GROWTH OF SHIGELLAE IN MONOLAYER TISSUE CULTURES

DOLORES F. GERBER AND H. M. S. WATKINS

Naval Biological Laboratory, School of Public Health, University of California, Berkeley, California

Received for publication May 22, 1961

ABSTRACT

GERBER, DOLORES F. (University of California, Berkeley) AND H. M. S. WATKINS. Growth of shigellae in monolayer tissue cultures. J. Bacteriol. **82**:815–822. 1961.—The influence of environment on virulence, antigenicity, and antibiotic susceptibility of infectious agents is well recognized. The development of tissue culture monolayer techniques has stimulated new interest in manifestations of these attributes at the cellular level.

Shigella flexneri and *S. sonnei* were grown intracellularly in Henle epithelial cell monolayer tissue cultures; uptake of the bacteria was induced by 50% normal horse serum in the infection medium. Since preliminary experiments indicated that streptomycin in the extracellular fluid depressed intracellular multiplication of these organisms, the antibiotic was later omitted from the tissue culture growth fluid. Extracellular multiplication of shigellae was controlled by thorough washing of the infected monolayers and replacement with fresh medium at 2-hr intervals during incubation.

Intracellular growth patterns of two *S. flexneri* and two *S. sonnei* strains were established. Differences in the capacity of strains to adapt to the intracellular environment was reflected in the number of organisms subsequently produced, and appeared to be associated with the degree of intracellular resistance to streptomycin. No such variation was seen in cultures grown in broth.

Microscopic examination of stained monolayers showed that intracellular bacterial growth was confined to the cytoplasm, which became filled with organisms prior to cellular disintegration.

Biological implications of the variable response of *Shigella* strains to the intracellular environment are discussed.

Infectious agents grown in vivo or in tissue cell systems frequently differ from the same organisms grown in lifeless culture media, in respect to virulence, antigenicity, and susceptibility to antibiotics (McDermott, 1958, 1959; Smith, 1958; Smadel, 1960). As early as 1916, Smyth, noting that the usual culture media afforded conditions for growth of bacteria quite unlike those available in the living animal host, investigated the growth characteristics of avian tubercle bacilli in chick embryo tissue explants. He suggested the possibility of using infected tissue cultures to differentiate pathogenic and nonpathogenic organisms. Smyth's (1916) studies, and subsequent work by others with tissue explants infected with mycobacteria, have been summarized by Fell and Brieger (1947). The usefulness of tissue explants in the exploration of host-parasite relationships on the cellular level is limited by the presence in explants of an unstable variety of cells subjected to undefined and uncontrolled metabolic interactions and possessed of varied capacities for reaction with a given infectious agent.

The development of techniques for the propagation of viruses in tissue culture monolayers (Dulbecco and Vogt, 1954) comprised of individual cell types stimulated fresh inquiries concerning cellular infection with bacteria. Shepard (1955, 1957*a*,*b*, 1959) has employed HeLa cell monolayers in studying the intracellular growth characteristics of virulent and avirulent mycobacteria and has surveyed the efficacy of sera from several animal species in selectively inducing cell penetration by a number of other bacterial pathogens. Braude and Siemienski (1960) utilized intracellular infections of monkey kidney epithelial cell cultures by *Proteus* and *Escherichia* in elucidating the role of bacterial unrease in the pathogenesis of experimental pyelonephritis. Finally, Merriott, Shoemaker, and Downs (1961) have explored the growth characteristics of strains of *Pasteurella tularensis* of varying degrees of virulence grown intracellularly in several tissue culture cell lines.

This report describes a method developed for

the intracellular cultivation of *Shigella flexneri* and *S. sonnei* in Henle 407 human intestinal epithelial cell monolayers and presents evidence of a differential capacity in *Shigella* strains for growth and for resistance to streptomycin during intracellular residence.

MATERIALS AND METHODS

Growth and maintenance of Shigella strains. Four *Shigella* strains were extensively used: Flexner strain B_3F, received from S. B. Formal, Army Medical Research Institute, as strain 43-G-100; Flexner strain $B_{4b}M$, isolated from an acutely ill monkey at the Naval Biological Laboratory (NBL); Sonne strain D_I(DG), isolated from an accidentally infected worker at NBL; and Sonne strain D_I347, received from A. S. Brown, California State Department of Public Health as strain Ewing 1196.

Strains were maintained at 4 C in the lyophilized state, or for periods up to 3 months as stab cultures in beef heart infusion (BHI) semisolid agar. Colonies were inspected periodically for characteristic morphology, both by reflected and transmitted light (Cooper, Keller, and Walters, 1957), and were tested for serological specificity with group- and type-specific antisera and for antigenic smoothness by stability in 1:500 acriflavine solution (Pampana, 1933).

BHI broth shake cultures grown at 37 C for 24 hr were routinely employed to seed tissue cultures. The numbers of viable bacteria in broth cultures and in suspensions of disrupted infected tissue cells were determined from BHI agar plates inoculated with 0.1 ml of decimal dilutions in 1% peptone-water, after overnight incubation at 37 C.

Tissue monolayers. The Henle 407 intestinal epithelial cell line was maintained in continuous serial passage by the method outlined by Henle and Deinhardt (1957); Eagle's Basal Medium plus 10% pooled normal human serum, containing streptomycin (10 μg per ml) and penicillin (10 units per ml), was changed every 48 hr on monolayers grown in rubber-stoppered 3-oz prescription bottles. For passage, 6- to 7-day-old monolayers were drained of nutrient and covered with 5 ml of 0.1% trypsin Difco Laboratories (Detroit, Mich.) solution diluted (1:250) with the phosphate buffered saline (PBS) of Dulbecco and Vogt (1954). The tissues were incubated at 37 C for 20 to 30 min with intermittent shaking after the monolayers loosened from the glass. Separated cells were further dispersed by gentle mixing with a pipette, and were then sedimented by centrifugation at 1000 rev/min for 5 min. For each experiment, 10-ml aliquots from a single suspension of cells pooled from 10 bottles were dispensed into each of 100 new bottles. Under these conditions, uniform monolayers incubated at 37 C were obtained in 6 to 7 days.

For microscopic examination of monolayers, cells were grown on coverslips in Leighton tubes (Microbiological Associates, Inc., Bethesda, Md.). The infected tissue preparations were washed three times in PBS, fixed in 10% neutral formalin in distilled water, and stained by the modified Giemsa method described by Eisler and Bevis (1953). Tissues stained by this method could be held for several months without fading.

Intracellular infection of cells. Initially, intracellular infection was induced by the method described by Shepard (1955, 1959): Washed (five times with PBS) monolayers were incubated at 37 C with an infection medium containing a known concentration of *Shigella* organisms. The infection medium consisted of normal inactivated (56 C, 2 hr) horse serum which had been diluted to 40% in PBS and shown to be free of inhibitory activity or of agglutinins for shigellae. After 2.5 hr, the tissues were washed three times with PBS; 10 ml of Eagle's medium plus 10% human serum, containing streptomycin (10 μg per ml) to suppress extracellular multiplication of bacteria, were added and incubation was continued. After preliminary experiments indicated that the presence of streptomycin in the extracellular fluid diminished intracellular multiplication of shigellae, streptomycin was omitted from the nutrient fluid. Extracellular multiplication was controlled by washing the infected monolayers three times with PBS and replacing the medium at 2-hr intervals during the course of incubation.

RESULTS

Reproducibility of viable intracellular organism determinations. The organisms were released from washed (five times with PBS) infected monolayers by treatment with 2 ml of 0.25% trypsin in PBS for 3 min at 37 C, followed by vigorous shaking by hand for 15 to 20 sec; incubation and shaking was repeated. Microscopic examination of such preparations showed no intact cells. Determinations of viable organisms were made on disrupted cell suspensions

from duplicate monolayers as well as on the last monolayer wash fluids, to assure that extracellular organisms did not interfere with determinations of intracellular organisms. Table 1 shows results of two typical experiments each with *S. flexneri* strains B_3F and $B_{4b}M$. The disruption procedure resulted in satisfactory replication of bacterial counts from duplicate tissue preparations. Henceforth, the number of intracellular viable organisms are reported as the average of single counts obtained from duplicate tissue preparations.

Effect of streptomycin on intracellular multiplication of Shigella. To compare growth curves of intracellular organisms when various concentrations of streptomycin were present in the extracellular fluid, monolayers were infected by incubating them at 37 C with 6 ml of an infection medium containing approximately 3 × 10^7 organisms per ml of strain B_3F. After 2.5 hr incubation, the infection medium was replaced by nutrient fluid containing streptomycin. Low concentrations of the drug in the nutrient fluid, while effective in suppressing multiplication of extracellular organisms, also inhibited intracellular multiplication; in the absence of streptomycin, intracellular multiplication increased by approximately 2 log within 6 hr of incubation (Fig. 1).

TABLE 1. *Intracellular viable organisms from two Shigella strains grown in Henle cell monolayer tissue cultures*

Time	Strain B_3F		Strain $B_{4b}M$	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
hr				
2.5	1.9×10^5*	2.8×10^6	2.8×10^7	5.8×10^6
	3.5×10^5	1.5×10^6	2.5×10^7	3.0×10^6
3.5	1.9×10^5	9.3×10^4	9.8×10^6	9.0×10^6
	5.8×10^5	2.8×10^4	9.5×10^6	7.0×10^6
5.0	8.3×10^5		3.3×10^7	
	6.5×10^5		3.0×10^7	
6.5	3.8×10^6	2.7×10^5	9.3×10^7	6.7×10^7
	1.9×10^6	2.9×10^5	1.0×10^8	5.3×10^7
9.0	1.0×10^7	1.3×10^6	1.5×10^8	1.4×10^8
	2.0×10^7	1.8×10^6	2.5×10^8	1.5×10^8

* Organisms per ml of disrupted tissue cell suspension.

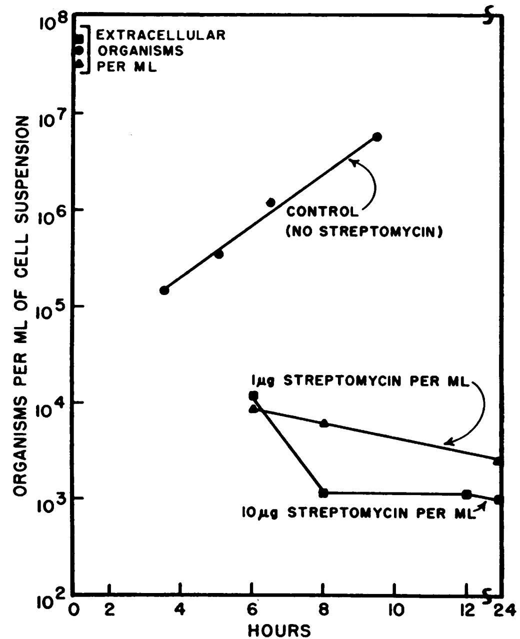

FIG. 1. *Effect of streptomycin upon intracellular multiplication of Shigella flexneri strain B_3F in Henle cell monolayer tissue cultures.*

Control of extracellular Shigella multiplication. Since shigellae grew freely in the streptomycin-free nutrient fluid utilized for Henle cell cultures, some method to differentiate intracellular and extracellular organisms was required for a study of the characteristics of the intracellular organisms. An alternative method to the use of an antibiotic in the nutrient fluid appeared to be frequent changes of nutrient fluid after thorough washing of the monolayers. Table 2 shows that the washing procedure consistently reduced the number of extracellular organisms to levels 2 to 3 log below corresponding concentrations of intracellular organisms. Accordingly, this means of removing extracellular organisms was employed during the determination of intracellular growth curves.

Intracellular growth curves of Shigella strains. The intracellular growth patterns of four *Shigella* strains were established by the methods described above. Two of the strains, *S. flexneri* strain $B_{4b}M$ and *S. sonnei* strain D_I(DG), were recent isolates from clinical dysentery; the other two, *S. flexneri* B_3F and *S. sonnei* D_I347, were laboratory strains of long standing. For comparative purposes, growth curves were also

determined for organisms multiplying in static BHI broth cultures (Fig. 2). During the initial period of intracellular residence, there was a decrease that varied with the strain in the number of viable organisms; thereafter, multiplication took place at a rate which was

TABLE 2. *Extracellular and intracellular organisms in monolayer tissue cultures*

Time	Organisms per ml*		
	Extracellular	Last wash fluid	Intracellular
hr			
2.5	4.7×10^7	4.0×10^4	1.0×10^7
3.5	2.5×10^5	1.9×10^4	4.0×10^6
5	3.7×10^5	5.9×10^4	1.3×10^7
6.5	1.7×10^7	6.4×10^3	4.0×10^7
9	1.3×10^8	1.8×10^6	2.5×10^8

* Average of duplicate bottle determinations.

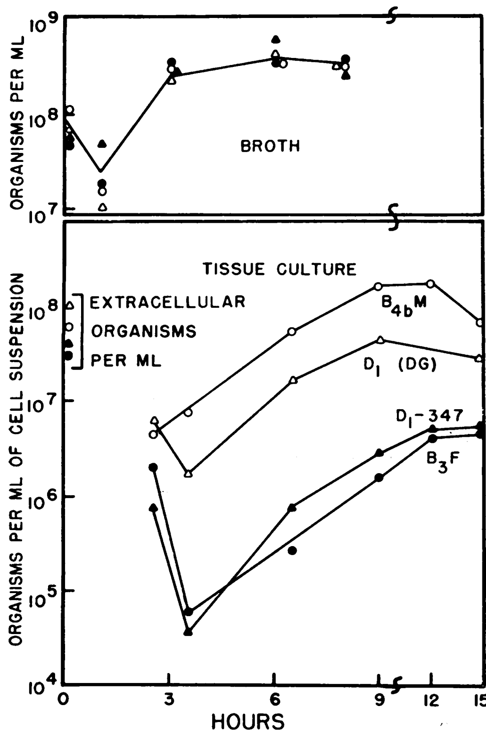

FIG. 2. *Growth of Shigella flexneri and S. sonnei in Henle cell monolayer tissue cultures containing antibiotic-free nutrient fluid, and in beef heart infusion broth.*

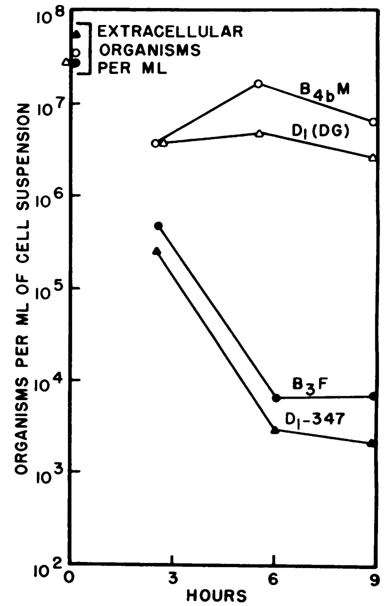

FIG. 3. *Effect of streptomycin upon intracellular multiplication of Shigella flexneri and S. sonnei in Henle cell monolayer tissue cultures.*

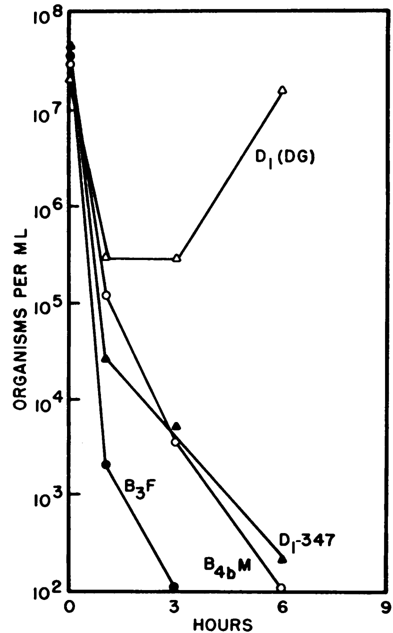

FIG. 4. *Effect of streptomycin upon multiplication of Shigella flexneri and S. sonnei in beef heart infusion broth.*

essentially constant for all strains, but then resulted in a considerable difference in the peak yields reached after 9 to 12 hr of postinfection incubation. Growth in broth, however, showed no such strain differentiation. All four strains under these conditions gave the same curve.

Microscopic examination of stained preparations of coverslip tissue cultures, which had been made simultaneously with the above bottle cultures used for the growth curve determinations, showed gross cellular deterioration at 12 hr incubation in tissues infected with the two high-yield strains $B_{4b}M$ and $D_I(DG)$; no cytopathogenicity was evident in tissues infected with the two low-yield strains after 15 hr incubation.

Sensitivity of Shigella strains to streptomycin. Similar determinations were made on the growth of these four *Shigella* strains in tissue cells and in BHI broth, in the presence of 10 μg of streptomycin per ml of medium. Figure 3 shows that the two high-yield strains while growing intracellularly were markedly less susceptible to streptomycin than were the two low-yield strains, the former showing peak yields approximately 3 to 4 log higher at 6 to 9 hr of incubation. In broth, no substantial differences were seen in the susceptibility to streptomycin of three of

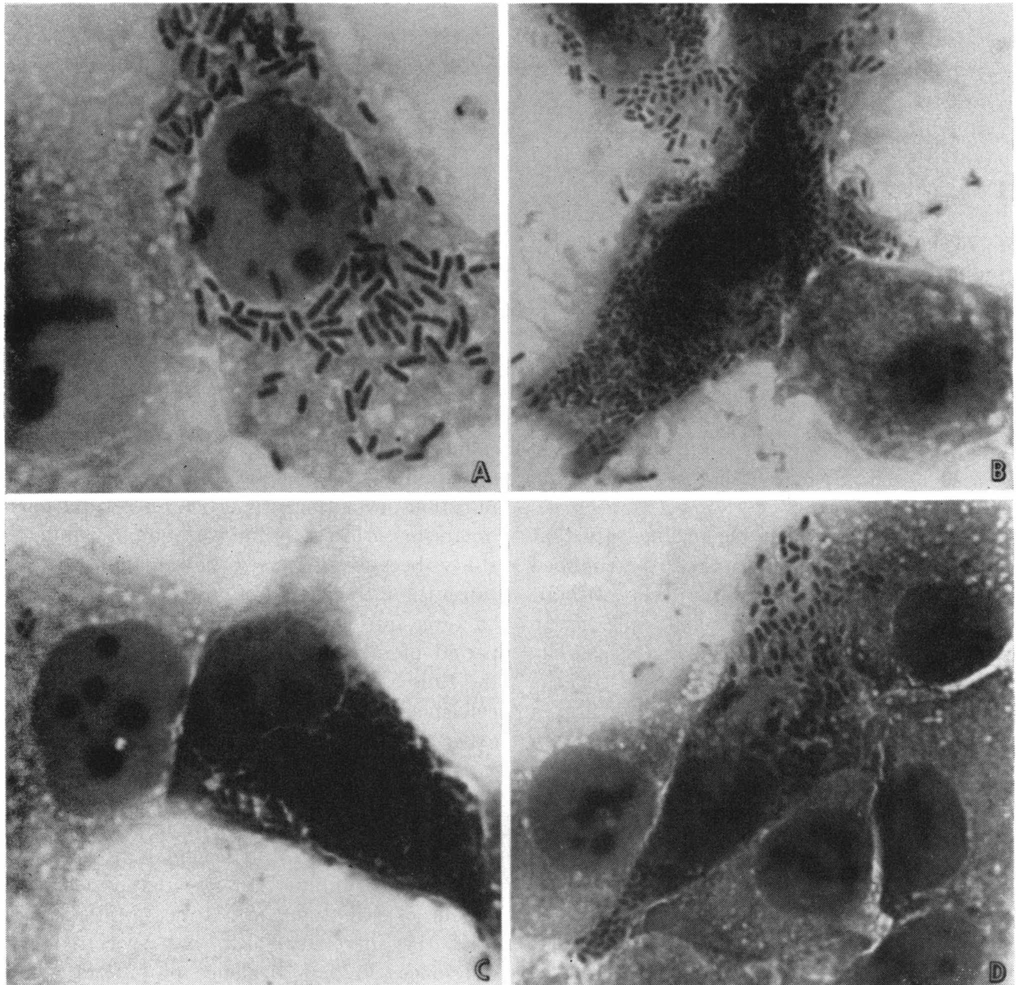

FIG. 5. *Henle epithelial cells infected with Shigella flexneri (A, B, C) and S. sonnei (D). Photograph at 900 × magnification; A, enlarged 2 ×.*

the strains (Fig. 4); the fourth strain, D_I(DG), had apparently become streptomycin resistant after a 3-hr lag period.

Microscopic appearance of infected Henle cells. Stained coverslip tissue preparations were examined at intervals during the intracellular growth cycle of the four *Shigella* strains studied. Cells representative of several stages of infection are illustrated in Fig. 5.

In tissues infected with the two low-yield strains, only an occasional intracellular organism could be found at any stage. In tissues infected with the two high-yield strains, however, infected cells were plentiful after 3.5 hr incubation. After 6 hr, larger numbers of bacteria were seen in infected cells. Cellular disintegration became widespread at 12 to 15 hr, and cells containing all degrees of bacterial multiplication were visible.

Figure 5A shows a cell in an early stage of infection in which active division of the elongated bacteria scattered throughout the cytoplasm is taking place. Figure 5B shows a cell, at a later stage of infection, filled with organisms, with beginning cytoplasmic disintegration and pycnosis and hyperchromatosis of the nucleus. Figure 5C contains a cell whose cytoplasm is so densely packed with organisms that the nucleus appears to be displaced and indented. These tissues were all infected with the Flexner strain B_{4b}M. In Fig. 5D, an essentially similar picture is seen of a cell infected with the Sonne strain D_I(DG).

All four photographs in Fig. 5 illustrate that bacterial multiplication appeared to be confined to the cytoplasm of individual cells without extension to immediately adjacent cells, many of which were entirely devoid of organisms. The paucity of bacteria in areas external to the sharply defined cell boundaries indicates, first, a remarkable capacity of the cells to maintain structural integrity in the presence of extensive intracellular bacterial multiplication, and, secondly, the efficacy of repeated washings of the tissue as a means for removing extracellular organisms.

Comparison of the cellular picture at successive intervals of incubation with the intracellular growth curves illustrated in Fig. 2, indicates that intracellular yields approximating 10^7 organisms per ml of tissue cell suspension were attained before an occasional infected cell could be seen; but when yields of 10^8 organisms per ml were obtained, densely infected cells in large numbers were evident. Cellular deterioration and the onset of cytoplasmic disintegration occurred earlier and was more extensive in tissues infected with strain B_{4b}M than in those infected with strain D_I(DG). This appeared to be the result of the higher yields obtained with the former strain.

DISCUSSION

The Henle cell was selected for use in this investigation because of its origin in the human embryonic intestinal epithelium; however, the shigellae apparently possess no specific tropism for this cell, inasmuch as intracellular infection of chick fibroblast monolayers was also obtained by the same technique. The latter tissue showed the same difference in the growth curves of strains B_{4b}M and B_3F.

The observation that a heavily infected tissue cell frequently lay immediately adjacent to other cells completely free of bacteria suggests (i) that infection did not spread from cell to cell, and (ii) a possible variation in cellular susceptibility to infection. Whether the percentage of infected cells might have been increased by use of another infection-promoting serum was not studied. Shepard (1959) noted that sera from different species varied in their capacity to induce infection of cells by different bacterial genera, and that horse serum had relatively poor infection-promoting properties for several microorganisms. Horse serum was employed in this study because it was believed that it would cause the introduction of only a small number of bacteria into the tissue cells during the initial period of incubation, with a resultant delay in the time at which progressive destruction of the cells in a monolayer would force termination of an experiment.

The diverse curves for the intracellular multiplication of several *Shigella* strains indicate that strains differ significantly in their capacity to adapt successfully to intracellular residence, and also in their ability to resist the deleterious effects of streptomycin. It has been reported that this diversity may be associated with differences in oral virulence of *Shigella* strains for monkeys (Watkins, 1960). No such strain variation in growth curves was manifested in the absence of tissue cells.

The observations of strain variability of *Shigella*, demonstrable only in an intracellular environment, are in accord with McDermott's (1958, 1959) suggestion that it is the adaptive capability of the individual organism that is the key factor in determining the effect of a particular environment upon the host-organism interaction. Others have reported the capacity of cells in tissue culture to differentiate between pathogenic and nonpathogenic organisms. Holland and Pickett (1956), using chick fibroblasts, and Stinebring and Kessel (1959), using guinea pig mononuclear cells, were able to distinguish virulent from avirulent *Brucella* species, while Shepard (1957*a*,*b*, 1958) had similar results with mycobacteria growing in HeLa cells. However, the diminished yields and variable response of *Shigella* strains in an intracellular location subjected to the action of streptomycin concentrations lethal to the same organisms growing extracellularly apparently was not a problem with the bacterial species studied by these workers.

Thayer and his associates (1957*a*,*b*) have reported on the failure of a number of antibiotics to affect gonococci growing intracellularly in tissue culture. On the other hand, Smadel (1960) has indicated that tissue cultures infected with *Salmonella typhosa* can be freed of infection with the proper antibiotic treatment. (After completion of work described here, Merriott et al. (1961) reported that streptomycin interfered with intracellular growth of *Pasteurella tularensis* in L cells.)

Microscopic examination of densely infected cells reveals remarkably little cellular deterioration, suggesting that the metabolic activities of both cell and organism may proceed for some time until mechanical pressure disrupts the former. This arouses speculation as to what contribution cellular enzymatic systems may make to bacterial ecology. Smadel (1960) has suggested the utilization of tissue culture systems for studies of the carrier state in certain infections. Such host-parasite models should receive attention for potential contributions to an understanding of the pathogenesis of localized diseases such as shigellosis.

ACKNOWLEDGMENTS

The authors wish to acknowledge the competent technical assistance of Violet Nesbitt.

This work was sponsored by the Office of Naval Research under a contract with the Regents of the University of California.

LITERATURE CITED

Braude, A. I., and J. Siemienski. 1960. Role of bacterial urease in experimental pyelonephritis. J. Bacteriol. **80**:171–179.

Cooper, M. L., H. M. Keller, and E. W. Walters. 1957. Microscopic characteristics of colonies of *Shigella flexneri* 2a and 2b and their relation to antigenic composition, mouse virulence and immunogenicity. J. Immunol. **78**:160–171.

Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines of poliomyelitis virus. J. Exptl. Med. **99**:167–182.

Eisler, D. M., and R. E. Bevis. 1953. Use of potassium cyanide in the Giemsa stain. A. M. A. Arch. Pathol. **56**:647–649.

Fell, H. B., and E. M. Brieger. 1947. The effect of phagocytosis on the growth and survival of avian tubercle bacilli in embryonic chicken tissue cultivated *in vitro*. J. Hyg. **45**:359–369.

Henle, G., and F. Deinhardt. 1957. The establishment of strains of human cells in tissue culture. J. Immunol. **79**:54–59.

Holland, J. J., and M. J. Pickett. 1956. Intracellular behaviour of *Brucella* variants in chick embryo cells in tissue culture. Proc. Soc. Exptl. Biol. Med. **93**:476–479.

McDermott, W. 1958. Microbial persistence. Yale J. Biol. and Med. **30**:257–291.

McDermott, W. 1959. Inapparent infection. Relation of latent and dormant infections to microbial persistence. Public Health Repts. U. S. **74**:485–499.

Merriott, J., A. Shoemaker, and C. M. Downs. 1961. Growth of Pasteurella tularensis in cultured cells. J. Infectious Diseases **108**: 136–150.

Pampana, E. J. 1933. Microbic dissociation. Detection of the "R" variant by means of a specific drop-agglutination. J. Hyg. **33**:402–403.

Shepard, C. C. 1955. Phagocytosis by HeLa cells and their susceptibility to infection by human tubercle bacilli. Proc. Soc. Exptl. Biol. Med. **90**:392–396.

Shepard, C. C. 1957*a*. Growth characteristics in HeLa cells of the rapidly growing acid fast bacteria, *Mycobacterium fortuitum*, *Mycobacterium phlei* and *Mycobacterium smegmatis*. J. Bacteriol. **73**:722–726.

Shepard, C C. 1957*b*. Growth characteristics of tubercle bacilli and certain mycobacteria in HeLa cells. J. Exptl. Med. **105**:39–48.

SHEPARD, C. C. 1958. A comparison of the growth of selected mycobacteria in HeLa, monkey kidney and human amnion cells in tissue culture. J. Exptl. Med. **107**:237–246.

SHEPARD, C. C. 1959. Nonacid-fast bacteria and HeLa cells. Their uptake and subsequent intracellular growth. J. Bacteriol. **77**:701–714.

SMADEL, J. E. 1960. Some aspects of intracellular infections. J. Immunol. **84**:1–5.

SMITH, H. 1958. The use of bacteria grown *in vivo* for studies on the basis of their pathogenicity. Ann. Rev. Microbiol. **12**:77–102.

SMYTH, H. F. 1916. The reactions between bacteria and animal tissue under conditions of artificial cultivation. IV. The cultivation of tubercle bacilli with animal tissue *in vitro*. J. Exptl. Med. **23**:283–291.

STINEBRING, W. R., AND R. KESSEL. 1959. Continuous growth of *Brucella abortus* in mononuclear phagocytes of rats and guinea pigs. Proc. Soc. Exptl. Biol. Med. **101**:412–415.

THAYER, J. D., M. I. PERRY, H. J. MAGNUSON, AND W. GARSON. 1957*a*. Failure of penicillin to kill phagocytized *Neisseria gonorrhaeae* in tissue culture. Antibiotics & Chemotherapy **7**:311–314.

THAYER, J. D., M. I. PERRY, F. W. FIELD, AND W. GARSON. 1957*b*. Failute of penicillin, chloramphenicol, erythromycin and novobiocin to kill phagocytized gonococci in tissue culture. Antibiotics Ann. 1956–1957, p. 513–517.

WATKINS, H. M. S. 1960. Some attributes of virulence in Shigella. Ann. N. Y. Acad. Sci. **88**: 1167–1186.