HeLa Cells and Histoplasma capsulatum. Phagocytosis and Subsequent Intracellular Growth

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In an earlier study (Shepard, 1955) it was found that tubercle bacilli were much more actively phagocytosed by HeLa cells when horse serum was incorporated into the tissue culture medium, in place of the more commonly used human or rabbit serum. By selection of a favorable horse serum it could be seen that a significant proportion of the tubercle bacilli in an inoculum could thus be introduced into the HeLa cells (Shepard, 1958a), where their subsequent intracytoplasmic growth took place rapidly and with characteristic morphology.

In a study of Histoplasma capsulatum and HeLa cells, Larsh et al. (1956) found that the mycelial phase converted to the tissue phase more readily in HeLa cell cultures than in artificial medium. In view of these findings a joint study was undertaken of the cytological aspects of the growth of this fungus in HeLa cells, especially as concerned the relationship between phagocytosis and the serum constituent of the tissue culture medium. It was soon evident that the use of human serum in the tissue culture medium led to more active phagocytosis of the yeast phase of H. capsulatum than did horse serum with known ability to promote phagocytosis of tubercle bacilli. These observations led to a survey of other animal sera, and of those tried, guinea pig serum was much superior. Guinea pig serum also was found to be satisfactory for the nutrition of the HeLa cells and this made it possible to devise methods for the rapid intracellular growth of H. capsulatum from small inocula. In addition to the research referred to (Larsh et al., 1956), the other published studies of the yeast phase of H. capsulatum in tissue culture cells appear limited to those of Randall and co-workers (1951, 1953a, b).

Materials and Methods

The tissue culture methods have been described in more detail elsewhere (Shepard, 1957). HeLa cells were grown on cover slips in Leighton tubes for 2 days in 40 percent human serum in BSS (balanced salt solution) (Hanks and Wallace, 1949) and then washed in BSS. Following this, 1 ml was added of a medium consisting usually of 20 percent serum of the desired species and 80 percent BSS containing the amino acids and vitamins recommended by Eagle (1955).

To study phagocytosis, a yeast phase suspension of H. capsulatum was stored in 10 percent formalin and washed in BSS 3 times by centrifugation just prior to use and adjusted to a turbidity corresponding to an optical density of about 0.55 at 600 mμ in a 19 mm tube in a Coleman Junior spectrophotometer. The inoculum was then introduced in 0.05 ml amounts. These experiments were terminated after 1 day at which time the cover slips were washed twice with BSS and fixed with neutral formalin. The tissue culture fluid was stirred and a smear made before it was removed from the cells. The cover slips and tissue culture fluid preparations were stained with giemsa. To estimate the amount of phagocytosis, the number of infected HeLa cells was counted in five standard representative fields. For this purpose an infected cell was defined as one appearing to have any contact at all with histoplasma organism. This method of estimating phagocytosis undervalued the differences between effective and noneffective sera, because many of the cells in a favorable medium, such as that containing fresh guinea pig serum, were multiply infected. Organisms attached to the cover slip in a position that appeared to contact the cell also contributed greater proportional amounts to the low scoring sera. The conditions of microscopy must allow the distinct resolution of the refractile cell wall of the yeast cell and its content of blue-staining protoplasm. This degree of
optical definition is necessary to differentiate the yeast phase of *H. capsulatum* from blue-staining spheres of similar size that may be found in normal HeLa cell cultures.

To study growth of *H. capsulatum* in HeLa cells, cultures of the fungus were employed that had been grown 3 days on Cozad's modification (unpublished) of the medium of Salvin (1950) and transported by air to Montgomery. Although some of these cultures contained a few short mycelia, growth in the HeLa cells appeared to be entirely in the yeast phase. A loopful of growth from the slant was suspended in BSS, and the clumps broken up with bulb and pipette. The number of yeast cells in the inoculum was counted in a hemocytometer and also by the examination of a drop produced by emptying a 2 µl pipette onto a glass slide. The dilution was adjusted so that a few hundred organisms were contained in this microdrop, and after fixation and staining the area could be examined carefully under oil immersion. A count was kept of the total number of organisms, and also of the number of aggregates. An aggregate was defined as 1 or more organisms that did not touch other organisms. Aggregates of 10 or more were rare, but those of 2 to 4 were frequent. Singles were most common, and the average number of yeast cells per aggregate was about 2.

The medium used for growth studies was 20 per cent fresh guinea pig serum and 80 per cent BSS containing the amino acids and vitamins recommended by Eagle (1955). The suspension of *H. capsulatum* in yeast phase was then introduced in 0.05 ml amounts. The guinea pig serum medium was used until the termination of the experiment and was changed every 2 days. The incorporation of 200 units of penicillin and 100 µg of streptomycin per ml did not noticeably affect the phagocytosis or subsequent growth of the organism, and these antibiotics were usually included.

All potentially dangerous work was carried out in an infectious disease hood.

**Results**

Phagocytosis. When the sera of different species were incorporated into the tissue culture medium, the amount of phagocytosis was observed to vary greatly according to species. All results shown in table 1 were obtained in one experiment, and were confirmed on other occasions. Horse serum was ineffective, and the particular lot giving the greatest phagocytosis of mycobacteria resulted in only 13 infected cells in 5 microscopic fields. This was in marked contrast to the results with the guinea pig serum, which gave 184 to 201 infected cells in 5 fields. The difference between the horse and the guinea pig sera is undervalued as stated above. Little phagocytosis was seen also with calf, monkey, and rabbit sera; and some human sera. Intermediate amounts of phagocytosis were observed with chicken, dog, and the remainder of the human sera. Some of the human sera were taken from patients with histoplasmosis, and some showed positive complement-fixing antibodies. One series of bleedings was available that in-
Inactivation of the sera lowered the amount of phagocytosis. For example, a fresh guinea pig serum giving 153 infected cells in 5 fields yielded only 4 infected cells in 5 fields when it was heated to 56°C for 30 min before being incorporated into the medium. Similarly, inactivation of a human serum from a stored pool lowered the amount of phagocytosis from 80 to 21.

The concentration of guinea pig serum giving the greatest phagocytosis was 20% in the medium described, one sample showing scores of 112, 171, and 96 at concentrations of 10, 20, and 40% per cent, respectively. The optimal concentration of serum might be found to vary with other samples, since the concentration of horse serum giving the greatest phagocytosis of tubercle bacilli has been found to vary with different lots. It is not feasible, however, to test the phagocytic effect of different concentrations of fresh guinea pig sera in advance and it has been necessary to adhere to 20% per cent in growth experiments. Also, this amount seems to be optimal for nutrition of the cells, and no difficulty has been encountered in keeping good monolayers of HeLa cells in this medium for the period of 6 days.

**Growth of H. capsulatum in HeLa cells.** Based on these observations, the procedure outlined in the section on materials and methods was developed for the growth of the yeast phase of *H. capsulatum*. In most experiments 10-fold serial dilutions of suspensions of living *H. capsulatum* were inoculated into the tissue culture fluid, and examinations made at daily intervals. The appearance of the growth is illustrated in figures 1–6. The growth spread from cell to cell so that by the 6th day after inoculation large plaques, or colonies, of infected cells had developed to a
size that frequently contained more than 100 organisms. It could be seen with careful focusing that the colonies consisted of the fungus in the yeast phase and darkly staining HeLa cells. The colonies could be detected under low power (about 100×), and higher power magnification (about 500×) with adequate resolution was used to see that the colonies actually contained *H. capsulatum*. The whole cover slip could be scanned in this manner, and a count of the colonies made. Although there were a few yeast cells to be found outside of the colonies by prolonged search with high power, these apparently originated from the colonies. In most cases, lengthy examination under high magnification of cover slips inoculated with dilutions greater than those yielding colonies did not reveal the fungus. Smears of the extracellular fluid revealed few yeast cells, and these were large groups of usually 20 or more organisms in the cultures receiving the most concentrated inoculum.

Examination of the monolayers growing in their tubes before fixation and staining showed changes similar to those seen in the stained preparations. The aberrations induced under these conditions made identification of the fungus difficult except with large aggregates of organisms.

*Minimal inocula and rates of growth of* *H. capsulatum*. The data of table 2 show that the minimal inoculum required to institute growth of *H. capsulatum* in HeLa cell cultures was 3 to 20 organisms, and 2 to 10 aggregates of organisms. By comparison, in experiment C about 1000 organisms were required for growth on Sabouraud's medium and about 100 on Cozad's medium.

Rates of growth were estimated by counting the total number of yeast cells in standard representative microscopic fields of cover slip preparations washed and fixed on successive days. The number on organisms in the extracellular fluid was disregarded since it was less than 1/100 of the
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Figure 5. Six days after inoculation with a higher dilution (10^{-3}) of the same suspension. The growth of H. capsulatum has occurred in isolated colonies or plaques, such as the one shown. 212 X

Figure 6. A greater magnification of the colony shown in figure 5. The dark mass consists of Histoplasma capsulatum and darkly staining HeLa cells. Coverslips may be scanned with low power magnification, and suspected colonies examined with higher magnification to see if they contain typical organisms. 850X

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<th>Expt</th>
<th>Count of Histoplasma per mL of Inoculum</th>
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<tr>
<td></td>
<td>(1) Hemocytometer</td>
</tr>
<tr>
<td>A</td>
<td>2.3 × 10^4 cells</td>
</tr>
<tr>
<td>B</td>
<td>5.9 × 10^4 cells</td>
</tr>
<tr>
<td>C</td>
<td>3.5 × 10^7 cells</td>
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A suspension was made from growth on Cozad’s medium, and the number of organisms estimated (1) by counts in a hemocytometer, (2) by examination of a 2μL drop dried on a slide, (3) by the number of colonies developing in HeLa cell cultures inoculated with high dilutions of the inoculum, (4) by observation of the highest dilution of inoculum resulting in growth and (5 and 6) by counts of the colonies developing on artificial media.

TABLE 2
Sensitivity of HeLa cell cultures in detecting the yeast phase of Histoplasma capsulatum

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number on the cover slip. The increase in number of organisms on successive days was accounted for by a generation time (doubling time) of about one day.

Phagocytosis and growth of Candida albicans in HeLa cells. Comparative studies of C. albicans were also carried out. It was found that this organism was also phagocytosed by HeLa cells in medium containing guinea pig serum, and that chicken, horse, human, monkey, rabbit, and sheep serum were less effective. When living inocula were used, rapid growth of C. albicans ensued. Growth in the tissue culture fluid seemed more pronounced than with H. capsulatum, and all HeLa cell cultures receiving C. albicans were overwhelmed in three days, including those receiving inocula diluted to contain about one organism. The microscopic morphology of C. albicans growing in HeLa cells would appear to allow its easy differentiation from H. capsulatum.

Discussion

In media that varied in the species of the serum constituent, marked differences in the amount of phagocytosis of H. capsulatum by HeLa cells were observed. The use of horse serum, which had been selected for its ability to give efficient phagocytosis of tubercle bacilli, resulted in very little phagocytosis of the fungus of the species examined, fresh guinea pig serum was markedly superior.

Monolayers of HeLa cells in a medium containing 20% of guinea pig serum in Eagle's medium provided a sensitive medium for the growth of H. capsulatum in the yeast phase. This would seem to provide a convenient model for the experimental study of H. capsulatum in human cells in tissue culture. Some of the results indicate that the system also might be useful in the diagnosis of histoplasmosis. Thus, under the conditions of these experiments, 3 to 20 organisms were necessary to produce growth in HeLa cells, and the growth was detectable in 6 days or less by microscopic examination of the cover slip cultures. The data of experiment C in table 2 indicate HeLa cell cultures were considerably more sensitive in detecting H. capsulatum than were the artificial media used. However, the results of Rowley and Huber (1955) show that artificial medium may be much more satisfactory and they found that as many as 80% of the aggregates in suspensions produced colonies under their experimental conditions. The work of Rowley and Huber and the present work were done with pure cultures grown on artificial medium. The usefulness of HeLa cell cultures for diagnosis will, of course, have to be decided by studies with clinical material. In this connection studies of tubercle bacilli in human sputum and their growth in HeLa cells have shown that a short digestion with trypsin was sufficient to dissolve the mucus so that the bacteria could be concentrated by centrifugation (Shepard, 1958a).

The procedure did not kill the numerous non-acid fast organisms present, but their growth in the HeLa cell cultures was controlled in most instances by penicillin. Preliminary experiments have shown that this trypsin solution has no significant effect upon the viability of H. capsulatum.

The basis underlying the phagocytosis-promoting activity of horse serum for mycobacteria and of guinea pig serum for H. capsulatum remains obscure. Nevertheless, the present study provides some information on this point. It was possible to study the effect of antibody on phagocytosis of H. capsulatum by HeLa cells by incorporating the sera of patients into the tissue culture medium; and no apparent relationship was noted to the course of the clinical illness or the presence of complement-fixing antibody. Also there seemed to be no relationship to the susceptibility of the species to histoplasmosis. Complement may play a role in the phagocytosis of H. capsulatum by HeLa cells, as suggested by the results with inactivation of guinea pig and human sera. However, no distinct effect on the uptake of tubercle bacilli resulted from inactivation of horse serum (Shepard, 1955).

The contrast in the effect of guinea pig serum and horse serum on the phagocytosis of H. capsulatum and Mycobacterium tuberculosis has suggested an experimental approach whereby it has proved possible to produce infections in HeLa cells with Pasteurella tularensis, Brucella suis, Salmonella typhosa, and Streptococcus pyogenes (Shepard, 1958b). These intracellular bacterial infections are also achieved by the incorporation into the tissue culture medium of serum of an appropriate species. Thus, although the mechanism is still unexplained by which the species of the serum is able to control the type of particle taken in by these cells, the specificity of
the reaction between microorganism, serum, and human cell offers attractive experimental avenues.

SUMMARY

Horse sera that had been selected for their ability to promote phagocytosis of tubercle bacilli by HeLa cells, when incorporated into the tissue culture medium, did not enhance phagocytosis of the yeast phase of Histoplasma capsulatum by these cells.

When sera of a number of species were studied as components of the tissue culture fluid, it was found that fresh guinea pig serum resulted in the greatest phagocytosis of the fungus. The sera of calves, monkeys, and rabbits were ineffective, and those of chickens and dogs were intermediate. Some human sera had intermediate value and some produced little phagocytosis.

In these preliminary experiments the sera of human patients with histoplasmosis did not seem noticeably more or less effective. There appeared to be no relationship to the antibody response of the patient, nor to the susceptibility of the species to the disease. Heating serum to 56 C reduced its ability to promote phagocytosis of H. capsulatum.

When a tissue culture medium containing 20 per cent fresh guinea pig serum was used for the HeLa cell cultures, they were found to be sensitive to small inoculum of H. capsulatum. An inoculum of 3 to 20 yeast phase organisms produced growth in monolayers of HeLa cells with colonies or plaques of infected cells that were detectable with low-power microscopy within 6 days.

REFERENCES


