NONACID-FAST BACTERIA AND HeLa CELLS: THEIR UPTAKE AND SUBSEQUENT INTRACELLULAR GROWTH[1]

C. C. SHEPARD

Communicable Disease Center, Public Health Service, United States Department of Health, Education, and Welfare, Montgomery, Alabama

Received for publication November 6, 1958

The growth of bacteria in cells requires first that they gain access to the cell. In the intact animal, bacteria are taken up by cells with natural phagocytic activity, the polymorphonuclear leucocytes and monocytes. Cells of the more convenient tissue culture systems of human cells, such as HeLa cells, do not normally take in bacteria when commonly used tissue culture media are employed. Fortunately, HeLa cells do take up bacteria when the appropriate serum is used in the tissue culture medium. Certain horse sera promote the uptake of mycobacteria and carbon particles (Shepard, 1955, 1957*a*), and guinea pig serum enhances the phagocytosis of *Histoplasma capsulatum* (Larsh and Shepard, 1958). The control exercised by serum constituent of the tissue culture medium is seen again in the present study, which is concerned with HeLa cells and several species of nonacid-fast bacteria. The bacteria chosen for study were primarily those causing natural human infectious disease. Since their attainment of an intracellular position was followed by intracellular growth, their true cytoplasmic position could be differentiated from attachment to the exterior surface of the cell or to the cover slip in the region of the cell. After bacteria had entered the cell, streptomycin could usually be employed to prevent extracellular growth without affecting the amount of intracellular growth.

MATERIALS AND METHODS

To study phagocytosis without intracellular growth, the following technique was used. HeLa cells were grown on cover slips in Leighton tubes for 2 days in human serum medium and then washed in Hanks balanced salts solution. One ml of medium containing 20 per cent of the species of serum and 80 per cent balanced salts solution containing the amino acids and vitamins recommended by Eagle (1955) was then placed on the cells. A 0.5-ml suspension of the microorganism was added. Growth of bacteria in the phagocytosis experiments was avoided by the inclusion of penicillin and streptomycin in the medium. The fungi used in phagocytosis experiments were killed by pretreatment with 10 per cent formalin, which was subsequently removed by centrifugal washing. After incubation at 37 C for 1 day, the cells were washed twice with balanced salts solution, fixed with neutral formalin, and stained by the Giemsa or Ziehl-Neelsen methods (Shepard, 1957*a*). The number of HeLa cells appearing to contain microorganisms or to contact them in any way was counted in five or more standard representative microscopic fields. This method of scoring is simple and rapid, but it understates the differences between sera, because (a) cells in higher scoring sera tend to develop multiple infections, (b) the occasional organism attaching to the glass and accidentally contacting a cell contributes more to low scoring sera, and (c) efficiently phagocytosing tissue cultures significantly decrease the concentration of microorganisms in the medium.

To study intracellular growth of the organisms it was necessary to have HeLa cells in prime condition when they were exposed to bacteria. The growth of many of the bacteria studied was so rapid in the fluid, that if they did not enter the cells promptly, the extracellular growth in a few hours was sufficient to alter the state of the tissue culture. The following schedule was found to provide cells in the required condition consistently. On Thursday, cultures in milk dilution bottles were started with 3 million cells per bottle, in medium consisting of 20 per cent human serum and 80 per cent Eagle's medium in balanced salts solution. The procedure using trypsin was that described by Puck *et al.* (1956). Extra arginine was added to a concentration of

[1] Presented in part at the meeting of the Federation of American Societies for Experimental Biology (Immunology Section), April 1958.

TABLE 1

List of microorganisms employed

Species	Media	Isolate	Source
1. *Histoplasma capsulatum*	Cozad's modification of Salvin's (1950)	Str	Dr. H. W. Larsh, Kansas City Field Station, Communicable Disease Center (CDC)
2. *Candida albicans*	Same	1539	Same
3. *Salmonella typhosa*	Tryptose agar	Pa 58, Ty 2	Dr. P. R. Edwards, CDC, Chamblee, Ga.
4. *Pasteurella tularensis*	GCBA (Downs *et al.*, 1947)	Sm, Norway 425F4G, 425F30G, Helena, 38	Dr. Max Moody, CDC, Chamblee, Ga. (From Dr. Cora Owen, Rocky Mountain Lab., Hamilton, Montana, see Bell *et al.*, 1955)
5. *Brucella abortus*	Tryptose agar	19	Dr. C. A. Manthei, Agricultural Research Service, Beltsville, Md.
B. melitensis	Same	16 M	Same
B. suis	Same	3 Boar	Same
B. suis	Same	2619	Dr. Moody
6. *Mycobacterium tuberculosis*	Loewenstein-Jensen	H37Rv	Dr. William Steenken, Trudeau Sanitarium, Saranac Lake, N. Y.
7. *Streptococcus pyogenes*, group A	Neopeptone infusion agar (5% rabbit blood)	DS 149-8 (type 12)	Dr. Elaine Updyke, CDC, Chamblee, Ga.
		SS 116 (type 47)	Same
		T31 (type 3)	Dr. Moody
		T61 (type 6)	Same
S. pyogenes, group C	Same	X	Isolated from rabbits blood. Typed by Dr. Updyke
8. *Staphylococcus aureus*	Tryptose agar	DA 16-7 (phage type 80/81)	Dr. Updyke
		DA 104-8 (7, 77)	
		DA 394-7 (81)	
		PS 29 (phage propagating strain)	

10^{-3} M, following the experience of Thomas *et al.* (1958) with spinner cultures, and its presence in such high concentrations in the bottle cultures has been very helpful in obtaining tube cultures in optimal condition on the following Wednesday. The medium was changed daily until Monday, when the bottle cultures were treated with trypsin and cover slip cultures started with 300,000 cells per Leighton tube in 20 per cent human serum in Eagle's medium in balanced salts solution. Extra arginine was not found helpful at this stage. The medium was not changed on Tuesday. On Wednesday, the cells were washed twice in balanced salts solution and an infection medium added, that is, 20 per cent serum of the appropriate species in Eagle's medium. After several hours, a change was usually made to growth medium, which was 40 per cent human serum and 60 per cent Eagle's medium, with added streptomycin to prevent extracellular growth (table 3). Before tubes were returned to the incubator after changes of medium, they were first warmed in a 37 C water bath and then placed on trays that had been warmed in the incubator. At appropriate intervals the tissue cultures were washed twice with balanced salts solution, fixed, and stained.

TABLE 2

Amount of phagocytosis by HeLa cells according to species of serum in tissue culure medium and species of microorganism added

	Chicken	Dog	Guinea Pig	Horse	Horse, Stored	Human, Stored	Monkey	Rabbit	Sheep
Experiment A									
Histoplasma capsulatum	49	89	100 (93)*	99	9	56	8	21	8
Candida albicans	56	100 (43)	78	42	7	14	30	5	7
Salmonella typhosa	32	100 (66)	47	18	11	2	3	53	18
Pasteurella tularensis	37	100 (141)	12	4	0	0	1	24	6
Brucella suis	100 (36)	56	67	19	3	25	3	22	14
Mycobacterium tuberculosis	100 (108)	48	80	11	43	3	6	27	11
Group D streptococcus	56	73	100 (124)	11	1	17	5	24	31
Experiment B									
Group A streptococcus	100 (89)	46	93	—†	88	72	—	62	—
Experiment C									
Staphylococcus	92	57	87	—	3	3	—	11	100 (262)

* The figure in parentheses is the number of infected HeLa cells counted in five standard representative microscopic fields in the highest scoring serum for that microorganism. The other figures are the relative percentages of this maximal score observed for the various serum species shown. Thus, for *Histoplasma capsulatum* 93 infected cells were counted in the five fields with guinea pig serum and 9 per cent of this number, or 8 infected cells, with stored horse serum. All sera were freshly drawn, except the two marked "stored," which had been kept for months in the refrigerator, and the two chicken sera in experiments B and C, which were mixtures of equal parts of fresh and inactivated sera (see text). Growth of the microorganisms during the experiment was prevented by antibiotics or pretreatment with formalin.

† — = Not done.

TABLE 3

Media and schedules used to observe intracellular growth

Microorganism	Serum in Infection Medium	Time in Infection Medium	Serum in Growth Medium	Time to Fill HeLa Cell
Pasteurella tularensis Sm strain	Dog	3–6 hr	Human	24 hr
Brucella abortus (19) *B. melitensis*	Guinea pig	3–6 hr	Human	48 hr
B. suis	Guinea pig	3–6 hr	Human	24 hr
Salmonella typhosa	Dog	3 hr	No change	3 hr
Staphylococcus	Chicken	2 hr	Human	5 hr
Streptococcus:				
Group A	Chicken	2 hr	Human	5 hr
Group D	Guinea pig	2 hr	Human	<24 hr
Mycobacterium tuberculosis	Horse	1 day	Human	5 days
Histoplasma capsulatum	Guinea pig	3 days	No change	2–3 days

Measurements of intracellular growth rates. In the earlier studies, the intracellular growth rates of tubercle bacilli were estimated from microscopic observations of the size of the bacterial groups that developed in the cells (Shepard, 1958*a*, *b*). This method was necessary because the tubercle bacilli grew in groups that could not be dispersed for conventional counting methods. The nonacid-fast bacteria, however, were readily dispersed by breaking up the HeLa cells so that colony counts were possible.

An experiment with *Pasteurella tularensis* illus-

TABLE 4

Measurements of growth rates of Pasteurella tularensis in HeLa cells

Date	Strain	I Time	II B/TC	III Generation Time
		hr		*hr*
12/9/57	Sm	8	1.8×10^6	—
		12	1.3×10^7	1.4
		24	1.8×10^8	3.2
	425F4G	8	2.4×10^6	—
		12	7.6×10^6	2.4
		24	1.5×10^8	2.8
12/16/57	Sm	5	1.2×10^5	—
		8	2.5×10^5	2.8
		12	1.4×10^6	1.6
		24	9.9×10^7	2.0
	425F4G	5	5.1×10^5	—
		8	5.1×10^5	∞
		12	5.1×10^5	30.0
		24	1.4×10^7	2.6

* I. Time after addition of bacteria; II. Number of bacteria counted per tissue culture tube; III. Generation time accounting for increase in II during the preceding interval.

At the time indicated (I) the cells were removed from the glass, disrupted and the bacteria counted by plating out. The infection medium in both experiments contained the mixture of heated and fresh chicken serum. Change to growth medium (10 μg streptomycin per ml) was done at 6 hr in the first experiment and at 3 hr in the second.

trates the method employed (table 4). HeLa cells in an infection medium consisting of 20 per cent dog serum and 80 per cent Eagle's medium were exposed to an inoculum of 3.8×10^7 organisms of the Sm strain for 6 hr. The tissue culture fluid was then removed from the cells, the cells washed twice with balanced salts solution, and 1.0 ml of growth medium (40 per cent human serum, 60 per cent Eagle's medium, and 10 μg streptomycin per ml added. Two hours later three tubes were washed three times with balanced salts solution, the cover slips being lifted each time in order to remove the streptomycin. Formalin fixative was added to one tube so that its cover slip could be stained later and examined microscopically. To each of the other two tubes was added 1.0 ml of trypsin solution (Puck *et al.*, 1956) to remove the cells from the glass. Microscopic observation showed complete removal of the cells in each instance. The cells in both tubes were dispersed with bulb and pipette and transferred to one glass cup of the Mickle apparatus. The cup contained about twenty 3-mm glass beads. It was vibrated for 15 sec with an amplitude of about 5 mm. The contents were removed, diluted by 10-fold increments, and 0.01 ml of each dilution plated out on glucose cysteine blood agar (Downs *et al.*, 1947). The number of colonies counted was greater than 40 in almost every instance. The calculation of generation times is greatly facilitated by the tables of logarithms to base 2 of Finney *et al.* (1955).

The period of 15 sec vibration was based upon experiments with HeLa cells in which *P. tularensis* had grown 24 hr. After 7 sec, smears showed shreds of cytoplasm, some containing bacteria, still attached to the nuclei, but after 15 sec, the nuclei bore very little cytoplasm and almost no bacteria. No further change was apparent after 30 sec, but after 60 sec, the number of nuclei decreased. The smears showed that the bacteria that were not still trapped in the cytoplasm were well dispersed at all intervals. Colony counts of the samples rose 5- to 10-fold following disintegration, and did not change significantly between 7 and 60 sec.

In measurements of intracellular growth rates, the following controls were performed each time colony counts were made: (a) smear of tissue culture fluid to see that bacteria had not grown extracellularly in the fluid, (b) the usual stained cover slip preparation to see that the microscopic picture of bacteria in the cells developed as expected, and that the bacteria had not grown extracellularly on the glass, and (c) a smear of the solution in the Mickle apparatus to see that the cells were well disrupted and that the bacteria were well dispersed, that is, that they were present chiefly as single organisms. The results were satisfactory in every case.

The cultures of microorganisms and the media employed for their growth are shown in table 1. The nonacid-fast bacteria used for inoculum in HeLa cell cultures were grown about 16 hr, and a loopful removed from the slant and suspended in balanced salts solution with the aid of bulb and pipette. The preparation of inocula of *Mycobacterium tuberculosis* was described earlier (Shepard, 1957*a*), as was that of *H. capsulatum*

(Larsh and Shepard, 1958). It is worth reemphasis that the suspensions of microorganisms must be very well dispersed, since clumps are poorly phagocytosed, and thus contribute disproportionately to the extracellular growth. Filtration through filter paper (Shepard, 1957*b*) was carried out as routine for inocula of tubercle bacilli. It was also found helpful with clumped suspensions of streptococci but did not contribute much to the other bacterial suspensions. Control smears were made of each inoculum.

RESULTS

Phagocytosis. The selective action of sera of different species in promoting the phagocytosis of microorganisms of different species is shown in table 2. The greatest phagocytosis of *H. capsulatum* and *Candida albicans* was observed with guinea pig and dog sera, of *Salmonella typhosa* and *P. tularensis* with dog serum, and of staphylococci in sheep serum. Group *D* streptococci were phagocytosed most frequently in guinea pig serum, but group A streptococci were ingested frequently in all the sera tested. These results have been confirmed in essence in many similar experiments, and not much difference has been seen between different lots of sera from the same species.

In addition to the type of experiment described, some of the sera were compared for their ability to promote intracellular infections of the type illustrated in the figures. Four or five of the high scoring sera for a bacterium were compared as constituents of the infection medium, and the schedules of table 3 were followed. The results confirmed the findings in table 2, and the differences between serum species were even more marked in the case of *H. capsulatum*, *S. typhosa*, and *P. tularensis*. With *M. tuberculosis*, however, the stored horse serum, a sample that had been selected previously for its ability to promote ingestion of tubercle bacilli, was found to yield the most completely intracellular infections, and much of the growth seen following the use of chicken and guinea pig serum was at least partially extracellular.

Except where indicated, the sera of table 2 were fresh samples. Recent studies (*unpublished*) of the effect of heating fresh sera have shown that the phagocytosis-promoting activity of some species is decreased and others increased by heating to 56 C for 30 min. Unexpectedly it was found that a mixture of equal volumes of heated and fresh chicken sera was much more effective than either alone in aiding phagocytosis of Brucella. The mixture of heated and fresh chicken serum is about as effective as the best sera in table 2 for *P. tularensis*, *M. tuberculosis*, group A and D streptococci, and staphylococci, and somewhat simplifies the collection of sera for a day's experiment.

Intracellular growth. (1) Microscopic observation:—In table 3 are given the media and schedules found most suitable to produce growth of the microorganism in HeLa cells. In most cases the infection medium contained 20 per cent of the species of serum giving the most frequent phagocytosis as shown in table 2. The number of cells becoming infected could be increased by more concentrated inocula or by longer periods of exposure, and in some experiments almost all of the cells were infected. In most of the experiments to be described, less than 10 per cent of the cells were infected. The growth medium was 40 per cent human serum plus 60 per cent Eagle's medium for most bacteria. Experiments with *P. tularensis* showed that the number of cells developing the full-blown picture shown by figure 1 was a function of the growth medium. When the concentration of human serum was 10, 20, or 60 per cent, or when the amino acids or vitamins were not added, the number of such cells was decreased. Results with other nonacid-fast bacteria were not so sensitive to variations in growth medium.

P. tularensis grew well in HeLa cells and the well developed picture illustrated in figure 1 was seen in 24 hr. Usually 10 μg of streptomycin was present in the growth medium, but concentration of several hundred μg did not affect intracellular bacterial growth. This organism does not grow well in the tissue culture media, and satisfactory microscopic pictures were seen in the absence of the drug. The water strains (strains of lesser pathogenicity for rabbits or guinea pigs), did not grow as rapidly and by 24 hr had not yet filled the cells. No differences among the water strains were noted. The completely nonpathogenic strain 38 did not grow at all in the cells under the same conditions.

Brucellae entered the cells very well in chicken serum medium, but subsequent intracellular growth was not seen. Because Braun (1949) had reported that dissociation of brucellae occurred

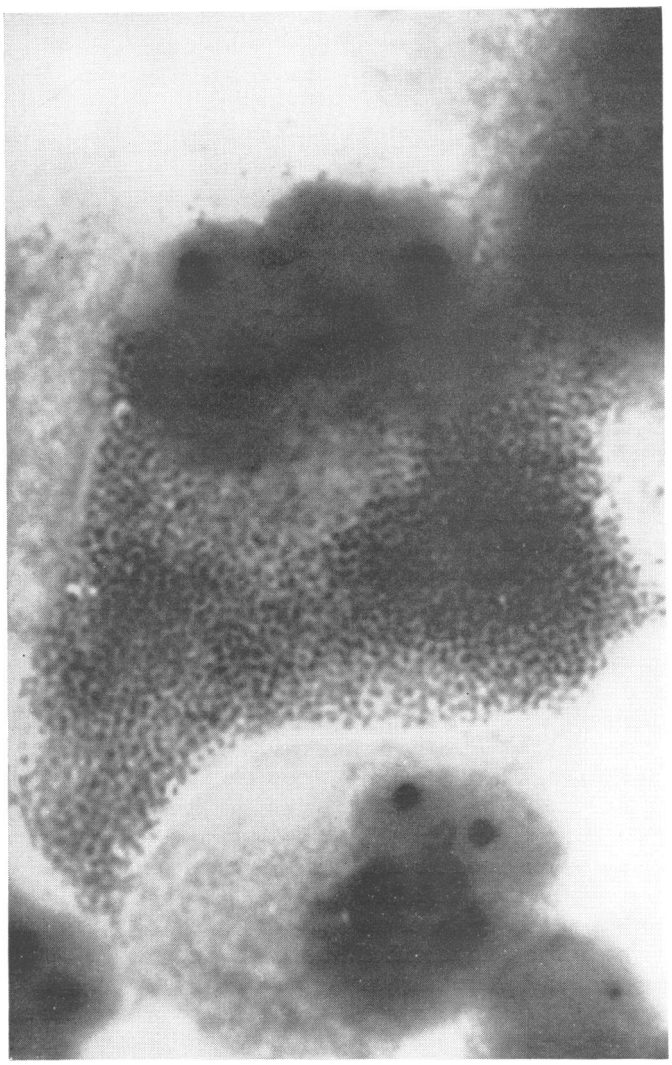

Figure 1. Growth of *Pasteurella tularensis* (Sm) in HeLa cells 24 hr after inoculation of tissue culture. Growth extends throughout the cytoplasm but leaves a juxtanuclear zone relatively free, 2190×.

in chicken serum, an infection medium containing guinea pig serum was tried. Rapid growth then ensued in the cells in medium containing streptomycin. *Brucella suis* filled the cells in 24 hr (figure 2), whereas *Brucella melitensis* (figure 3) and strain 19 of *Brucella abortus* required about 24 hr longer. The picture observed with HeLa cells resembles strikingly the figures published by Theobald Smith (1919) of brucellae infected epithelial cells of bovine fetal membranes.

S. typhosa gave the result, illustrated in figure 4, 3 hr after inoculation into an infection medium containing dog serum. Typical nests of short bacilli developed in positions that appear to be intracellular and juxtanuclear. The extracellular growth of *S. typhosa* in dog serum medium is in well developed chains and is easily distinguished from the apparently intracellular nests. It has not been possible, however, to see these groups after any change in the medium, and even shaking of the tube results in their disappearance. For these reasons growth in cells in medium containing streptomycin has not been seen. No differences were noted between strains Pa58 and Ty2.

In experiments with staphylococci, the infec-

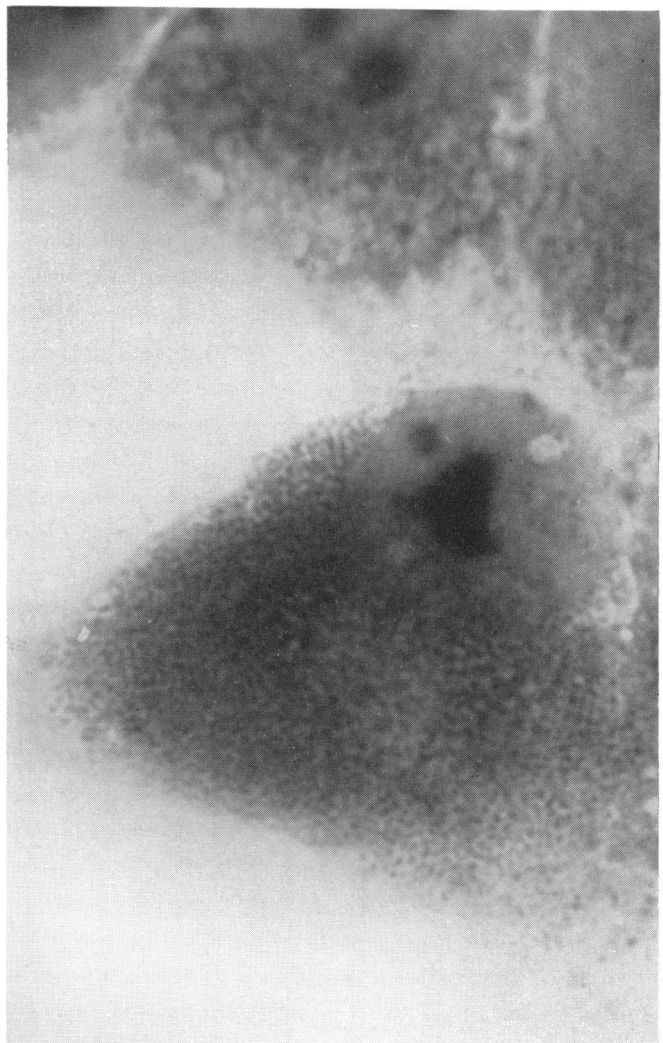

Figure 2. Growth of *Brucella suis* (3 Boar) at 24 hr. The cytoplasm becomes well filled with brucella species also, 2190×.

tion medium usually contained the mixture of heated and fresh chicken serum. The minimal inhibitory concentration of streptomycin for certain of the recently isolated epidemic strains overlaps the toxic concentration of this drug for HeLa cells. Intracellular growth of less resistant cultures was observed, and strain DA 16-7 grew intracellularly in the presence of 1024 μg streptomycin per ml, and only 64 μg prevented extracellular growth. Experience with staphylococci is as yet limited, and the results should be regarded as provisional. Figure 5 is the result in an experiment with strain PS 29 (sensitive to 4 μg streptomycin per ml) in the presence of 200 μg per ml in the growth medium.

Studies of group A streptococci have been done mostly with an infection medium containing the mixture of heated and fresh chicken serum. Figure 6 is a result at 6 hr. Five hours is a more favorable time for observation because there are more infected cells present and the amount of bacterial growth per cell is about the same. The intracellular growth of group A streptococci is usually in chains, but it also develops in clusters of cocci. No differences were noted among the group A strains.

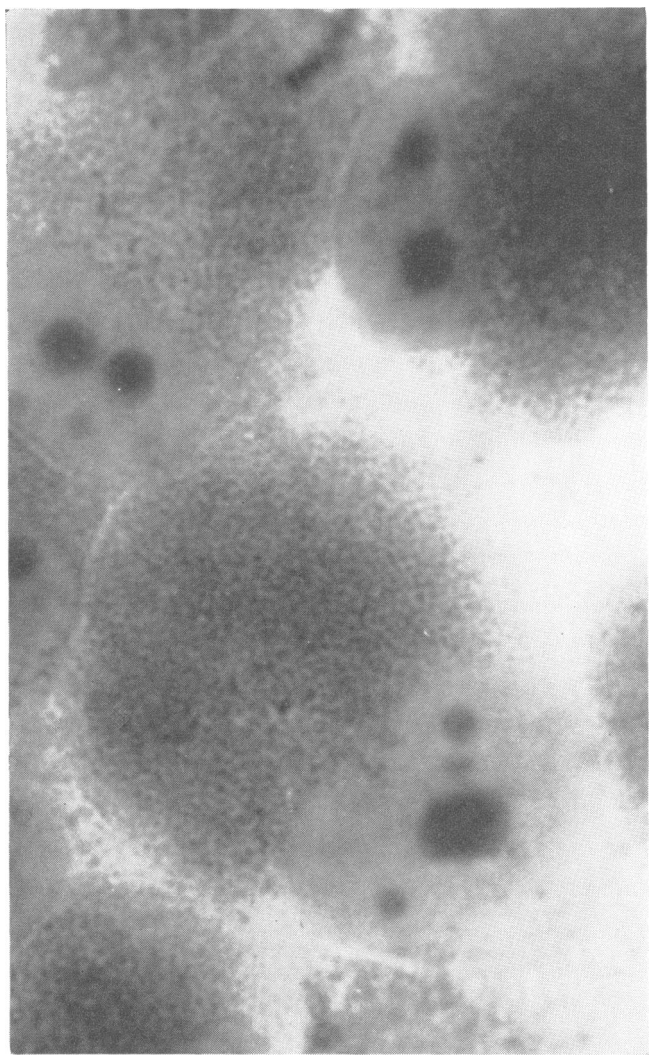

Figure 3. Brucella melitensis (16M) at 48 hr, 2190×

Guinea pig serum was much more effective in promoting uptake of group D streptococci, and the subsequent intracellular growth only partially filled the cell in 24 hr. The group D culture was never observed to form intracellular chains.

Results with *M. tuberculosis* have been described previously (Shepard 1955, 1957*a*). An infection medium containing the mixture of heated and fresh chicken serum is more efficient than the selected horse serum in promoting phagocytosis of mycobacteria grown in Tween-albumin medium. It is much inferior to the selected horse serum for the isolation of tubercle bacilli from sputum specimens (Shepard, 1958*a*), apparently because its enhancing effect is specific for mycobacteria.

The results with *H. capsulatum* have been described elsewhere (Larsh and Shepard, 1958). Continued maintenance of the tissue cultures in medium containing guinea pig serum results in the formation of plaques or colonies, thereby facilitating the detection of small numbers of yeast-phase organisms in the inoculum.

(2) Intracellular growth rates measured by colony count:—The method described under

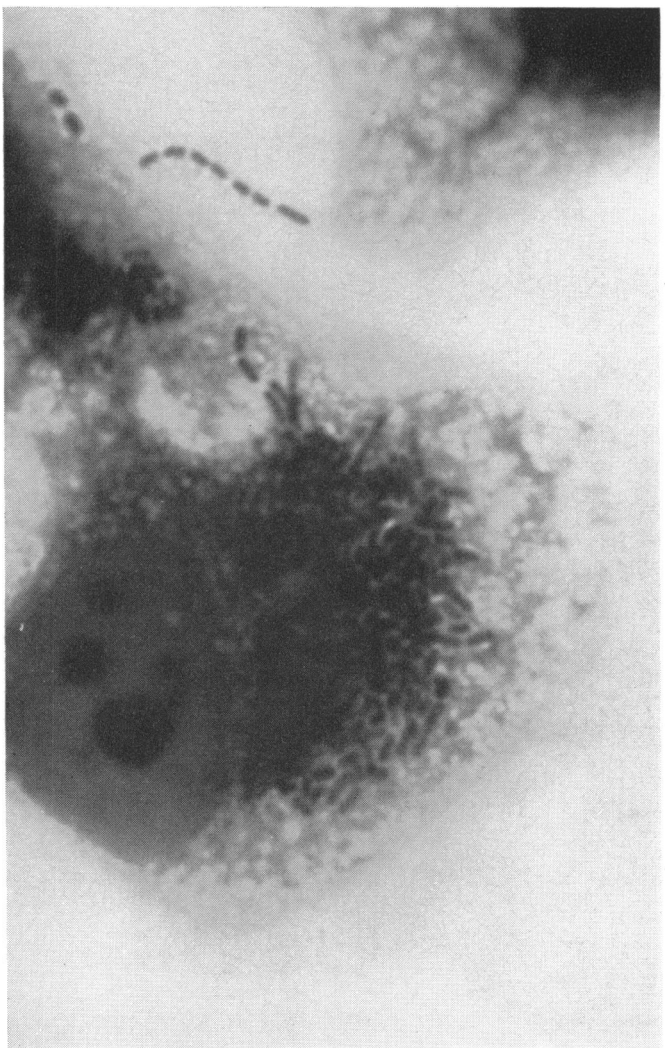

Figure 4. Salmonella typhosa (Pa 58) at 3 hr, 2190×

Materials and Methods was used to measure the growth rates of *P. tularensis* and brucellae in HeLa cells in medium containing streptomycin. Results of two experiments with *P. tularensis* are shown in table 4. The most rapid growth appeared to occur with generation time of 1.5 to 2.0 hr with strain Sm (fully virulent), whereas with strain 425F4G, the shortest generation time was about 2.5 to 3.0 hr. The nonpathogenic strain 38 showed no increase in colony count, although the notoriously poor plating efficiency of this strain rendered reproducible experiments impossible.

Similar experiments with brucellae yielded intracellular generation times of about 4 hr for *B. suis* (3 Boar), and about 6 hr for *B. melitensis* and strain 19 of *B. abortus*.

(3) Intracellular growth rates estimated by filling time:—In working with each bacterial species for the first time it was necessary to work out its schedule with some accuracy in order to obtain the results illustrated in the figures. In general, an accuracy of 20 to 30 per cent was required. If the cover slips were fixed too early the amount of intracellular growth was much less than that illustrated, and if they were fixed

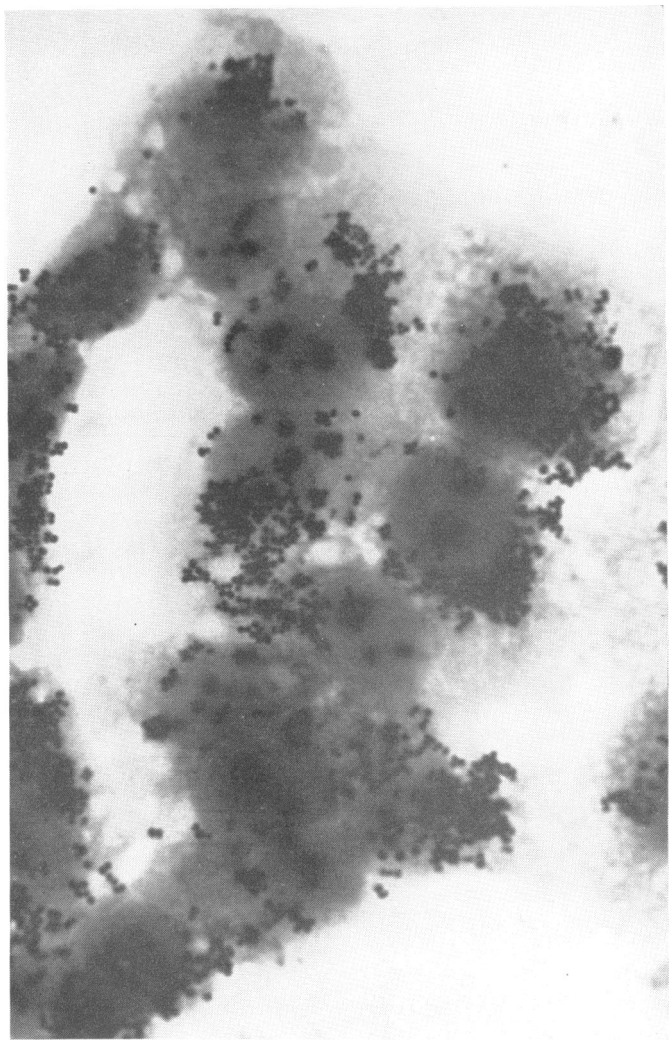

Figure 5. Staphylococcus aureus (PS29) at 5 hr, 875×

too late many of the infected cells rounded up and became pyknotic or were lost from the glass.

Filling time refers to the interval that gives the optimal microscopic picture without reference to the actual fraction of the cytoplasm of the cell that is occupied by bacteria. These times are given for the natural pathogens in table 4, together with the generation times of the same bacterial species in bacteriological media. In the last column of the table is shown the ratio of the filling time to the generation time. The ratios fall in the range of 7 to 8, except those for *P. tularensis* and *B. suis*. The difference of the ratios for these two species from the others is accountable chiefly by the greater numbers of these bacteria that develop within a given cell. The direct measurements of generation times of *P. tularensis* and *Brucella* species together and the value found previously for tubercle bacilli in HeLa cells (Shepard, 1958*a*, *b*), as estimated microscopically, are also given in table 5. The constancy of the ratios in the last column together with the direct measurements of intracellular generation times indicate that all of these bacterial pathogens are able to grow at rapid rates in these cells, even though they represent bacterial species that differ widely in their physiological properties.

Some reservation should be held for the intracellular growth rates of group A streptococci and

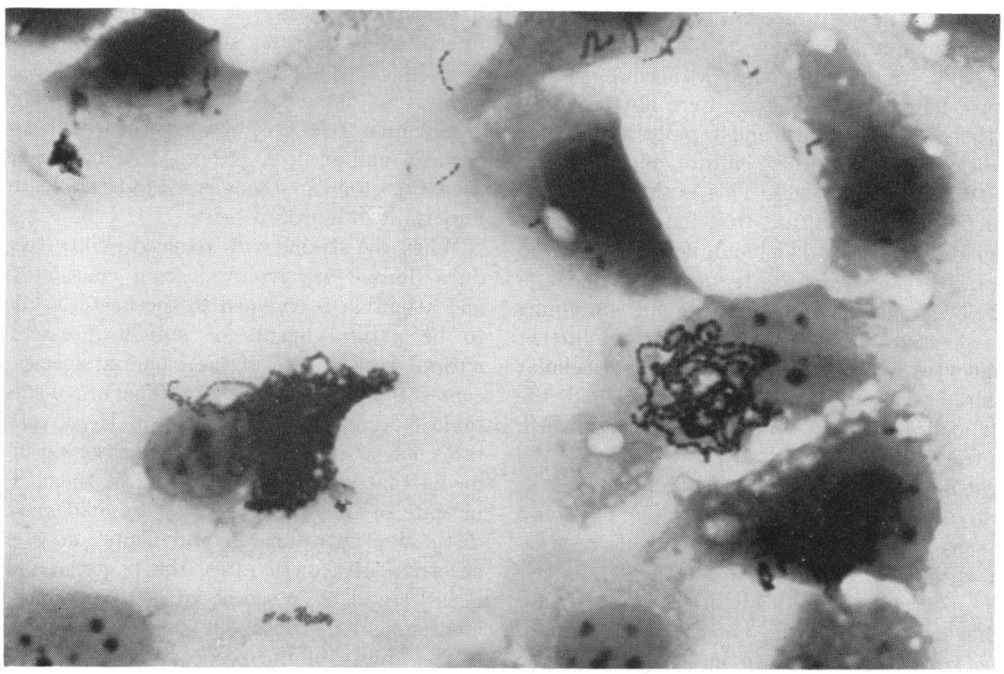

Figure 6. Group A streptococci at 6 hr, 875×

TABLE 5

Bacterial pathogens grown in HeLa cells arranged according to their rates of growth in HeLa cells

Bacterium	I Generation Time, Bacteriological Medium	II Generation Time, HeLa Cells	III Time to Fill HeLa Cells	IV Ratio III:I
Salmonella typhosa	24 min (1)	—	3 hr	8
Streptococcus group A	36 min (2)	—	5 hr	8
Pasteurella tularensis	1.7 hr (3)	1.7 hr	24 hr	14
Brucella suis	—	4 hr	48 hr	(12)*
Mycobacterium fortuitum	—	—	2–3 days	—
M. marinum (*M. balnei*)	11 hr (4)	—	3 days	7
Yellow bacilli	—	—	5 days	—
M. tuberculosis	18 hr (4)	24 hr (5)	5 days	7
M. ulcerans	2 days (4)	—	16 days (5)†	8

I. Generation time in bacteriological medium, from literature; II. Generation time calculated in HeLa cells; III. Time required to fill HeLa cells, to degree illustrated in figures: IV. Ratio, III:I.

(1) Mason, 1935; (2) Sherwood *et al.*, 1952; (3) Calculated from data of Won (1958); (4) Leach and Fenner, 1954; (5) Shepard, 1957*a*, *b*.

* Ratio, III:II.

† Results in monkey kidney tissue culture cells.

staphylococci since the late microscopic picture in the infected cells did not seem to change as rapidly as would be indicated by the generation times of these organisms in bacteriological medium.

DISCUSSION

The growth of nonacid-fast bacteria in HeLa cells follows the pattern observed previously for the mycobacteria (Shepard, 1957*a*). First is the obvious matter of cell entry, here again

regulated by the serum constituent of the tissue culture medium. Many of the bacteria studied were able to grow in the extracellular tissue culture fluid, so that if they were not taken up by the cells, they grew and rapidly altered the condition of the tissue culture medium and damaged the HeLa cells. This is the usual situation in a tissue culture that has become contaminated. On the other hand, if bacteria gained entrance to the cells, the tissue culture medium could be changed in most cases to one containing human serum and streptomycin, so that further phagocytosis would be minimal, extracellular bacterial growth would be stopped, and the tissue culture cells would be optimally nourished.

Intracellular bacterial growth then followed at rates governed by the pathogenicity of the strain and its natural growth rate. The term pathogenicity is used with considerable reservation since it is frequently used without reference to the operation carried out in its measurement. Pathogenicity for human beings may be judged by associating the type of human disease with the type of bacteria isolated, by observation of accidental laboratory infections, and only infrequently by the operation used to measure pathogenicity in laboratory animals, that is, the injection by defined routes of graded doses of bacteria. Earlier work was concerned with the relationship between growth rates in tissue culture cells and ability to produce disease among strains of tubercle bacilli of varying degrees of virulence (Shepard, 1947*a*, 1958*b*), and among the rapidly growing mycobacteria (Shepard, 1957*b*). In the present study, strains of *P. tularensis* followed the same pattern in that the fully pathogenic strain Sm grew more rapidly in HeLa cells than any of the water strains, and strain 38 did not grow at all in these cells. The behavior of these same strains in laboratory animals has been reported by Bell *et al.* (1955). The fully virulent strains, such as Sm, are lethal for mice and rabbits even in very small doses; the water strains kill mice but not rabbits with such doses, whereas strain 38 does not kill these animals even though inoculated in high concentrations. The history of infection of laboratory personnel seems also to fall in the same order, since fully virulent strains caused severe and even fatal infections before effective antibiotics were available, and strain 38 has been handled with impunity for years in many laboratories preparing diagnostic antigens. At least one laboratory infection with a water strain has been observed in an unvaccinated person, and it was a relatively mild, influenza-like illness.

A similar relationship was observed between group A and group D streptococci; organisms of the latter group are thought not to be etiologically important in human disease.

When the strains with reduced ability to produce disease are removed from consideration, and attention is confined to the bacteria known to be natural agents of human disease, the natural growth rate of the bacterial species becomes the dominant factor. The organisms of table 5 were all able to grow in HeLa cells at rates not far removed from those observed in the media that are optimal for each of them. Thus in spite of their heterogeneity, as evidenced by distinctive nutritional requirements and a wide spectrum of growth rates, the bacterial pathogens were all very successful in the cytoplasmic environment of this tissue culture cell.

The ability of the tissue culture cells to discriminate between these naturally pathogenic bacteria and their less pathogenic relatives supports the conception that the intracellular environment is decisive in the natural disease also. The cells in which bacteria are found in the intact animal are usually the natural phagocytes of the body, and the importance of these cells in controlling the outcome of an infectious disease has been generally accepted since the days of Metchnikoff. He proposed that the phagocytes contain cytases, enzymes capable of digesting bacteria that enter the cytoplasm (Metchnikoff, 1907), and much experimental work has been based upon the hypothesis that the phagocytic cells are uniquely endowed with antibacterial factors, which may be extracted from cells and demonstrated by action in cell-free media (reviewed by Suter, 1956, and by Skarnes and Watson, 1957). The results with HeLa cells direct attention to the pH, O_2 tension, and other nonspecialized biochemical qualities that would exist around a bacterium in the cytoplasm of an actively metabolizing cell. The influence of biochemical factors in infectious disease has been emphasized by Dubos (1957).

Since the HeLa cells also provide a location for the multiplication of viruses, a relationship between the viruses and the bacterial pathogens is suggested. The basic differences in the methods

of reproduction that probably exist, at least for the smaller viruses, seem, however, to separate widely these two categories of disease agents. Even an evolutionary relationship would have to be distant, although a regressive type of evolution (Luria, 1953) of a bacterium in a living cell would seem to provide unique opportunity for the development of a dependent relationship.

The amount of bacterial growth that develops in HeLa cells with these pathogens, many of of which are thought to possess toxic properties, is surprising. It may be that the relative innocuousness of the bacteria depends upon their physiological age, since they were present in the cell during or shortly after this logarithmic growth phase when breakdown of bacteria would be minimal.

ACKNOWLEDGMENT

The excellent technical assistance of Mrs. Nannett Jackman and Mrs. Betty L. Gray is gratefully acknowledged.

SUMMARY

The uptake by HeLa cells of different species of bacteria was observed to be promoted selectively by the sera of different species of animals, when the sera were incorporated in the tissue culture medium at a concentration of 20 per cent. Dog serum promoted the uptake of *Pasteurella tularensis* and of *Salmonella typhosa*, guinea pig serum group D streptococci and *Histoplasma capsulatum*, chicken and guinea pig serum *Brucella abortus*, *Brucella melitensis*, and *Brucella suis*, and guinea pig and sheep serum staphylococci.

A tissue culture medium containing 20 per cent of the appropriate serum was used to infect the cells with a particular bacterium, and in most cases it could be followed by a medium containing 40 per cent human serum to depress phagocytosis, and also containing streptomycin to prevent extracellular growth. Intracellular growth of the bacteria could then be observed. The natural pathogens grew in the cells at rates related to their optimal rates on bacteriological media, and strains of lower pathogenicity grew more slowly.

REFERENCES

BELL, J. F., OWEN, C. R., AND LARSON, C. L. 1955 Virulence of *Bacterium tularense*. I. A study of the virulence of *Bacterium tularense* in mice, guinea pigs, and rabbits. J. Infectious Diseases, **97**, 162–166.

BRAUN, W. 1949 Studies on bacterial variation and selective environments. II. The effects of sera from *Brucella*-infected animals and from normal animals of different species upon the variation of *Brucella abortus*. J. Bacteriol., **58**, 299–305.

DOWNS, C. M., CORIELL, L. L., CHAPMAN, S. S., KLAUBER, A. 1947 The cultivation of *Bacterium tularense* in embryonated eggs. J. Bacteriol., **53**, 89–100.

DUBOS, R. J. 1957 Metabolic interrelationships between host and parasite. In *Host-parasite relationships in living cells*, pp. 172–190. Charles C Thomas, Springfield, Ill.

EAGLE, H. 1955 The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. J. Exptl. Med., **102**, 37–48.

FINNEY, D. J., HAZLEWOOD, T., AND SMITH, M. J. 1955 Logarithms to base 2. J. Gen. Microbiol., **12**, 222–225.

LARSH, H. W. AND SHEPARD, C. C. 1958 HeLa cells and *Histoplasma capsulatum*. Phagocytosis and subsequent intracellular growth. J. Bacteriol., **76**, 557–563.

LEACH, R. H. AND FENNER, F. 1954 Studies of *Mycobacterium ulcerans* and *Mycobacterium balnei*. III. Growth in semi-synthetic culture media of Dubos and drug sensitivity *in vitro* and *in vivo*. Australian J. Exptl. Biol., **32**, 835–852.

LURIA, S. E. 1953 *General virology*. John Wiley & Sons, New York.

MASON, M. M. 1935 A comparison of the maximal growth rates of various bacteria under optimal conditions. J. Bacteriol., **29**, 103–110.

METCHNIKOFF, E. 1907 *Immunity in infective diseases*. Cambridge University Press, London.

PUCK, T. T., MARCUS, P. I., AND CIECURA, S. J. 1956 Clonal growth of mammalian cells *in vitro:* Growth characteristics of colonies from single HeLa cells with and without a "feeder" layer. J. Exptl. Med., **103**, 273–284.

SALVIN, S. B. 1950 Growth of the yeastlike phase of *Histoplasma capsulatum* in a fluid medium. J. Bacteriol., **59**, 312–313.

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 of tubercle bacilli and certain other mycobac-

teria in HeLa cells. J. Exptl. Med., **105,** 39–48.

SHEPARD, C. C. 1957*b* Growth characteristics in HeLa cells of the rapidly growing acid fast bacteria, *Mycobacterium fortuitum*, *Mycobacterium phlei*, and *Mycobacterium smegmatis*. J. Bacteriol., **73,** 722–766.

SHEPARD, C. C. 1958*a* A study of the growth in HeLa cells of tubercle bacilli from human sputum. Am. Rev. Tuberc. Pulmonary Diseases, **77,** 423–435.

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

SHERWOOD, N. P., RUSSEL, B., BOWMAN, K., AND OTT, J. 1952 Studies on streptococci. IV. A study of the relationship of certain factors in streptococcal infections to the LD_{50} dose of streptococci. J. Infectious Diseases, **91,** 246–259.

SKARNES, R. C. AND WATSON, D. W. 1957 Antimicrobial factors of normal tissues and fluids. Bacteriol. Revs., **21,** 273–294.

SMITH, T. 1919 A characteristic localization of *Bacillus abortus* in the bovine fetal membranes. J. Exptl. Med., **29,** 451–456.

SUTER, E. 1956 Interaction between phagocytes and pathogenic microorganisms. Bacteriol. Revs., **20,** 94–132.

THOMAS, W. J., ZIEGLER, D. W., SCHEPARTZ, S. A., AND MCLIMANS, W. F. 1958 Use of arginine to eliminate medium changes in tissue culture systems. Science, **127,** 591–592.

WON, J. D. 1958 New medium for the cultivation of *Pasteurella tularensis*. J. Bacteriol., **75,** 237–238.