SHORT COMMUNICATIONS

Enhancement of Growth of *Leptospira icterohaemorrhagiae* by Tissue Cell Cultures

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INTRODUCTION

Tissue cultures are widely used to study the relationship between various bacteria and tissue cells. Previous studies concerning growth of leptospires in cell culture have elucidated no stimulatory growth factors (Ellison *et al.* 1965; Harrington & Sleight, 1966; Miller, Miller & White, 1966; Finn & Jenkin, 1973). Our results indicate that the cell lines (Clone B, BHK 21 and HK) and primary cells (PMK, CEF) may markedly enhance the growth of leptospires in some media.

METHODS

Test organisms. Avirulent and virulent strains of *Leptospira* serotype *icterohaemorrhagiae* were used. The avirulent Wijnberg strain was obtained from Dr Esther Shenberg, WHO/FAO Leptospirosis Reference Laboratory, Israel Institute for Biological Research, Ness-Ziona, Israel. The virulent strains were isolated from kidneys of *Rattus norvegicus*, generally used when freshly isolated and transferred only a few times in Korthof's medium (Korthof, 1932). After 6 to 8 days in Korthof's medium a culture was washed (3 times) in test medium and resuspended in it for use as inoculum.

Tissue culture media. The test media were prepared according to Eagle (1939) (Eagle's medium); Morgan, Morton & Parker (1950) (medium 199); and Melnick (1955) (LH medium). Eagle's modified medium (Eagle's medium containing tryptose and a twofold concentration of amino acids; EM medium) was prepared according to Stoker & MacPherson (1961). Unless otherwise stated, 5% (v/v) haemolysed sheep serum, inactivated at 56 °C for 30 min, was added to all media. Sera containing 0.05% or less haemoglobin were considered as non-haemolysed. To obtain haemolysed serum, a 5% (v/v) stock solution of packed sheep erythrocytes in distilled water was added to non-haemolysed serum to reach a final concentration of 0.5 or 0.25% haemoglobin in whole serum. The balanced salt solutions (BSS) were prepared according to Hanks & Wallace (1949) or Earle (1943/4).

Cell lines and primary tissue cultures. Hamster embryo (Clone B) was prepared according to Yaniv & Gotlieb-Steimatzky (1970). For a monolayer, 150 000 cells/ml were used in EM medium supplemented with 10% (v/v) calf serum. Baby hamster kidney 21 (BHK 21) cells were prepared according to MacPherson & Stoker (1962). For a monolayer, 150 000 cells/ml were used in EM medium supplemented with 10% (v/v) calf serum. Human kidney

(HK) cells were grown in medium 199 supplemented with 10 % (v/v) calf serum. For a monolayer, a concentration of 150 000 cells/ml was used. Primary monkey kidney (PMK) vervet cells were prepared according to Rappaport (1956). For a monolayer, 300 000 cells/ml were used in LH medium supplemented with 3 % (v/v) inactivated calf serum. Chick embryo fibroblasts (CEF) were prepared according to Lindenmann & Gifford (1963). For a monolayer, 400 000 cells/ml were used.

Stock cultures of cell lines were periodically examined for mycoplasma by the method of Chanock *et al.* (1962). No antibiotics were added to the various solutions or tissue culture media.

Inoculation of cell monolayers with leptospirae. The cell monolayers (2–3 day cell lines or 7 to 8 day primary tissue cultures) were washed three times with the medium tested. To each tube containing 1.8 ml medium, 0.2 ml leptospiral culture was added to obtain a final concentration of 10^5 , 10^4 or 10^3 bacteria/ml. In all trials at least quintuplet cultures in tissue culture tubes (Leighton type; Bellco Glass Incorporated) were used.

Incubation. Unless otherwise stated, the inoculated cultures were incubated at 30 °C in a 5 % CO₂ incubator.

Measurement of growth. Leptospirae were counted in a Petroff Hausser counting chamber using a dark-field microscope at $\times 200$ magnification. Cultures were diluted 1:2 with 0.4 % formaldehyde in saline (0.85 % NaCl). The counts of organisms expressed in Table 1 are the average of three culture tubes.

RESULTS

The growth of leptospirae in tissue culture media

The tissue culture media, Eagle's, 199, and LH, were tested for the growth of *Leptospira icterohaemorrhagiae* Wijnberg. Only Eagle's medium supported a steady growth of leptospirae from an inoculum of 10^3 organisms/ml. The growth was abundant and similar to that obtained in Korthof's medium. In medium 199 poor growth was obtained and only from an inoculum of 10^5 organisms/ml, and in LH medium there was no growth from either of the above inocula.

Lack of growth in LH medium was investigated by cultivating strain Wijnberg in Korthof's medium with the addition of 10 % (v/v) haemolysed sheep serum and ingredients similar to those found in LH medium. The results showed that MgCl₂, MgSO₄ and CaCl₂, in concentrations similar to those found in LH medium, did not inhibit leptospiral growth. However, there was a strong inhibitory effect with 0.5 % lactalbumin hydrolysate, a relatively smaller effect with 0.85 % NaCl and a still smaller influence with 0.035 % of NaHCO₃.

Growth of leptospirae in the presence of culture cells

The growth of leptospirae in media with and without tissue cells was compared. In the first experiments, Clone B tissue cells were used. In Eagle's medium, considered the most suitable of the three media previously tested, leptospirae grew well either with or without Clone B cells. However, Eagle's medium supplemented with non-haemolysed sheep serum produced a very poor yield in medium alone and a relative enhancement in association with cell line Clone B. The experiment was repeated with LH medium and there was a similar enhancing effect of Clone B cells on leptospiral growth that was especially marked since this medium failed to support growth without the cells. Haemoglobin, which has an enhancing effect on leptospiral growth in Eagle's medium, has only a slight influence, if any, on growth of leptospirae in LH medium. In further experiments strain Wijnberg was grown in associa-

Table 1. *Effect of primary tissue cells and cell lines on the growth of Leptospira icterohaemorrhagiae Wijnberg in Eagle's ME and LH media*

Expt no.	Growth system	10 ⁻⁶ × No. of organisms (counts/ml medium)	
		Eagle's	LH
1	Medium alone	78	0
	Medium + BHK 21 monolayer	96	72
	Medium + PMK vervet monolayer	60	55
2	Medium alone	58	0
	Medium + CEF monolayer	52	46
3	Medium alone	68	0
	Medium + BHK 21 monolayer	88	94
	Medium + HK monolayer	72	60

Inoculum: 10⁸ organisms/ml. Eagle's and LH media were prepared on the basis of Hanks' BSS and supplemented with 5 % (v/v) sheep serum and 0.0125 % haemoglobin. Values are the highest obtained of the periodic 4 day readings.

tion with BHK 21 tissue cells in Eagle's medium, medium 199 and LH medium; all media contained haemolysed sheep serum. In the absence of tissue cells, leptospirae grew well only in Eagle's medium, whereas in the presence of BHK 21 cells the leptospirae grew effectively in all three media. Other cell cultures were tested for the enhancement of growth of strain Wijnberg in Eagle's and LH media, both prepared on Hanks' BSS. The results of three experiments are summarized in Table 1. In both these media primary tissue cultures (CEF and PMK) had less of an enhancing effect than the tissue lines (BHK and MK) on the growth of strain Wijnberg; indeed there was no growth enhancement in Eagle's medium with primary tissue cultures and the leptospiral yield was even less than that obtained in the same media without tissue cells. However, primary cell cultures as well as tissue lines make rich leptospiral growth possible in LH medium, although the growth was higher with cell lines than with primary cells. No marked differences were observed in response to the growth-enhancing factor between avirulent strain Wijnberg and freshly isolated virulent rat strains of serotype *icterohaemorrhagiae*. The growth-enhancing factor for leptospirae was not secreted from the tissue cells into the medium, but was bound to the cells. Part of the enhancing factor, however, passed to the supernatant after cell extraction by freezing and thawing and did not lose its activity after filtration through a 0.45 µm Millipore filter.

DISCUSSION

The growth-enhancing factor of the cells for leptospiral growth was demonstrated in two of three media tested. The third Eagle's medium, which was optimal for leptospiral growth, produced different results under various conditions. When Eagle's medium was supplemented with haemolysed serum, in medium alone or in association with cell lines Clone B or BHK 21, the leptospirae grew abundantly. The latter results are very similar to those obtained by Miller *et al.* (1966) in the culture of serotype *pomone* in Eagle's medium, with primary foetal bovine kidney and human embryonic skin muscle fibroblast cell cultures. Eagle's medium supplemented with non-haemolysed serum produced poor growth in medium alone. In association with cell line Clone B the growth was enhanced but inferior to that obtained in ME medium supplemented with haemolysed serum (with or without

tissue cells). In the two other media, 199 and LH, the leptospirae grew well and produced a rich yield only in association with cell cultures. It is worth emphasizing that in LH medium in the presence of tissue cells, leptospiral growth was abundant, whereas there was a complete lack of growth in medium alone. Serum of various mammals containing no more than 0.05 % haemoglobin actively inhibited leptospiral growth. Haemoglobin neutralized this inhibition (Lindenbaum & Eylan, 1971; Lindenbaum, 1972). Sheep's non-haemolysed serum, which does not favour leptospiral growth in Korthof's or Eagle's medium, supports rich leptospiral growth in LH medium with tissue cells. Concentrations of a 0.5 % hydrolysate of lactalbumin and 0.85 % NaCl in Korthof's medium inhibited the growth of virulent and non-virulent strains of *L. icterohaemorrhagiae*. Since these materials are natural ingredients of LH medium, they might be responsible for the lack of growth of leptospirae in this medium. Some differences were found between the effect of cell lines (Clone B, BHK 21, HK) and primary cell cultures (PMK, CEF) on enhancement of leptospiral growth. These differences may be due to the various growth and metabolic properties of the two kinds of cells tested.

The leptospiral growth-enhancing factor in the cell culture is either not secreted into the medium or is secreted in such small amounts that it does not permit leptospiral growth in the supernatant from cell cultures in LH medium. Our results were analogous to those obtained by Carski & Shepard (1961) relating to mycoplasma. They found that tissue culture medium, in the absence of cells, was not sufficient to grow mycoplasma even when the medium remained in contact with a HeLa monolayer for three days.

The activity of the leptospiral growth-enhancing factor might explain the phenomenon of the attachment of leptospirae to the cells, as shown by Miller *et al.* (1966). Our results indicate that leptospiral growth on the cell wall may be due to an unknown enhancing substance rather than to the deposition of mucopolysaccharide acid on the surface of fibroblastic tissue culture cells. Unlike the growth-enhancing factor for leptospirae, the mucopolysaccharide acid was secreted into the cell culture media (Morris & Goodman, 1960). However, our findings were obtained mainly in experiments with epithelial cells, whereas those of Miller *et al.* (1966) are related to fibroblastic cells. It seems that the growth-enhancing factor for micro-organisms in tissue cells was found in the growth of leptospirae and mycoplasma because of their slow propagation and metabolic characteristics, which, in contrast to other bacteria, do not cause rapid destruction of tissue cells.

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