Limited Multiplication of *Mycobacterium lepraemurium* in Cell Cultures. (23657)

JOHN H. WALLACE, STEPHEN D. ELEK,* AND JOHN H. HANKS
Leonard Wood Memorial Laboratory, Department of Bacteriology and Immunology, Harvard Medical School

Since the demonstration of *M. lepraemurium* and its recognition as the causative agent of rat leprosy(1,2), this intracellular parasite has been an object of unusual interest with the problem of human leprosy. Like the human leprosy bacillus, it has thus far defied all efforts at cultivation *in vitro*, whether in bacteriological media or in cell cultures(3). Zinsser and Carey were the first to report the intracellular multiplication of this organism in spleen explants *in vitro*(4). Evaluation of this claim as well as of subsequent ones by other investigators is difficult. Though previous studies in this laboratory† have demonstrated high infectiousness in *M. lepraemurium* maintained in rat fibrocytes for 30 days, there were no significant increases in bacterial numbers. The problems involved in making microscopic determinations in cellular systems containing mycobacteria are considered in detail elsewhere(5). Metabolic studies(6,7,8) have elucidated many of the peculiarities of *M. lepraemurium*; especially the disruption of its endogenous metabolism and infectiousness by the serum concentrations previously employed for growing tissue cells *in vitro*. *In vivo* experiments have shown that during logarithmic growth, when bacterial numbers double in 8-10 days, bacilli are being destroyed in some infected cells while flourishing in others(9). This observation is possibly explained by the experimental finding that only 15-20% of freshly harvested bacilli are metabolically active.†

The present paper reports a 2-3 fold increase of *M. lepraemurium* in cell culture systems in which the following circumstances prevailed: 1) Cells containing a high incidence of active microorganisms were assured by explanting spleen cells from mice at a time when the bacilli were in the logarithmic phase of growth. 2) For *in vitro* infection by means of bacillary suspensions, each phagocytic cell was permitted to acquire on the average some 10 bacilli which had been protected in serum albumin and yeast supplement(6). 3) The probable impediment imposed by inhibitors in serum was minimized by nurturing the cells in fluids containing low concentrations of body fluids. All supplements were known to exert no deleterious effects on the metabolism of the bacilli. 4) Bacillary numbers were determined before and after incubation by combining the microorganisms from culture supernates with those liberated from disrupted cells and making direct microscopic counts.

Materials and methods. M. lepraemurium: The Wells strain was used in cells of infected mouse spleens without prior storage. Suspensions of the Hawaiian strain of *M. lepraemurium* were prepared from infected rat testes. Following light centrifugation, these bacilli in 20% tissue homogenates were refrigerated in 5% albumin and 5% Difco yeast supplement B. *Tissue culture.* Spleen tissue of mice infected with the Wells strain of *M. lepraemurium* for 3-6 weeks *in vivo* was minced and "Mag-mixed" in pancreatin 0.25% for 30 minutes. Suspended cells were decanted from undigested tissue, spun lightly to remove enzyme and extracellular organisms, and resuspended in a medium composed of mouse serum 10%, human ascitic fluid 10%, Eagle's amino acids, vitamins, and glutamine(10), chick embryo extract 1%, and BSS. Aliquots were frozen for control bacillary counts and distributed to tubes for incubation at 34°C. Stock cells of clone 929, Earle's L strain of mouse fibrocytes,‡ were maintained in: horse

* St. George's Hospital Medical School, University of London.

† J. H. Hanks—unpublished observation.

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serum 2%, Eagle's mixture, Bacto-peptone 0.5%(11), Waymouth's trace minerals§, M/560 Na_2HPO_4, and Hanks' BSS. These cells were infected by combining equal amounts of a 0.5% tissue suspension of the Hawaiian strain of *M. lepraemurium* in medium with a pancreatinized suspension of cells (10^6/ml) from stock cultures. Aliquots of this mixture (0.2 ml) were distributed to 15 mm tubes in which 12 mm round cover slips had been seated in 0.3 ml of medium. After incubation at 34°C for 24-48 hours, cells were pancreatinized from the coverslips, centrifuged lightly to separate intact infected cells from damaged cells and extracellular bacilli, and again pooled. Aliquots containing 100,000 infected cells were distributed to fresh cover slips and incubated at 34°C. The day of replanting was regarded as day zero. Aliquots also were frozen for subsequent control observations and bacillary counts. *Bacillary counts.* In early experiments, cells were pancreatinized from 1-3 cultures, pooled with supernatant fluids, and centrifuged at 3300 x G for 30 minutes. Supernatant fluids were removed to 0.1 ml per sample and sediments were frozen and thawed 3 times and aspirated vigorously through a small needle to encourage cell rupture. 0.001 ml of each resulting suspension was spread on five 5 x 5 mm areas on microscope slides by means of an Agla micrometer syringe. Films were heat fixed, Ziehl-Neelsen stained, and differentiated in sulfuric 4%-methylene blue(12). Bacilli were enumerated in 20 randomly chosen oil immersion fields in 2 to 5 films/sample. The differences between counts were analyzed by the paired replicate method of Wilcoxon(13). In later experiments, analyses were made of the counts recorded in each of 15 fields across the diameters of microspots delivered to slides by means of a standard 0.7 mm platinum loop. This method has been described and analyzed by Hanks(5). The homogeneity and reproducibility of samples was improved as follows: Bile 0.05% was incorporated in the pooled samples in 10 mm lusteroid tubes prior to high speed centrifugation. After the volumes of concentrates had been adjusted to 0.1 ml per sample, these tubes were packed

§ Personal communication.

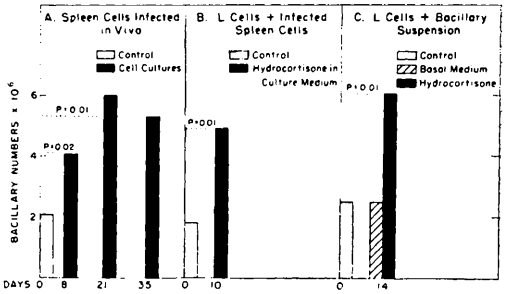

FIG. 1. Multiplication of *M. lepraemurium* during incubation in cell cultures.

in a 10 KC Raytheon ultrasonicator (water depth around tubes = 15 mm) and oscillated at 150 micro-amperes for 2 minutes. The incorporation of bile is necessary to prevent abnormal distributions of bacilli during the drying of such homogenates on films. Reliable fixation necessitated exposure of flame-fixed slides to formalin fumes for 8 minutes prior to staining and differentiation.

Results. Multiplication of *M. lepraemurium* in cells *in vitro* has been initiated under 3 circumstances of cellular infection and within 2 cell types.

The first type of experiment consisted of direct explantation of spleen cells from mice infected with the Wells strain of *M. lepraemurium*. Results shown in panel A, Fig. 1, represent initial counts compared with those made after incubation for 8, 21, and 35 days. Since bacillary numbers nearly doubled within 8 days, proliferation during this period occurred at the maximal rates observed during infection of susceptible hosts. After 21 days, the counts were 2.8 x the original. After 35 days the counts were beginning to decline, possibly because of deterioration of the cell populations between the 21st and 35th days. Rees and Wong at the National Institute for Medical Research, London, have obtained similar results with spleen explants from *M. lepraemurium* infected mice.§

An interesting modification of the foregoing experiment consisted in ascertaining whether infected mouse cells could be employed as a means of transferring *M. lepraemurium* to an established line of fibroblastic cells. Preliminary studies showed that during incubation for 48 hours in low serum medium appreciable numbers of the inadequately

maintained mouse spleen cells were phagocyted by L cells. In the conduct of such experiments the *in vitro* infected L cells were pancreatinized after 48 hours, pooled, and redistributed to new coverslips. At this time aliquots were also stained for observations of original cell-bacillary relationships, and other aliquots were frozen for subsequent control bacillary counts. Microscopic study of incubated cultures demonstrated that some of the L cells were successfully parasitized while others obviously had destroyed mycobacteria. Counts in such systems demonstrated no bacillary increases despite the fact that the medium contained a low concentration of serum and the bacilli had not been exposed to extracellular environment. Apparent bacillary success in some cells was offset by the resistance and/or high metabolic capacity of others.

Grossfeld has described modifications of cell metabolism by hydrocortisone and has shown that the compound permits cultured cells to survive without feeding for significantly longer periods than controls(14). The above experiment in which spleen cells were combined with L cells was repeated. However, after pancreatinization and pooling of infected L cells, aliquots were replanted in low serum medium containing hydrocortisone‖ at the level of 0.2 mg/ml. As shown in Panel B, Fig. 1, after 10 days incubation the numbers of bacilli in the modified L cells had increased to approximately 2.5 times the initial numbers. Further incubation did not result in significant increase of *M. lepraemurium*.

In the previous experiments, bacilli were protected from extracellular environment during transfer to *in vitro* systems. Trials were then made by adding to L cells the Hawaiian strain of *M. lepraemurium* which had been suspended and refrigerated in albumin and yeast supplement. These organisms maintained their microscopic hydrogen transfer capacity during their acquisition by the L cells.¶ Hydrocortisone (0.2 mg/ml) was incorporated in the medium used for replanting a portion of the infected cells. Panel C, Fig. 1, representing the average of several determinations, shows that the bacillary increase obtained by this procedure resembled that already described. After 14 days incubation the increase of bacterial numbers was 2.7 times the original counts. Again prolonged incubation resulted in no further increase. The counts did not rise when hydrocortisone was omitted from the medium.

Because of the limited increases in bacillary numbers in the foregoing experiments, attempts were made to demonstrate: (a) that the more prolonged freezing of bacilli in control samples might permit attrition by ice crystals or make the organisms more susceptible to sonic disruption, and (b) that breakage of the longer bacillary elements into shorter lengths during their residence within cells might cause an apparent rather than a true rise in counts. A series of critical studies showed that bacillary numbers remained constant during freezing and thawing and during freezing for periods longer than those reported; and also that decreases in bacterial lengths played no part in elevation of the counts.

The present demonstration of limited multiplication of *M. lepraemurium* in cells *in vitro* probably has been made possible by adherence to the considerations outlined in the introduction. A definition of the exact role of these and other factors must await the development of cellular systems which are more favorable to continuous cycles of cellular infection.

Summary. Quantitative determinations have revealed significant multiplication of *M. lepraemurium* in explanted spleen cells which had been infected *in vivo;* also in the L strain of mouse fibrocytes infected *in vitro,* provided the metabolism of the latter was altered by means of hydrocortisone. During periods of 8-10 days, the rate of multiplication closely approximated the maximal rates observed in susceptible animals.

‖ Through the courtesy of Dr. J. C. Richards, Merck Sharp & Dohme Research Laboratories, Division of Merck & Co.

¶ Unpublished data.

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Relationships between Bacterial Resistance to Serum and Penicillin. (23658)

JACOB G. MICHAEL AND WERNER BRAUN
Institute of Microbiology, Rutgers, State University, New Brunswick, N. J.

Despite numerous studies on differential resistance of bacterial species and strains to bactericidal effects of normal sera, only little information is available on the genetic aspects of bacterial sensitivity or resistance to serum factors(1-3). Where such information has been collected, antigenic alterations of the bacteria usually accompanied alterations in "serum resistance." Recently, there has been a resurgence of interest in bactericidal systems, due to recognition of the role of properdin(4-5) in such phenomena. Therefore, a renewed effort has been made to study certain genetic aspects of bacterial resistance with particular emphasis on closely related strains of similar antigenicity. In the course of these studies, changes in serum resistance without qualitative antigenic alterations and an inverse correlation between resistance to penicillin and to bactericidal factors of normal human serum were observed. These observations form the subject of the present report.

Methods. Smooth cultures of *Shigella dysenteriae* #377, and *Escherichia coli* SPI, sensitive to the properdin system(4), were obtained from Dr. R. J. Wedgwood. These cultures proved extremely heterogeneous on the basis of colonial morphology when inspected by oblique lighting(6). Single colony isolates were used to establish substrains with greater colonial homogeneity, displaying identical antigenic* and fermentation characteristics. The susceptibility of these strains to normal human serum, obtained from a single donor, was determined by exposing a known number of bacteria (approx. 5000) for various periods of time at 37°C to serum diluted with ABA buffer (Michaelis buffer, pH 7.5, containing albumin(4)). The concentration of serum in buffer varied from 1:6 to 1:2.5 depending upon the sensitivity of the strain tested, but the total volume during exposure time was always 1.2 ml. Bacteria for testing were harvested from 18-hour tryptose agar slants and plated on tryptose agar (in 0.1 ml aliquots) following exposure to serum. Prior to plating and prior to incubation at 37°C the bacterial suspensions in serum were kept in an ice-bath. Sera were stored at –40°C. All tests were done at least in duplicate and duplicate samples of replicate cultures were assayed.

Results. Six colonial types were recognized when the original culture of *Sh. dysenteriae* was inspected on McConkey agar. Four of these yielded stable subcultures differing in their resistance to the bactericidal effects of normal human serum; the other 2 isolates proved to be unstable in regard to colonial morphology and antigenic characteristics and were not studied further. Table I demonstrates typical differences in serum resistance between the most sensitive (S_3) and most resistant (S_1) of the stable substrains isolated from the original heterogeneous culture.

When attempts were made to convert mem-

* Based on reactions with specific antiserum and acriflavine.