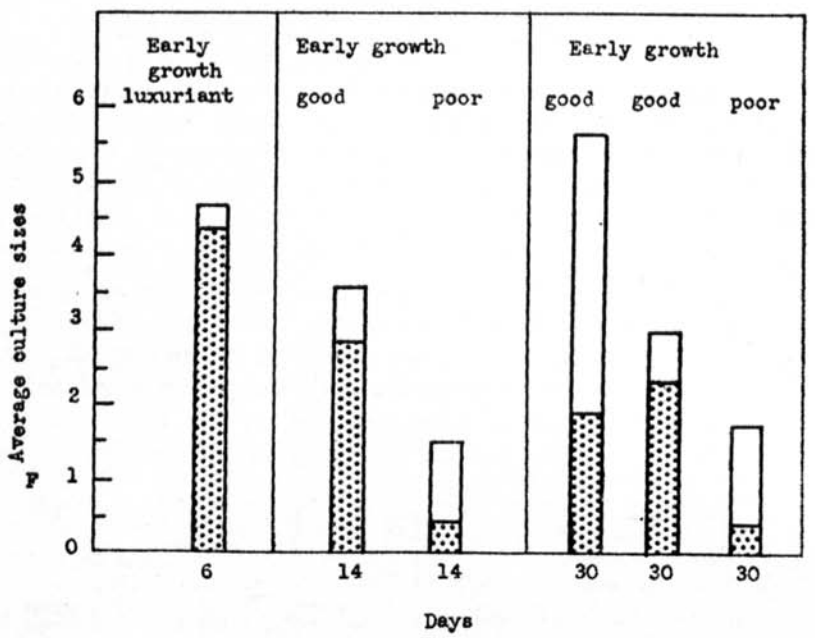

Figure 1. Influence of early growth and of subsequent degree of growth on the proportion of cells containing bacilli. Stippling indicates the proportion of cells with bacilli.

The influence of the subsequent growth rate was also revealed in the same experiment. Cultures which had enjoyed a good early outgrowth but later grew slowly or became "stalled" still contained about 75 per cent of bacillated cells after 30 days. The cultures of this class which grew steadily and attained a large size after 30 days, though initially rich in bacilli, now contained only 33 per cent of bacillated cells. Cultures which were initially poor grew very slowly and without appreciable change in the degree of bacillation. After incubation for several weeks the greatest concentrations of bacilli were always found in the cells of cultures which had enjoyed an early luxuriant outgrowth, but which soon stalled as the result of calcification, incubation at 30°C., or of unknown factors. Among the cultures which grew to large size and contained a low incidence of bacilli, none gave evidence of any cells returning to their original, heavily-bacillated condition.

SUMMARY OF EXPERIMENTS

Experiment 1: Explants were prepared from a small cutaneous nodule. The patient, a female of 20 years, exhibited no suitable subcutaneous nodules, and innumerable lesions of the type biopsied. These were extremely hard, softened at the center, and often perforating through the skin. Ten cultures were prepared from washed suspensions of the macrophages which were liberated during cutting of the tissue explants, but none of these cultures permitted conclusions concerning cell or bacillary multiplication. This type of experiment with heavily-loaded round cells was not continued, since attempts to establish useful cultures of normal blood monocytes were almost universally unsuccessful at that time. The problem of handling monocytic cells, and of adequate controls on the fate of the bacilli, proved to be so highly specialized that work in this direction was deferred.

Forty-eight tissue explants (12 in each of 4 media) were maintained (with one renewal) in their respective media for 14 days, when all positive cultures were provided with media containing 15 per cent of embryo juice and some were placed at 30°C. to retard their growth rate. Since a high proportion of the cultures, especially those growing vigorously, were being lost by calcification, a second generation was prepared after 30 days. It was terminated after another 16 days, (total 46) due to threatening calcification.

Cultures of the first series were sampled for microscopic work after 6, 14, and 30 days. As described in connection with table 1, their analysis involved a serial arrangement in order of excellence for bacillated cells, and then a series of tabulations in order to see what features of the culture histories — whether early growth, subsequent growth rate, medium, incubation temperature, or incubation period — might explain the differences in the bacillary content of the cells. The conclusions concerning the greater bacillary content of cells which grew early and vigorously, the relation to the concentration of cells and bacilli in the explants, and the rapid decline of bacilli during constant cell growth have already been presented (see Table 1 and Figure 1).

The average percentages of cells containing bacilli fell continuously: 93 per cent at 6 days, 60 per cent at 14 days, 43 per cent at 30 days and 22 per cent at 46 days. As noted earlier (2), these decreasing percentages were associated with progressively fewer bacilli per positive cell.

Experiment 2. Twelve explants only were prepared from a subcutaneous nodule (obtained chiefly for other purposes) from a 60-year-old male patient. This rather limited experiment is cited because of the unexpectedly good outgrowth from tissues of advanced age, and because an unusual degree of growth stabilization (in $PL_{40}EM_5$) permitted maintaining the cultures in the original tubes for 56 days without complete cessation of growth.

During the first month the majority of the fibroblasts were small and finely branched. During the second month some of the cells became "balled," while others separated their connection with the original tissue and their processes were bent back as though they were repelled by the explants. These cultures at that time showed little of the matting or organization which characterizes fibroblast cultures, and no attempt was made to establish a second generation.

TABLE 2. *The percentages of cells containing bacilli during maintenance of fibroblasts in contact with lepromatous explants for 56 days (Experiment 2).*

Growth period (weeks)	Number of cultures sampled	Cells with bacilli	Cells without bacilli	Total cells counted	Per cent cells with bacilli
2	2	195	10	205	95.2
4	2	149	171	320	46.6
6	2	124	439	563	22.0
8	2	69	364	433	15.9

Cultures were sampled at intervals of two weeks, each sample being composed of two cultures, one which had shown rapid and another which had shown slow outgrowth. As may be seen in table 2 and figure 2, the percentages of cells with bacilli again fell continuously: 95 per cent at 2 weeks, 46 per cent at 4 weeks, 22 per cent at 6 weeks, and 16 per cent at 8 weeks.

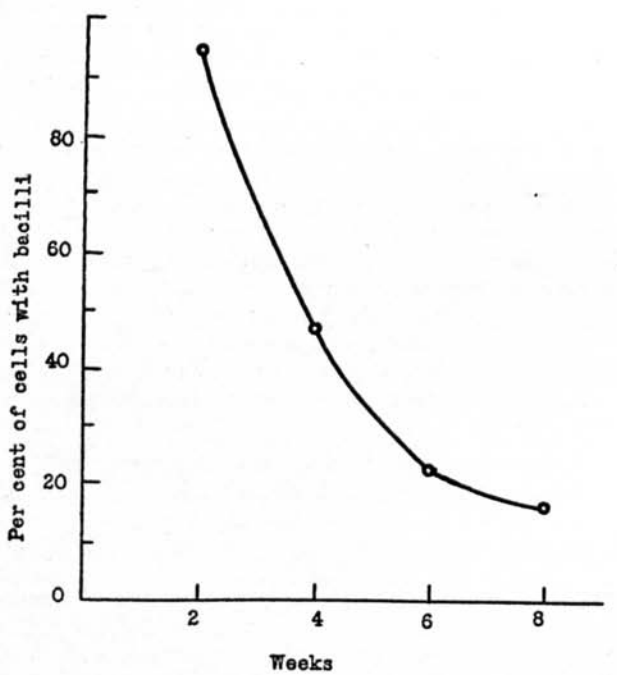

Figure 2. The proportion of cells containing bacilli during maintenance in contact with lepromatous explants for 8 weeks. (See Table 2).

Experiment 3. Since the proportion of cells containing bacilli decreased steadily during the growth of fibroblast cultures from lepromata, a method was sought to secure cells containing both leprosy bacilli and other inert particles, for in this way bacillary growth might be detected even though it lagged behind the rate of cell growth. The washed carbon from India ink was found useful for this purpose.

A subcutaneous nodule was obtained from a 25-year-old male patient,

13 days after the injection of 0.1 cc. of ink 1:4. This dosage of ink proved to be excessive; the nodule was inflamed 24 hours after the injection, and then rapidly returned to a normal appearance. By the time of biopsy, however, the patient stated that the nodule was disappearing, and this proved to be the case. Although the nodule was badly fragmented, 6 "white" and 6 "black" explants were placed in each of three media (36 cultures). The explants prepared from fragments lacking ink were very translucent when viewed in the cultures and were found to be almost devoid of cells and bacilli; the "inked" explants were jet black. Nevertheless, after 7 days of incubation, 9 of the cultures in PL_{25} (averaging F 1), 9 in $PL_{25}EM_5$ (averaging F 2.4), and 10 of those in $PL_{25}EM_{15}$ (averaging F 2.9) were growing. The cultures in PL_{25} contained no cells in which carbon was visible by low power magnification, those in $PL_{25}EM_5$ revealed a few such cells, while those in $PL_{25}EM_{15}$ exhibited many cells with coarsely flocculated carbon.

Since the control preparation had already revealed the virtual absence of bacilli, these cultures were maintained for only 14 days before being sampled to study their response to carbon. The original explants were rated for carbon density on a relative scale of 1 to 10. Among the black explants the microscopic ratings for carbon varied from 7 to 10, and the average ratings of those in each of the three media were comparable (see Table 3 and Figure 3). In PL_{25}, 29.2 per cent of the cells contained finely dispersed carbon, but not in sufficient concentration to permit detection by low power. In $PL_{25}EM_5$, 13.3 per cent of the cells contained carbon, usually in fine dispersion, but more rarely in large amounts and in coarse aggregates. This flocculated, "low-power" carbon was found to be an accurate indicator of the presence of bacilli in such cells. In $PL_{25}EM_{15}$, 8.2 per cent of the cells contained carbon; again, the bacillated cells accounted for the occurrence of flocculated carbon.

TABLE 3. *Influence of available carbon and of culture size on the percentages of cells containing carbon after 14 days. (Experiment 3).*

Medium	Average size of cultures*	Average carbon rating of explants		Cells with carbon	Cells without carbon	Total cells counted	Per cent cells with carbon
PL_{25}	F 3.6	9.0	"Black"	112	273	385	29.2
$PL_{25}EM_5$	F 4.0	8.0		45	292	337	13.3
$PL_{25}EM_{15}$	F 5.0	8.3		41	459	500	8.2
PL_{25}	F 4.0	2.0	"White"	36	264	300	12.0
$PL_{25}EM_5$	F 5.3	1.5		17	535	552	3.1
$PL_{25}EM_{15}$	F 4.9	1.7		25	695	720	3.5

* Each group contained three cultures.

The frequency and the amount of finely dispersed carbon in the early cells of individual cultures in a given medium could in general be correlated with its concentration in the original explants, or with the initial growth rate. After two weeks of active growth, however, the average percentage of cells containing microscopic carbon was related inversely to the average extent of culture growth (Table 3 and Figure 3), and was lowest in the media containing embryo juice.

Since some of the "white" explants revealed microscopic carbon in small amounts, they also were given a carbon rating. The lower portion of table 3 includes the results from three such cultures in each medium. The percentages of cells containing carbon in the three media were 12, 3.1, and 3.5 per cent respectively. It is again clear that the percentage of cells containing carbon was related directly to the carbon available, and inversely to the sizes of the culture when sampled. There is a striking resemblance between these results with inert particles and those already mentioned in connection with factors which influence the occurrence of leprosy bacilli in the cells from non-inked nodules.

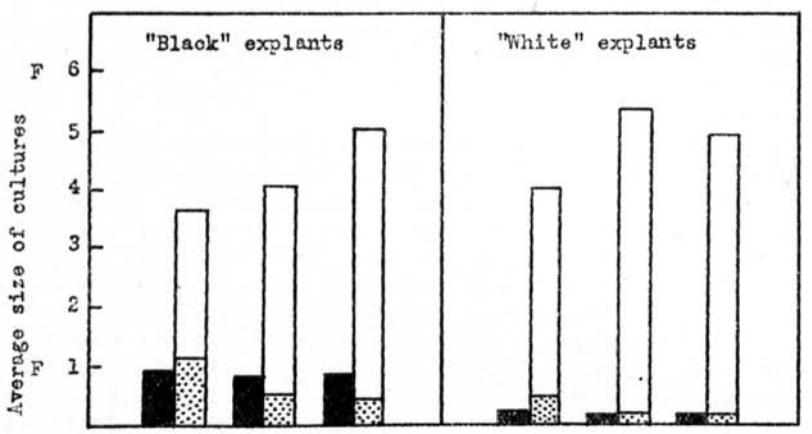

Figure 3. Influence of available carbon (solid blocks) and of culture size on the proportion of cells (stippled) with carbon (see Table 3).

Experiment 4: A small nodule was procured from a 34-year-old male, 24 hours after the injection of 0.05 cc. of ink 1:100. It revealed no inflammation. Fifteen "white" and 15 "black" explants were cultured in each of two media (total 60 cultures), one of which proved to be contaminated, so that only the explants in $PL_{25}EM_{15}$ may be considered. Six of the "black" and 3 of the "white" explants in this medium produced growth, the "black" explants producing superior early outgrowth.

Due to the small number of cultures, and unfortunately for accurate results, the first samples after 11 days included only 3 small "black" cultures, such as already have been shown to contain low concentrations of cells and bacilli at the outset. The remaining cultures maintained a slow but steady growth for 50 days, when the experiment was terminated.

TABLE 4. *Proportion of cells containing bacilli and carbon as related to the concentrations of these particles in the explants. (Experiment 4).*

Tissue cultures			Average rating of explants for		Per cent of cells with		Ratio of cells with carbon to bacilli
Growth period	Number sampled	Average size	bacilli	carbon	bacilli	carbon	
11 days	3	F 1.7*	2.0	3.5	8.5	11.0	1.3
50 days	3	F 4.0	4.0	6.0	7.2	14.6	2.0
50 days	3	F 4.6	6.0	0.0	7.3	0.0	

* This group of cultures did not represent the average degree of growth at 11 days, see text.

Among the cells examined after 11 days, 8.5 per cent contained bacilli and 11 per cent contained carbon (see Table 4 and Figure 4). After 50 days the average counts from the three "black" cultures revealed 7.2 per cent of cells with bacilli and 14.6 per cent with carbon. The three "white" cultures of comparable size contained 7.3 per cent of cells with bacilli.

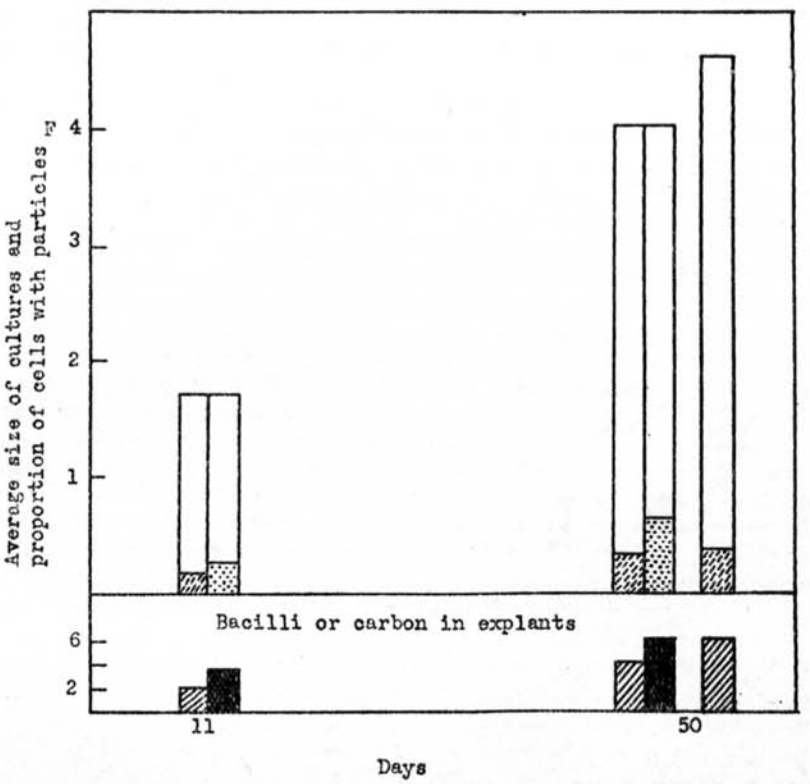

Figure 4. Relation of bacilli and carbon in explants to the proportion of cells containing these particles.

In the columns showing cell growth, small rods or stippling represent the proportion of cells with bacilli or carbon, respectively.

The anomalous increase in the proportion of cells with carbon must be attributed to two causes: the failure to include luxuriant cultures in the 11-day sample and the fact that the average carbon ratings of the explants in the 50-day cultures was almost twice that of the cultures sampled at 11 days. The percentage of cells containing bacilli decreased only slightly in the interim between the two samples, but the rating of the original explants for bacillary content revealed that here again the ill-advised selection of small cultures for the 11-day sample furnished explants with less than one-half of the bacillary content of the explants in the final samples.

The strict parallelism between the high carbon, as well as bacillary, content of the explants and the occurrence of luxuriant outgrowth, indicates a definite collection of carbon within 24 hours by the more cellular

portions of the injected nodule. The important relation between luxuriant outgrowth and a high concentration of both cells and bacilli has already been emphasized. The results with carbon are here shown to be analogous.

For each cell which contained bacilli there were 1.3 with carbon after seven days and 2.0 with carbon after 50 days. Since the explant ratings for bacilli in the 50-day samples were more than twice (and the carbon ratings less than twice) the ratings of the 7-day samples these ratios suggest that the bacilli were disappearing more rapidly than the carbon.

Experiment 5: A subcutaneous nodule was obtained from a 20-year-old male patient, eight days after the injection of 0.05 cc. of ink 1:40. There was no indication of inflammation following the injection, or in the biopsied nodule. The ink was found to be spread for a distance of approximately only one millimeter around the needle track, with the result that most of the explants included both white and inked areas. Twelve explants were cultured in each of the 3 media used in Experiment 3, but this time produced only 3, 5, and 4 slowly positive growths in the respective media. This patient may have been a poor source of tissues for explantation, since another nodule removed 30 days after the ink injection (two nodules were injected simultaneously) failed to produce a single outgrowth. The media employed were supporting excellent growth of cultures from other patients.

Examination of the living cultures confirmed the fact that visibly flocculated carbon occurred only in media containing embryo juice. One culture from each medium was sampled after one week. The averaged results revealed that 50 per cent of the cells contained bacilli and 61.4 per cent contained carbon (see Table 5). Similar cultures were sampled after four weeks, when the second series was established. It was found that 10 per cent of the cells contained bacilli and 17.7 per cent contained carbon.

The cultures of the second series were maintained in two groups, in $PL_{25}EM_5$ and $PL_{40}E\,M_5$, respectively; the higher concentration of serum was advantageous in protecting the plasma against digestion. These cultures were sampled after 5 weeks (total 9), when the third series was established. The averaged counts now revealed that bacilli could be found in 2 per cent of the cells and carbon in 3.1 per cent.

The third series was maintained for 5 weeks in $PL_{40}EM_5$ before it was terminated, since preliminary samples did not reveal bacilli or carbon within the cells examined. A count among 800 cells from the cultures sampled after a total of 14 weeks *in vitro* revealed only one with 3 small and indefinite acid-fast granules. Actually many more cells were observed than those recorded in the detailed counts, but none revealed carbon, acid-fast granules, or bacilli.

TABLE 5. *Decreasing percentages of cells with bacilli and carbon during serial cultivation for 14 weeks. (Experiment 5).*

Tissue cultures				Per cent of cells with		Ratio of cells with carbon to bacilli
Series	Growth period weeks	Number sampled	Average size	bacilli	carbon	
I.	1	3	F 3.0	50.0	61.4	1.2
	4	3	F 3.0*	10.0	17.7	1.8
II.	9	6	F 4.7	2.0	3.1	1.6
III.	14	6	F 3.5	0.0	0.0	

* Fibroblast chains much longer than at seven days.

The results of this experiment, which are summarized in table 5 and shown in figure 5, indicate very clearly that the bacilli and the carbon disappeared at comparable rates. The figures in the last column of the table, however, suggest again that the bacilli disappeared more rapidly than the carbon, since the ratio of cells containing carbon rose from 1.2 at seven days to approximately 1.7 in the counts made after four and nine weeks.

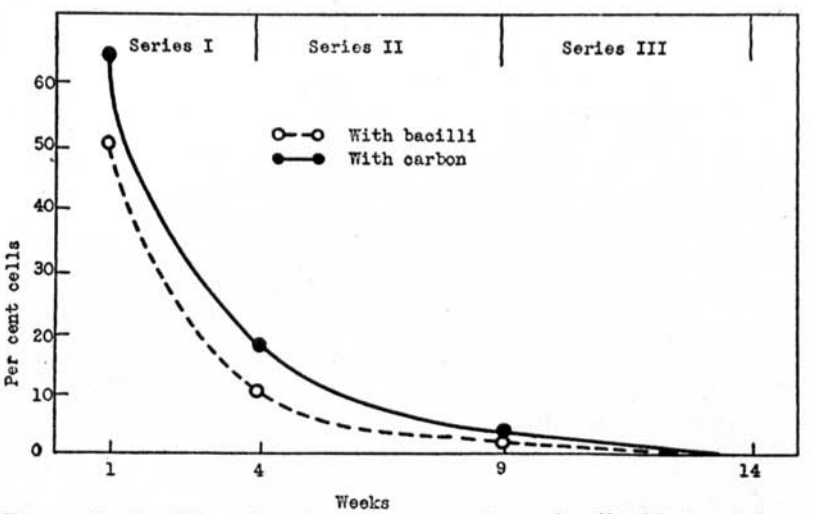

Figure 5. Bacilli and carbon disappear from the fibroblasts of lepromata at similar rates.

INFLUENCE OF pH AND CELL ACTIVITY ON DISAPPEARANCE OF BACILLI

In connection with two of the experiments with tuberculoid lesions (7), the substitution of a supplementing solution (SS) for embryo juice was noted to avoid the problem of plasma opacity, to support cell growth at approximately one-half the rate in embryo juice media, and to provide an oxidation potential more favorable to the survival of cells in the explants. The following experiment was taken as an occasion to inquire further into the influence of carbon dioxide pressures and of cell growth, on the fate of leprosy bacilli.

Experiment 6: A large nodule was obtained from a 19-year-old female patient, four days after the injection of 0.05 cc. of washed ink 1:500. This proved to be the most suitable dosage of ink employed, since it was entirely taken up by the tissue cells within the four day period and was found to be slightly less abundant than the bacilli at the outset.

One group of cultures was initiated in $PL_{33}SS$ and incubated under 30 mm. CO_2; these cultures developed very slowly but continuously. Six such cultures were sampled after 2 weeks and averaged 78 per cent of cells with bacilli and 75 per cent with carbon. At that time the CO_2 pressure in a representative sub-group of cultures was increased to 60 mm.

After 6 weeks, 6 cultures from the regular group (30 mm. CO_2) averaged 61 per cent of cells with bacilli and 53 per cent with carbon (see Ta-

ble 6 and Figure 6). The sub-group with doubled CO_2, on the other hand, revealed only 18 per cent of cells with bacilli, and these were extremely pale. Due to somewhat increased flocculation of the carbon, which make it less available for distribution among the cells, the proportion of cells with carbon had fallen to 31 per cent. By comparison with the incidence of bacillated cells, the ratio of cells with carbon had practically doubled.

TABLE 6. *Percentages of cells containing bacilli and carbon during slow cell growth in serum and SS, including the effect of extra carbon dioxide and of more rapid growth in the presence of embryo juice.*

Medium	Growth period (weeks)	Number of cultures sampled	Average size	Average per cent of cells with		Ratio of percentages with carbon to bacilli
				bacilli	carbon	
$PL_{33}SS$ (30 mm. Co_2)	2	6	F 2.0	78.4	75.4	0.9
	6	6	F 2.6	60.6	52.9	0.9
	12	5	F 2.4	33.3	38.8	1.2
Subgroup of above (60mm. CO_2)	2nd to 6th	5	F 2.3	17.6	30.7	1.7
$PL_{33}EM_5$ (30 mm. CO_2)	2	6	F 3.2	51.4	47.2	0.9
	6	5	F 5.3	21.3	23.6	1.1

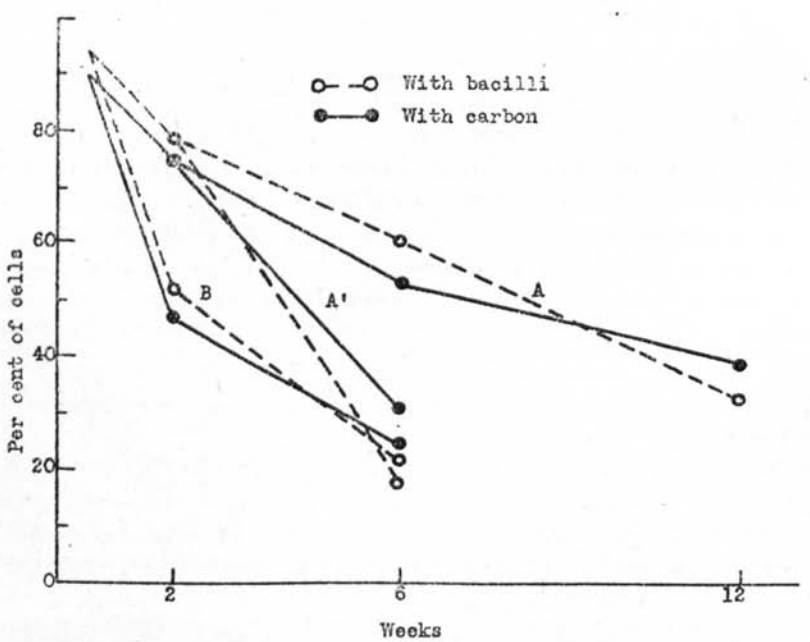

Figure 6. Bacilli disappear from cells more quickly when the pH is depressed by CO_2 (A') or growth is stimulated by embryo juice (B) than from cells growing slowly. (A).

Fainter lines are extended to the left of the first actual samples at 2 weeks in order to indicate that all the cultures were from a common source.

Seven cultures in $PL_{33}SS$ and under 30 mm. of CO_2 were continued. After 12 weeks, only 4 viable cultures remained and the experiment was terminated. Every culture now revealed more cells with carbon than with bacilli, the average proportions being 33 per cent of cells with bacilli and 39 per cent with carbon.

A second group of cultures started in $PL_{33}EM_5$ grew more luxuriantly. It provided samples after 2 and 6 weeks, but the remaining cultures were lost due to overgrowth or to calcification before the end of 12 weeks. To have transplanted these cultures would have made impossible the desired comparison with the control group. The samples after 2 weeks revealed 51 per cent of cells with bacilli and 47 per cent with carbon, while those after 6 weeks contained 21 per cent of cells with bacilli and 24 per cent with carbon. The curves for this control group (see Table 6 and Figure 6) resemble those of the preceding experiments.

To recapitulate: the proportion of cells with bacilli early in this experiment exceeded the proportion with carbon. This situation was found to be inverted in favor of superior carbon percentages within 6 weeks in cultures in media containing embryo juice, and much more strikingly inverted under an atmosphere containing twice the usual CO_2. In dilute serum and SS, the inversion was first observed only after 12 weeks *in vitro*. Thus a slightly alkaline reaction and lowered cell reactivity, though maintaining a higher proportion of cells with bacilli for the longest time, did not prevent their eventual disappearance at a rate greater than that for the control carbon particles.

DISCUSSION

This study of the possible growth of leprosy bacilli in tissue cultures from lepromata revealed that the fate of the bacilli was similar to that of the accompanying carbon particles, except that the bacilli were less durable. Within 24 hours after the injection of ink the carbon was found to be concentrated in the more cellular and heavily-bacillated explants, those likely to show early outgrowth of heavily-loaded cells. The degrees of phagocytosis was determined largely by the numbers of particles and of cells per unit volume of tissue, just as *in vitro* phagocytosis by leucocytes (6). It was influenced also by the physiological activity of the fibroblasts as indicated by the stimulating effect of embryo juice on the acquisition of particles by the cells of new cultures. This was in keeping with the observation of Carrel and Ebeling that greater quantities of neutral red were taken up by active chick fibroblasts than by those growing slowly (7).

After the early period of outgrowth, the proportion of cells containing carbon or bacilli was modified chiefly by the rate at which the particles were divided among the growing cells and only slightly, if at all, by continuous conveyance as in the case of macular and tuberculoid lesions (2). This was due in part to the fact that the great masses of bacilli in the nodules made the explants less hospit-

able to persisting or re-invading cells and in part to the complete separation of new outgrowth from the original explants in preparing the first subcultures. The curves obtained with leproma fibroblasts that were attached directly to original explants never showed the delayed or secondary rise which was encountered in some of the experiments with leprid tissues and attributed to more continuous transport of the bacilli.

Since carbon particles were observed (8) to stimulate chick fibroblasts, and since high cell activity was observed in this and a preceding work (2) to hasten the destruction of the bacilli, the possibility was considered that carbon might be an unfavorable factor in such experiments. There was, however, no evidence that carbon stimulated the human cultures used. The curves indicate that the bacilli disappeared at about the same rate in Experiments 1 and 2, without carbon, as in Experiments 3, 4, and 5, with carbon present. A note to follow this paper gives evidence that high concentrations of carbon do not interfere with the development of experimental rat leprosy.

Although the results of this and the preceding experiments do not elucidate the conditions necessary for the multiplication of the leprosy bacillus, they do afford information concerning several factors which influence the destruction of the micro-organisms in the fixed cells of connective tissue. In the presence of small to moderate numbers of bacilli, fibroblasts from leprid lesions segregate the bacilli into digestive vacuoles and reduce them to an acid-fast debris. This phenomenon was most striking in the fibroblasts from the reticular layer of the skin. This is the skin layer in which Wade (9) observed an especially activated histological response during positive lepromin reactions. When containing extremely small numbers of bacilli or when growing indolently, the fibroblasts from leprids exerted no recognized response to, or action on, the micro-organisms. The fibroblasts from lepromata were indifferent to high bacterial content, and it was only in the most stimulating media that they were capable of any obvious response to the bacilli. Although under these circumstances, they did not bring about a prompt destruction of the bacilli, the cells were now able to flocculate ink and to dispose of the bacilli more rapidly than cultures which were kept at a minimal rate of growth. A doubling of the usual carbon dioxide pressures was shown both here and in a preceding paper to hasten the disappearance of the bacilli.

Fibroblasts are not regarded by most leprologists as host cells of the bacilli and their immunological importance in any disease remains as yet undefined. The foregoing observations, none-the-less, are evidently related to several pathological, immunological, and

physiological features of the disease. The destructive response of fibroblasts from leprids is excited by small or moderate numbers of bacilli and brings to mind the lepromin-positiveness in the macular and tuberculoid types of the disease, the relatively marked cell response to the small numbers of bacilli which occur in the natural lesions, and the epithelioid cell pathology as evidence that bacilli are being destroyed and their phosphatides being dispersed. The lack of such response in fibroblasts from lepromata is associated with the contrasting features of the lepromatous disease.

The peripheral distribution of the disease in all patients is doubtless dependent on the same conditions which influence the fate of bacilli in cultivated fibroblasts. The internal organs are characterized by higher cell populations, a greater incidence of mitotic cells, a more active metabolism, and a lower pH than that of the peripheral tissues. In tissue cultures, modification of the growth rate and of the pH permits one to vary the physiological conditions along similar lines and with corresponding effects.

The relation between cell activity and the destruction of bacilli, and the occurrence of typical leprous lesions only in the most quiescent tissues of the body, make it necessary to question whether multiplication of the bacilli should be expected under the conditions used. The art or science of tissue cultivation, since its inception, has been directed toward obtaining the maximal activity and proliferation of the cultures. Though it was possible to reduce the growth rate by employing a temperature of 34 degrees C., and still further by the use of the supplementing solution to replace embryo juice in several instances, the suitability of these cells as a nutritional background for the bacilli cannot be regarded as having been adequately tested without reproducing more closely the physiological status of the skin itself.

SUMMARY AND CONCLUSIONS

1. Fibroblasts from human lepromata were maintained *in vitro* for intervals of 7 to 14 weeks. Irrespective of whether the new growth was left attached to the original explants or was subcultured in successive series of tubes, the proportion of cells containing bacilli (and the content per cell) decreased continuously.

2. By the use of carbon from India ink as inert control particles, all the quantitative relationships between the bacilli and the cells were duplicated, except that the bacilli disappeared more rapidly than the carbon.

3. The bacterial and carbon content of young cultures were found to depend on the concentration of particles and cells in the

explants and on the luxuriance of early outgrowth, while the concentrations of particles in older cultures of comparable early histories was related inversely to the degree of cell growth.

4. A temperature of 34 degrees C., slow growth, low cell metabolism, and a slightly alkaline medium permitted maintaining bacilli in the cells in apparently good condition for long intervals, but did not prevent an eventual inversion of the bacillus-carbon ratios. More active cell metabolism, or a lower pH, accelerated the disappearance of the bacilli. Analogous differences in physiological conditions differentiate the group of peripheral tissues in which leprous lesions are common from the internal organs in which the bacilli are rare or of abnormal appearance.

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. The fate of leprosy bacilli in fibroblasts cultivated from macular and tuberculoid lesions. Internat. J. Leprosy. 15 (1947) 31-47.

(3) TIMOFEJEWSKY, A. D. Explantationsversuche von leprösem Gewebe. Arch. f. exper. Zellforsch. 9 (1930) 182-202.

(4) SUWO, M. and KIN, S. Ueber Kulturen von leprösem Gewebe *in vitro*. I. Mitteilung. Gewebskulturen von Hautknötchen bei Menschenlepra. Internat. J. Leprosy. 7 (1939) 57-66.

(5) SMITH, D. T., WILLIS, H. S., and LEWIS, M. R. The behavior of cultures of chick embryo tissue containing avian tubercle bacilli. Am. Rev. Tuberc. 6 (1922-23) 21-34.

(6) HANKS, J. H. Quantitative aspects of phagocytosis as influenced by the number of bacteria and leucocytes. J. Immunol. 38 (1940) 159-176.

(7) CARREL, A. and EBELING, A. H. The fundamental properties of the fibroblast and the macrophage. I. The fibroblast. J. Exper. Med. 44 (1926) 261-284.

(8) HANKS, J. T. Attempts to infect chick embryos and chick tissue cultures with bacilli from human lepromatous lesions. Internat. J. Leprosy. 15 (1947) 70-77.

(9) WADE, H. W. (Personal communication)