

ATTEMPTS TO INFECT CHICK EMBRYOS AND CHICK
TISSUE CULTURES WITH BACILLI FROM HUMAN
LEPROMATOUS LESIONS

by

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During studies on the fate of leprosy bacilli in the fibroblasts of lepromatous lesions (1), occasion was taken to utilize the bacilli from any extra portions of the nodules in trying to establish experimental infections in chick embryos and chick tissue cultures. Due to the two different types of experimental material, the literature, methods, and results pertaining to each will be summarized in separate sections.

Leprosy Bacilli in Developing Chick Embryos

The susceptibility of chick embryos to a remarkable variety of bacteria, viruses, and Rickettsiae and their lack of bactericidal power (2) suggest that they offer as great likelihood as any non-human tissue of being susceptible to leprosy infection.

Moore (3) inoculated the bacillus of rat leprosy, three types of tubercle bacilli, and several strains of saprophytic acid-fast micro-organisms into the chorio-allantoic membrane of 12-day chick embryos by means of the Goodpasture-Budding technique, and six days later used the embryos for study or for further passage. He found that the rat leprosy bacillus and the human and bovine tubercle bacilli decreased in numbers during passage, while avian tubercle bacilli and the saprophytes increased.

Inoculation of the chorio-allantois or other developed organs appeared undesirable on account of the relatively short incubation periods remaining before the hatching of chicks. Cox's method (4) of injecting Rickettsiae into the yolk sac minimizes this objection and afforded the possibility that the bacilli might be distributed throughout the embryo and reach any sites favorable for multiplication earlier during the incubation.

Small groups of eggs were injected as material was available from different nodules until a total of 24 developing embryos had been studied. The age of the embryos at inoculation varied from three to seven days, and the period of further incubation varied from five to sixteen days. Fourteen of the inoculated eggs were incubated at 37 degrees C., while 10 were maintained at 34 degrees C. The concentration of bacilli in the inoculums covered a range from moderate numbers to dosages which killed embryos and prevented

their inclusion in the series. Microscopic examination of 200 to 250 fields per smear from the yolk, yolk sac, chorio-allantois, liver, spleen, breast muscle, and whole minced pulp in most instances failed to reveal any bacilli. To avoid difficulty with the acid-fast and eosinophilic granules which occur in normal embryos (eyes discarded), tissues smeared from embryos must be fixed by heat rather than formaldehyde and must also be decolorized thoroughly. On several occasions large numbers of bacilli were found in the yolk fluid, but apparently always from the site of the original inoculum. Following more careful homogenization of the yolk, these concentrations were avoided.

One chick was allowed to hatch from these eggs and was autopsied 26 days later. No bacilli were discovered during examination of 200 fields in smears from each of the following organs: heart, liver, spleen, lungs, inguinal lymph nodes, testes, ear lobe, leg muscle, and breast muscle.

These observations on the inoculation of chick embryos with leprosy bacilli, though not extensive, failed to suggest that the bacilli were collected preferentially or were growing in any organ or tissue.

Leprosy Bacilli in Chick Tissue Cultures

The inoculation of leprosy bacilli into autolyzing tissue media composed of embryo pulp and juice has in the past resulted in three discordant types of results. McKinley and Verder (5, 6) and Soule (7) reported microscopic growth of human leprosy bacilli in embryonal chick tissues minced in Tyrode's solution. Salle (8) with short-term chick cultures and in minced embryonic tissues, and Walker and Sweeney (9), using minced rat embryonal tissue, recovered from human and rat sources facultatively acid-fast diphtheroids which grew readily on bacteriological media. Holt (10) and Lowe and Dharmendra (11) reported that leprosy bacilli did not multiply in such media.

The fate of leprosy bacilli in chick tissue cultures during prolonged maintenance in the original vessels has apparently not been studied hitherto. The cultures were derived from the breast muscle of 10- to 15-day chick embryos or of 5- to 10-day-old chicks, not simply as a classical source, but because of the numerous publications of Japanese workers on the transmission of human and rat leprosy to the pectoral muscle of chickens.

Ota and Sato (12) reported infection of rats with the bacilli recovered from the breast muscle of a hen six and one-half months after the injection of a single large dose of rat leprosy bacilli. In other reports of similar results (13), this simple procedure was complicated by multiple heavy injections, the last of which may have been given a questionable short time prior to the tests in rats. Watanaba and Nonaka (14) found that the lesions resulting from a single injection regressed after three months and exhibited small granular bacilli. Nonaka (15) was unable to

effect a serial passage of rat or human type bacilli through chickens. Rat leprosy bacilli recovered from 15-day lesions were incapable of infecting rats. Nagai and Hayashi (16), using nodule fragments, observed regression of the lesions within 10 months, and were not convinced of bacillary multiplication. Ota and Nitto (17) subsequently reported successful passage of human leprosy bacilli through seven series of hens as a result of adding kiesleguhr, trypan blue, and potassium iodide to the original bacillary suspensions and to the material used for each transfer. Primary lesions prepared with bacilli and the "aggravating mixture" progressed for at least one year, while the lesions produced by the later serial transfers subsided more quickly. Heated suspensions of breast muscle from the second and fifth serial passage produced lepromin or lepromin-like reactions (18).

Methods: Cultures were prepared and maintained in 13 mm. serological tubes by methods already described. The media were of differing compositions in the various experiments, usually being combinations chicken serum (CH) with Simm's serum ultrafiltrate (SF) or with fresh (Em) or pasteurized embryo juice (EmP). Three temperatures of incubation were employed: 37 degrees C., 34 degrees C., and room temperatures which varied from 29-31 degrees C. Under these conditions the intervals between patching of the plasma and renewal of the media were approximately 2-3 days, 7 days, and 60-90 days, respectively. The most satisfactory control on the original number and disposition of the bacilli consisted of sampling and fixing a portion of the cultures in each experiment after they had grown for 3-10 days, and storing them in a dry atmosphere until the other cultures had been incubated for the chosen intervals.

Results: Several of the experimental conditions, though satisfactory for the cultivation of chick tissues, may be summarized briefly and dismissed as unsuited to the purpose of the present experiments. These shortcomings involved, first, the difficulty of getting the bacilli and the cells in uniform or intimate contact at the outset, and second, the problem of trying to maintain fairly stable cell populations if an extremely slow bacillary growth rate were to be demonstrated.

Attempts to provide for phagocytosis of bacilli by incorporating a small island of bacilli in the plasma immediately beside each explant proved disappointing. The fibroblasts grew through these bacillated areas with singular unconcern for the bacilli, usually without any modification of the circular outline of the tissue colony. Among 80 cultures prepared in this way, only 3 or 4 showed a balling up of fibroblasts in this region, but this phenomenon was not due to phagocytosed bacilli and its rarity attests to the non-toxicity of the leprosy bacilli. In one such culture, 800 cells were studied in detail, which revealed that 3 fibroblasts had undoubtedly ac-

quired bacilli: 1 with several bacilli and 2 with one bacillus each. The macrophages in chick tissue cultures were usually to be found in the original explant area near the center of the cultures, and they did not migrate sufficiently to encounter the bacilli.

Immersion of the explants during many hours in solutions containing both bacilli and serum resulted in the adhesion of inconstant numbers of bacilli, and did not bring about an early or important entrance of bacilli into the cells.

More suitable combinations of the bacilli or of carbon particles with the entire cell population was obtained by injecting these particles into embryonic breast muscle a short time prior to preparing explants, or by similar injections into hatched chicks from which the tissues were explanted after a few days. Since the tissues filtered out, and retained near the center of the injection site, the coarser clumps of bacilli and most of the carbon, the distribution in tissues biopsied within a few days was not uniform, and cells containing small numbers of single bacilli were to be found only around the margins of the infiltrated areas. The original explants usually contained variable clump sizes and numbers of bacilli. This method permitted observing the response of both macrophages and fibroblasts to the bacilli.

The usual method of maintaining cultures at 37 degrees C., by repeatedly replacing a fluid phase of embryo juice, necessitated frequent patching of the plasma and induced such rapid growth of the cultures that they had to be divided very frequently. The bacilli disappeared rapidly by dilution, and the method was soon abandoned. At 34 degrees C., the growth rate was retarded but it proved to be impossible to retain a given series of cultures in the original tubes for more than a few weeks. In such short intervals, it was impossible to gain an adequate idea of the possible changes in bacterial numbers, or to maintain the prolonged contact desired between the bacilli and the originally infected cells. Incubation at room temperature produced an unexpected longevity even if the cultures were not renewed or patched for periods as long as 90 days. The cultures under this condition were the only ones in which the rate of cell development was adapted to studying a micro-organism of slow or doubtful multiplication. Several typical experiments are summarized below.

A mixture of washed leprosy bacilli and of washed carbon from India ink was injected into the breast muscles of a 2-day chick, and recovered 5 days later. Three groups of explanted cultures were maintained at 37 degrees C. Those in CH₈Em₁₅ exhibited relatively few macrophages and unsatisfactory phagocytosis. The fibroblasts grew so rapidly that the culture could not be kept in the original

tubes for more than 14 days. Those in $\text{CH}_8\text{SF}_{33}$ revealed more active macrophages, but fell into poor condition after about 2 weeks. Those in CH_{33} early showed in living preparations a remarkable collection of carbon in the round cells at the center of the cultures and, in stained cultures, many bacilli along with the carbon. Within less than 20 days practically all of the carbon and bacilli had been collected by round cells. Bodies in living preparations which were suspected of being giant cells were found upon sampling to be clusters of individual cells around heavily-loaded macrophages. After 31 days the ratio of bacilli to carbon appeared lowered, the individual bacilli paler, and the outlines of bacilli in clumps less distinct.

Two similar groups of cultures were maintained at room temperatures for 2 months in $\text{CH}_8\text{EmP}_{15}$ and in CH_{33} without renewal of the media. The lower temperature and the use of heated embryo juice proved much more favorable than the fresh embryo juice used above to the persistence of round cells, which were recognized in both media for approximately one month by their content of sharply segregated carbon. By this time, cultures in $\text{CH}_8\text{EmP}_{15}$ lost their centers by softening of the plasma, while those in CH_{33} became too dense for clear observation. Upon patching and renewal, or re-imbedding, of the cultures after 60 days the bacilli and carbon made their appearance for the first time in a goodly proportion of the fibroblasts. The extracellular bacilli remaining no longer exerted any attraction for the cells, and normal cells did not cluster around those containing bacilli. No individual bacilli could be found, while those in clumps were fused and indistinct.

Subcultures from this series were maintained for a second period of 60 days without renewal of the medium. Although the growth (now almost entirely fibroblasts) was much slower than in the first series, no bacilli were found at the end of 4 months.

The general results of two additional experiments at room temperature, employing explants from chick embryos, may be summarized as follows: (a) Pasteurized embryo juice or serum ultrafiltrate with 15-20 per cent of chicken serum proved to be the type of media most suited to the persistence of active round cells in cultures of the greatest longevity; (b) during the storage period the macrophages often persisted for 60 days, while fibroblasts were consistently maintained for 90 days without change of the medium; (c) the bacilli and the carbon were at first found almost exclusively in the macrophages and later in the more persistent fibroblasts; (d) the presence of either kind of particles acted as a mild stimulant which increased the digestion of the plasma, hastened the disappearance of the round cells, and measurably reduced the longev-

ity of the fibroblasts; and (e) the leprosy bacilli showed no signs of proliferative activity and disintegrated more rapidly than in neutral bacteriological media. Occasional clumps of bacilli lying free in the plasma remained in better condition than those inside of cells.

During the study of these cultures, it was found necessary to discriminate between leprosy bacilli and two kinds of artefacts which occur in cultures of chick fibroblasts. These were chromatin threads, which often suggested blue micro-organisms, and acid-fast lipid vacuoles in the outermost fibroblasts in the cultures. Chick fibroblasts differ from human fibroblasts in being remarkably prone to lipid accumulation, especially during growth in moderate or high serum concentrations and during the first month in unchanged media at room temperature. Lipid vacuolation in serum media, e.g., CH₃₃, occurs to a degree which is fatal to the outermost cells. This process is similar to the clipping of a hedge, and accounts for the density of growth and the restricted radii of cultures in serum media. Such vacuoles with acid-fast material were recognized by their peripheral distribution and lack of carbon (the bacilli and carbon were segregated near the centers), by their occurrence in normal control cultures, and by the rise and fall of their incidence with the varying degrees of lipid vacuolation.

Although these experiments were not carried out under conditions which permit a quantitatively accurate statement that the bacilli failed to multiply, it must be emphasized that none of the results suggested their multiplication, while a decreasing incidence or actual disintegration of bacilli was usually observed. The interval during which the bacilli can be kept in developing chick embryos is too short to justify any conclusion except that it is not a useful method. The high carbon dioxide content, acidity, and cell activity of the embryo are physiological conditions very different from those in the human organs that are involved in leprosy infections. These are also the conditions in which the bacilli disappear most rapidly during the cultivation of fibroblasts from tuberculoid or lepromatous lesions in humans.

Attempts to infect either chick or human fibroblast cultures by the addition of bacilli to the plasma, or by immersion of explants in bacillary suspensions have proven unsatisfactory. Fibroblasts from both sources, when growing into or through masses of bacilli, engulfed disappointingly few. Human fibroblasts have been shown to be capable of a remarkable conveyance of bacilli when growing from naturally infected tissues. Suitable infection of chick tissue cultures necessitated injections of the bacilli into the tissues prior to the preparation of explants. These observations suggest that

fibroblasts take up particles from the central colony area more readily than through their growing tips.

Insofar as the results of the present work are related to the question of bacillary multiplication in the pectoral muscle of adult chickens, they must be taken to support the observations of Watanabe and Nonaka, Nonaka, and of Nagai and Hayashi, whose studies disclosed an eventual disappearance of the lesions rather than bacillary proliferation. The two cell types cultivated from the embryonic and the young chick breast muscles were certainly those upon which a successful infection of the adult tissues might depend, and they were obtained at a time when the natural resistance of fowls is at its minimum.

SUMMARY AND CONCLUSIONS

Bacilli from leprous nodules were used to infect developing chick embryos, tissue cultures from chick embryos, and newly hatched chicks.

There was no evidence that the micro-organisms grew during the brief interval between the injection of bacilli into chick embryos and the hatching of chicks.

When the bacilli were injected into chick tissues prior to the preparation of explants for cultivation, the bacilli occurred almost exclusively in the macrophages during the existence of these cells and only later in the more persistent fibroblasts. The bacilli appeared to be no more toxic than carbon particles. Either kind of particles stimulated the cells enough to hasten the digestion of the plasma and to reduce measurably their longevity during continuous growth in non-renewed media. The bacilli within cells disintegrated more rapidly than those outside.

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