AN ATTEMPT TO CONFIRM GROWTH OF *MYCOBACTERIUM LEPRAE MURIUM* ON CHORIOALLANTOIC MEMBRANE OF LIVE CHICKEN EMBRYOS

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R. Noel and Soeur Marie Suzanne have reported that *Mycobacterium leprae murium* multiplies on the chorioallantoic membrane of the developing chick embryo and that, following three passages on membranes, the bacilli produced typical rat leprosy within 27 days after intratesticular inoculation (4, 5). In view of the fact that there exists no accepted *in vitro* method of cultivating this mycobacterium, the importance of confirming or refuting these observations is evident.

Evaluation of the results of Noel and S. Marie Suzanne requires accurate information on two points: (*a*) Does the chorioallantoic membrane yield more bacilli than the number inoculated; and (*b*) are the recovered bacilli more infectious than the aliquots which were inoculated? The present communication summarizes data obtained by microscopic enumeration of the bacilli and by measurement of the relative infectiousness of the inoculated and the recovered bacilli.

METHODS

Infected rat testes were homogenized in M/75 Na_2HPO_4 and centrifuged at low speed to provide clarified supernates equivalent to 5 per cent tissue concentration. This supernate was diluted to a concentration of 0.1 per cent for the initial control microscopic counts. The chorioallantoic membrane of each 9-day embryo was inoculated with 0.1 ml. of the 5 per cent suspension. Infected membranes were homogenized in 5 ml. of buffer per membrane, with the result that the suspensions recovered from the eggs corresponded also to a 0.1 per cent concentration of the bacterial suspension. The data obtained by microscopic means included the original number of bacilli inoculated, and the number occurring in first-passage membranes after 4 and 10 days. In serial-passage experiments, the bacilli were counted at the end of each 7-day passage interval. The preparation of homogenates and standard films, and the methods of staining and enumeration, were according to the more recent methods of Hanks and Backerman (3).

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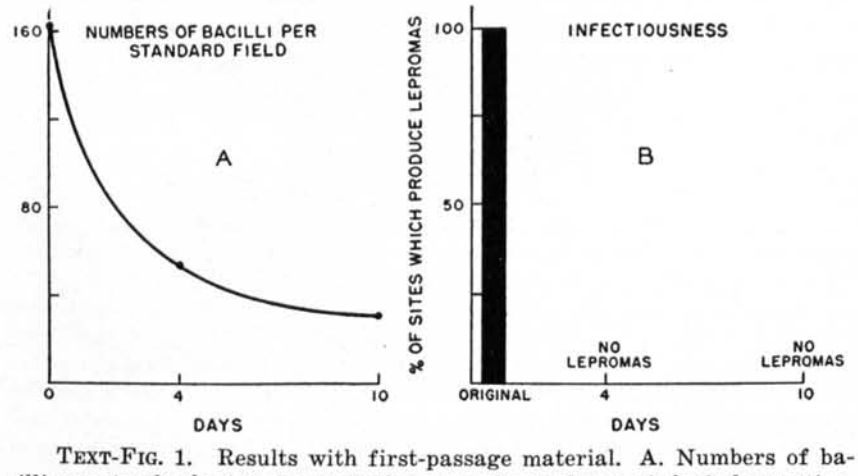
[1] The opinions or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department.

The relative infectiousness of bacilli during the first passage was measured by inoculating rats in six skin sites, employing the rotating patterns of Hanks and Backerman (2). Since comparisons were confined to the original suspensions, 4-day membranes and 10-day membranes, each aliquot was tested in duplicate in each rat. Data for each suspension, therefore, was obtained from 12 inoculation sites. Inoculation sites were palpated monthly, while final data were collected after six months.

Serial passage experiments were conducted similarly, except that possible persistence of infectiousness was ascertained by intratesticular inoculation, in accordance with the procedure of Noel and S. Marie Suzanne. Because of the increasing difficulty of finding microorganisms in the standard dilution, four rats were inoculated intratesticularly with second passage material.

RESULTS

Typical results during a first passage of *M. leprae murium* on chorioallantoic membranes are illustrated in Text-fig. 1. It is apparent that the numbers of bacilli declined steadily throughout the 10-day period of observation, and that infectiousness was not demonstrated after the bacilli had been on the membranes for only four days. At the termination of the observations, positive lesions had existed in the control sites for 30 weeks.



TEXT-FIG. 1. Results with first-passage material. A. Numbers of bacilli per standard microscopic field during the 10-day period of observation. B. Results of inoculations with suspensions of inoculated chorioallantoic membranes.

In the serial passage experiments, the results were similar except that the numbers of bacilli declined more rapidly. Membranes of the second passage did not contain sufficient bacilli for enumeration at the standard dilution, while membranes of the third passage appeared to be devoid of bacilli at this dilution.

Bacilli were demonstrated, however, in more concentrated suspensions of second-passage membranes, but not in third-passage membranes.

In serial histological sections of testes inoculated with second-passage material and biopsied after 27 days it was not possible to find acid-fast bacteria. After 5 months, aspiration of other testes again failed to show any acid-fast organisms.

DISCUSSION

This attempt to confirm the results of Noel and Soeur Marie Suzanne reveals again the dangers of relying on qualitative observations on the presence of bacilli or on the production of histologic lesions in mycobacterial diseases. The quantitative methods employed in the present study indicate, on the other hand, that assessment of the fate of bacilli in such circumstances may be accomplished readily and conveniently.

Quite aside from the factual data obtained in the present study, there are certain features of the original work of Noel and S. Marie Suzanne which are incompatible with their conclusions. In the first place, if the murine leprosy bacilli had actually been adapted to the embryonic membranes or tissues, the observations should not have been terminated in the third passage. Continuous transmission would have been too tempting to be neglected. In the second place, an interval of 27 days in rat testes, especially without further transmission in rats, does not constitute evidence that typical rat leprosy has been caused by membrane-passed bacilli. In the third place, the maximal period of incubation on suitably developed membranes of chick embryos is limited to 10 days.

In order for the disease to be transmitted serially by means of 20 per cent membrane emulsions, the numbers of bacilli would need to increase at least five or more times during each 10-day interval. The only existing quantitative data indicates that in the most susceptible tissue (testis) of the natural host, approximately 12 days are required for a doubling of the numbers of bacilli (5). On this basis alone, it should be evident that neither murine nor human leprosy bacilli are capable of serial passage in any type of tissue which must be homogenized and diluted every ten days.

With respect to interpretation of the findings in this study, it may be recognized that the recovery from 4-day membranes of fewer bacilli than the number inoculated would not necessarily indicate an inability of the bacilli to survive or multiply in the

test environment. Early destruction of inferior bacilli, a lag period prior to the onset of growth, and/or the need for preliminary adaptation to a new tissue could explain this decline below the initial numbers. However, if the bacilli were to multiply sufficiently to exceed the original numbers by the end of ten days, it would be necessary for these early losses to be overcome by growth at a fantastic rate. In order to reach a favorable conclusion concerning the adaptability of murine leprosy bacilli to chick embryonic membranes it would only be necessary that the bacilli retain their infectiousness and that their numbers should rise significantly during the period between four and ten days. It would not be necessary that this rate of accumulation should exceed the normal rate for known susceptible rat tissues.

The experimental observations, however, do not provide data in support of these minimal—and reasonable—requirements. In the first place, the numbers of bacteria continued to fall between the fourth and tenth days. In the second place, their infectiousness had been so severely damaged that no lesions had been produced by any of the experimental aliquots two months after all control sites were positive. There appears to be no alternative but to conclude that the chorioallantoic membrane of chick embryos is an extremely unfavorable environment for murine leprosy bacilli.

SUMMARY

1. An attempt to confirm the work of R. Noel and Soeur Marie Suzanne has shown, (*a*) that *Mycobacterium leprae murium* suffers a marked loss of infectiousness during only four days on the chorioallantoic membrane of chicken embryos; and (*b*) a continuous decline in the numbers of bacilli which can be recovered.

2. Methods and criteria have been presented for evaluating such claims.

RESÚMEN

El autor trató de confirmar el reporte de R. Noel y Souer Marie Suzane que los bacilos de la lepra murina se reproducen en la membrana corioalantóica del embrio del pollo y que su infectividad se retiene aún después de tres cultivos consecutivos. Recuentos de los bacilos en suspensiones de inoculaciones del primer pase después de cuatro a diez dias demostraron gran disminución. Mas aún, después de cuatro días dichas suspensiones no produjeron ni lesiones dérmicas en las ratas inoculadas, ni se multiplicaron en el testículo. Membranas del segundo pase tenían demasiado pocos bacilos para enumeración en diluciones standard, aunque

algunos fueron hallados en suspensiones más concentradas. Membranas del tercer pase no contenían bacilos.

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