

## A STUDY OF THE BACILLI IN TISSUE CULTURES OF LEPROMATA IN SERUM MEDIA

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Quantitative inquiry into changes in the numbers of leprosy bacilli following the inoculation of suspensions of lepromatous tissue into a variety of special bacteriological media did not offer any suggestion of multiplication (1). A similar study of the bacilli in small cubes of nodule tissue incubated in or on bacteriological media, or in tissue culture solutions, also failed to reveal an increase in their numbers (2), even after repeated renewal of the nutrient solutions. In view of the decisively negative results, which agree with the general experience of many earlier workers, it has seemed desirable to examine the behavior of leprosy bacilli in tissue cells maintained by *in vitro* cultivation.

The possibility of using tissue cells *in vitro* as hosts for leprosy bacilli has been investigated by several workers. The first work along this line was with the bacillus of rat leprosy. Zinsser and Carey (3) reported intracellular multiplication of this organism in young spleen tissue maintained in rat serum. Friedheim (4) cultured infected rat spleen in rat plasma and rat embryo extract. The bacilli were not observed to multiply, and disappeared in subcultures. Lowe and Dharmendra (5) infected bits of skin and spleen of embryonic rats and cultured them in chicken plasma and rat embryo extract. Most of the cultures were incubated for seven days without change of medium, though a few were replanted at three-day intervals for 30 days. Growth of the bacilli was not detected. Kudicke and Vollmar (6) cultivated infected rat lepromata, lymph nodes, spleen, and sarcomata for periods of a few weeks in rat and chicken plasma supplemented with chick embryo juice. They found that 22 days was the extreme interval after which rats could be infected with the cultures. Although they confirmed many of the observations of Timofejewsky, to be discussed, they pointed out that the bacilli found in the outgrowths or in the plasma could have been carried from the heavily-loaded explants by growing or migrating cells. Suwo and Kin (7) cultivated young lepromata of rats and reported microscopic growth of the bacilli in fibroblasts and epithelioid cells, and in the medium itself.

Fraser and Fletcher were mentioned by Lowe and Dharmendra (5) as the first to use tissue cultivation in the study of human leprosy, but their work has not been available to the writer.

Timofejewsky (8) cultured two human leprous nodules in rabbit and human plasma supplemented with human embryo extract. The tissues grew normally for about ten days, but died in the third week. The deterioration of the cultures was attributed to a tremendous overgrowth of bacilli, which

were said to continue multiplying in the plasma after the death of the cultures. Suwo and Kin (9) also prepared, from each of 25 human leprous nodules, 20 to 30 tissue cultures which they embedded in chicken plasma and supplied with chick embryo juice every five days. Good growth of cells occurred for about ten days, after which fatty degeneration of the fibroblasts and rounding up of fibroblast-like cells ensued; the cultures were dead in 18 to 20 days. These authors also believe that the leprosy bacilli grew in the tissue cultures and in the surrounding medium.

With the exception of Zinsser and Carey, previous investigators have used tissue extracts rather than serum for the cultivation of leprous tissues. Carrel and Ebeling (10) showed long ago that blood monocytes and tissue macrophages of the chicken subsist best in serum or plasma media. Baker (11) says that embryonic juices or proteoses kill these cells at concentrations suitable for the multiplication of fibroblasts. Though fibroblasts grow best in higher concentrations of tissue juice or proteoses, they also require serum for continuous multiplication (12). Parker (13) has maintained chick fibroblasts for a year in single flasks by renewals with 33 per cent homologous serum. Although it was not known what medium, if any, might favor the migration and persistence of human tissue macrophages from explanted nodules, serum media appeared a plausible choice in this first study.

Though cultures were maintained for somewhat longer periods than in the work of earlier investigators, it was not possible to settle the question of bacillary multiplication in growing cells. The chief interest in the present communication lies in the demonstration by objective methods that the bacilli did not multiply in tissue fragments surrounded by living fibroblasts for intervals approximating one month.

#### METHODS

*Preparation and maintenance of tissue cultures.* — Nodules, obtained and controlled for sterility as in previous studies (1, 2) were cut into fragments of about 1 mm. for explantation. These explants, usually 12 for each of the three or four media in each experiment, were rinsed in balanced salt solution lacking sodium bicarbonate.\* Each explant was then taken into a pipette containing dilute (1-5 per cent) chick embryo juice and (one drop) transferred to one drop of 50 per cent chicken plasma previously dispensed in 13 mm. serological tubes.\*\* The tube was spun between the hands and slanted almost horizontally so that the explant lay just above

\* Composed as follows (Grams per liter): NaCl, 0.8; KCl, 0.4; CaCl<sub>2</sub>, 0.2; MgSO<sub>4</sub>, 0.7; H<sub>2</sub>O, 0.1; KH<sub>2</sub>PO<sub>4</sub>, 0.2; Na<sub>2</sub>HPO<sub>4</sub>, 0.2; H<sub>2</sub>O, 0.025; glucose, 2.0; and phenol red, 0.02. This solution was sterilized by autoclaving. When employed in media, 1 cc. of isotonic (1.4 per cent) NaHCO<sub>3</sub> was added to each 10 cc. of salt solution or of components other than serum, to make a final concentration of 0.125 per cent. The serum and all nutrient solutions containing NaHCO<sub>3</sub> were handled and stored under 50 mm. CO<sub>2</sub> to prevent alkalization, and were incubated under 25 mm. CO<sub>2</sub> to provide an initial pH of approximately 7.6.

\*\* Chicken blood was taken in a 10 cc. syringe containing 0.2 cc. of 10 per cent sodium citrate, 0.2 per cent final concentration. The plasma was stored in plain glass tubes.

the curvature at the base of the tube. After coagulation of the plasma, 0.5 cc. of the chosen medium was added to each culture. The small amount of plasma permitted almost complete change of the medium at each renewal (each three days) and provided so little chicken serum that the initial cultures were not washed to remove heterologous protein.

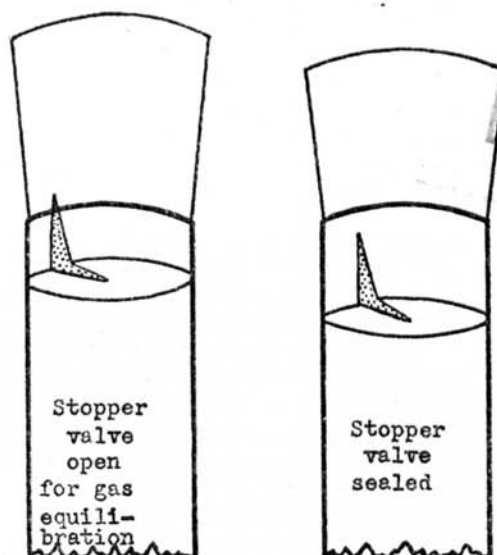


Figure 1. Stopper valve for opening and sealing vessels in which special gas tensions are required.

Paraffined, fissured cork stoppers (Figure 1) were inserted and the cultures assembled in Novy jars for the addition of 25 mm.  $\text{CO}_2$  to the atmosphere. After equilibration for three hours, or overnight, the neck of each tube was warmed and the stopper pushed down to a tight seal. The cultures were incubated in slanting position, at  $37^\circ\text{C}$ .

Following the work of Carrel and Ebeling and of Baker, the media designed as possibly favorable to macrophages was composed of 50 per cent human serum\* with 0.01 per cent added nitrogen from Witte's peptone or other protein digests recommended by Baker.

*Cytological study.*—Information regarding the cytology of the nodules and tissue cultures was obtained from three sources: unstained cultures, spreads of cell suspensions, and tissue sections.

For direct examination of these cultures with standard microscopes, the curvature of the tubes offered less interference than does the great depth of Carrel flasks. A magnification of  $\times 24$  was used for measuring the culture diameters, of  $\times 100$  for routine examination of cultures, and of  $\times 200$

\* Thanks are due to two small boys who repeatedly supplied blood for this purpose. To each 10 c.c. of serum, 0.5 cc. of  $\text{NaHCO}_3$  was added, also 0.2 cc. of a concentrated solution of phenol red and glucose to provide 0.002 per cent and 0.2 per cent, respectively.

for observing cells of special interest. None of these permitted discerning bacilli in living preparations.

The suspensions of cells which were liberated during the cutting of the original lepromata, and also by mincing tissue cultures on glass slides, were spread on slides and dried. The films were fixed with formaldehyde vapor for five minutes, stained with carbolfuchsin, and counterstained with methylene blue or by Wright's method. Although these preparations did not show the original relationships among the cells, they gave an opportunity for detailed study of the individual cells and their bacillary content.

Small portions of the original nodules, and entire tissue cultures, were fixed in formol-Zenker, imbedded in paraffin, and sectioned. The sections were stained with hematoxylin-eosin for a study of the cells and with carbolfuchsin and methylene blue for bacilli.

*Estimation of bacterial numbers.*—Single tissue culture explants (about 1 cu. mm.) were too small to be suspended by the methods used in the bacteriological studies for counting bacilli. In order to ascertain their bacillary content, the individual explants or cultures were placed on a glass slide underneath a lens and cut with a very sharp scalpel until 100 to 200 minute pieces or "chips" were obtained, the number of chips from each explant being made as constant as possible throughout a given experiment. The chips were so small that they were scarcely visible to the naked eye. They were fixed on the slides by heat rather than formaldehyde and stained as if they were ordinary bacteriological smears.

TABLE 1.—*Illustration of method of classifying tissue chips with regard to bacillary content, and of calculating score for each explant.*

Per cent of chip occupied by bacilli	Class and factor	Number of chips in each class	Number of chips x factor	Score (Degree of positiveness)
0	0	0	0	
1-25	1	15	15	
26-50	2	35	70	
51-75	3	30	90	
76-100	4	20	80	
Totals		100	255	$\frac{255}{100} = 2.6$ *

\* In a scale of 0 to 4+, this figure denotes the position of the result between 2+ and 3+, i.e., 2.6.

The average bacillary content of the chips from each explant was determined as shown in table 1. The chips were recorded as negative if no bacilli were seen with the low power objective or in five oil-immersion fields. In rating the positive chips, each was examined under low magnification, to decide what proportion of its area was occupied by masses of bacilli, the immersion objective being used repeatedly to check on the correctness of the low-power observations. The number of chips receiving a given rating (0 to 4+) was multiplied by the corresponding factor (see Table 1), and the total of the figures thus obtained was divided by the number of chips examined. The score thus obtained represented, on a scale of 0 to 4+, the average degree of positiveness of each explant. In the ex-

ample shown in table 1, the average degree of positiveness was 2.6+. Hereafter such values will be considered simply as a score, and the results may be judged by comparing the average scores of groups of control and cultivated tissues.

### RESULTS

Tissue cultures from lepromata obtained from 4 patients were maintained in good condition for periods of from fourteen to thirty-four days. Several features of these experiments are summarized in table 2.

TABLE 2.—*Summary of history of tissue cultures studied.*

Feature	Experiment			
	1	2	3	4
Age of patient (years)	35	36	56	20
Percentage of explants which grew	55	59	63	75
Period of active outgrowth (days)	10-14	11-13	11-17	11-14
Period of renewal of medium (days)	14	17	38	36
Type of cells in growth zone	Fibroblasts	Fibroblasts and a few small round cells.	Fibroblasts	Fibroblasts and a few small round cells
Reason for termination of experiment	Used on 16th day for histological study	Degeneration of fibroblasts 14th-18th days.	Degeneration of fibroblasts 15th-34th days.	Degeneration of fibroblasts 18th-30th days.

In each experiment the proportion of explants which grew, the longevity of the cultures, and the appearance of the fibroblasts appeared to be influenced more by an inherent growth capacity than by the age of the patient, the media employed, or the concentration of bacilli. The fibroblasts first made their appearance in three to six or ten days, and generally reached a maximum growth after about ten to fourteen days. As a general rule, degeneration was first apparent in the more rapidly growing and maturing cultures after about fourteen days, at a time when others were still growing. In experiments 3 and 4 some of the cultures remained in viable condition for as long as thirty-four and thirty days, respectively.

In only two experiments (2 and 4) were significant numbers of small round cells noted in the growth zone during the early life of the cultures. These cells were probably of blood origin and survived for only a few days. The large, heavily-loaded macrophages of the leproma were apparently unable to migrate out into the plasma. A remarkable rounding-up of fibroblasts was usually asso-



ciated with degeneration of the older cultures. Though suggesting a transformation of fibroblasts to macrophages (see Timofejewsky) it was, in reality, a terminal process.

Although the ratios of cell types, and the appearance of the cells loaded with bacilli, differed in each case the observations in Experiment 1 may be used to illustrate the general findings. The spreads of cell suspension from the fresh nodule showed only moderate numbers of free bacilli or globi. Though special studies would be required to identify all of the cell types, the following proportions and classes of cells were quite definitely recognizable: (a) About 60 per cent were macrophages, all but the smallest of which were literally stuffed with bacilli. Being freed from the compression of the tissues, these cells assumed a rounded shape on the slides and were recognized more readily than in tissue sections. (b) About 25 per cent resembled the fibroblast-like cells mentioned by Timofejewsky and by Suwo and Kin (histioids of Wade, possibly undifferentiated mesenchymal cells of Maximow) and contained an extremely variable number of bacilli. (c) About 2 per cent were typical fibroblasts, almost universally devoid of bacilli. (d) Occasional polymorphonuclear and other leucocytes were found, usually lacking bacilli.

After incubation of the explants for 16 days, similar cell suspensions showed enormous numbers of globi and bacilli scattered over the slides, and a complete disappearance of all cell types except fibroblasts. These cells were abundant; the majority were devoid of bacilli, while a lower proportion contained moderate numbers of micro-organisms.

Sections of the original nodule showed the usual leprous granuloma, composed of the cells mentioned above. The relationship of bacilli to the different cell types could not be determined as accurately as in the cell spreads. In corresponding sections of tissue cultures at 16 days, no differentiation was necessary, since there was only a single cell type. Except for fibroblasts in the vessel walls, where the canals may have provided for aeration and nutrition, the original explants had undergone necrosis, and the bacilli were left in great numbers throughout the residuum of the original leproma tissue. Among the actively growing and numerous fibroblasts at the margins of the explants there were definitely fewer bacilli than inside the original explants. Only one fibroblast out of 20 or 30 contained bacilli, and almost always in small numbers. It was sometimes observed that fibroblasts would grow through masses of bacilli without ingesting them.

As intimated, the cultures from the nodules used in the other three experiments possessed distinctive characteristics, but all of

the observations confirmed the fact that the majority of the bacilli were simply deposited in the explants by the death of their host cells. The most persistent cells were fibroblasts which usually contained few or no bacilli. The concentrations of bacilli in the growth zone and in the plasma was always much lower than in the central explants.

Estimations of the bacillary content of cultivated tissue explants were made in three experiments (See Table 3). In Experi-

TABLE 3—*Comparison of bacillary content of control explants and of explants in positive tissue cultures.*

Experiment number	Control Scores		Scores in positive cultures	
	Original control explants	Explants incubated without growth	After growth for	
			10-20 days	21-35 days
2	2.1	1.6	0.7	
3	2.3		2.6	2.1
4	2.4			2.5

ment 2, the average score of eight control tissues was 2.1; that of 10 explants incubated but failing to grow was 1.6; while the score of 12 tissues which grew for 14 to 18 days was 0.7. The explants in these cultures were extremely soft, and liberated many more bacilli into the plasma than could be explained by the migration of cells. This loss of bacilli during the cultivation and sampling of the softened tissues is believed responsible for the progressively lowered ratings.

In Experiment 3, the control group of 11 explants averaged 2.3, while nine positive cultures which were rated within the first 20 days averaged 2.6. The cultures which grew for the longer period of 21-34 days averaged 2.1. When the positive cultures were divided into two groups on the basis of poor growth and good growth, scores of 2.4 and 2.3, respectively, were obtained. Thus the bacillary content of the central part of the cultures was not dependent on the period or extent of fibroblast growth.

The results of Experiment 4 were summarized by pooling the scores of fourteen original control tissues with those of six explants which failed to grow; these gave an average of 2.4. The eighteen explants in cultures examined after 21, 32, and 41 days averaged 2.5. This nodule gave rise to a greater number of migrating round cells than any other. The cultures that exhibited both round cells and fibroblasts scored on the average 2.9, while those with only fibroblasts rated 2.2. Due to variations in the values for the individual explants, the difference between these averages is not significant.

## DISCUSSION

The results of the present study differ in several respects from those recorded by Timofejewsky and by Suwo and Kin. Since Timofejewsky used human and rabbit plasma, and neither he nor Suwo and Kin renewed the liquid phase with fluids containing serum, considerable softening of the plasma around the explants must have occurred. This liquefaction may possibly explain the high incidence of rounded cells containing bacilli, a feature entirely absent in these experiments. Differences in cytological observations may also be ascribed in part to the greater maturity of the cultures examined in the present study. Timofejewsky examined many early preparations on cover slips and Suwo and Kin based their chief observations on cultures after five to ten days. In the present study an attempt was made to learn what cell types persisted in a viable state as possible continuous hosts for the bacilli. The cultures were studied only after two weeks or later. The majority of fibroblasts remaining after such intervals were devoid of bacilli, while the positive fibroblasts contained only moderate numbers of micro-organisms.

The earlier workers explained the turbidity which developed in the plasma of their older cultures as due to a tremendous growth of the bacilli after the tissue cultures died. In the present work a similar opacity resulted from attempts to incorporate 10 or 15 per cent of chick embryo juice in the serum media. This opacity disappeared during the staining of sampled cultures, and obviously could not be attributed to bacilli.\* In the second experiment, which revealed the greatest numbers of bacilli in the plasma, the explants were soft and were shown to have lost many bacilli during the course of the experiment.

In considering the question of bacillary growth, it is necessary to distinguish between multiplication which might occur in the original explants and that which might take place in the new cells of the zone of outgrowth. The sampling and scoring method employed involved the recording of the bacillary content of thousands of tissue chips from the original and cultivated explants in each experiment and is believed capable of detecting a doubling of the original numbers of bacilli. The average scores failed to provide evidence of bacillary multiplication. The original explants underwent necrosis, leaving the majority of the bacilli without host cells. By more exact counting methods than those applicable here, it has been shown that the bacilli do not increase in numbers in devital-

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\* The precipitate has been identified as composed chiefly of calcium phosphates. Further details will be published later.



ized tissue slices in tissue culture solutions (2). The earlier workers apparently referred to multiplication of bacilli in the zone of outgrowth and later in the plasma. Since many bacilli are carried into the outgrowth and the plasma by the cells, the occurrence of bacillary multiplication in the zone of cell outgrowth cannot readily be proven or denied in primary cultures from lepromatous tissue. It seems wiser to reserve final judgment until the fate of the bacilli in growing fibroblasts has been studied by quantitative methods, and in serial subcultures.

#### SUMMARY AND CONCLUSIONS

1. Tissue cultures from four lepromata were grown and maintained in a viable condition in serum media for periods of from 14 to 34 days.
2. The infected macrophages died early in the history of the cultures and deposited their bacilli within the necrotic mass of the original explants.
3. The only cells that persisted throughout the life of the cultures were fibroblasts, which ordinarily contained no bacilli or only a few.
4. Quantitative rating of the explants with respect to their bacterial content did not reveal an increase in the total mass of bacilli.
5. The development of turbidity in the plasma of older cultures was found not to be attributable to multiplication of bacilli in this site as claimed by earlier workers.

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