The Growth of *Mycoplasma bovigenitalium* in Cell Cultures

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**SUMMARY**

A strain of *Mycoplasma bovigenitalium*, designated M 120, was grown, and produced a marked cytopathic effect (c.p.e.) in calf-, pig-, and monkey-kidney cell cultures. The c.p.e. was characterized by enlargement of the cells, the appearance of intracytoplasmic inclusions and partial destruction of the cell monolayers. A similar c.p.e. was produced in tissue-culture cells following the inoculation of mycoplasma 'toxins'.

Comparative growth studies of strain M 120 in calf-kidney cell cultures and in tissue-culture medium alone showed that the organism grew more readily in the presence of cells. The rate of virus production and appearance of c.p.e. of infectious bovine rhinotracheitis (I.B.R.) virus was delayed in cultures previously infected with the M 120 strain of mycoplasma as compared with normal cultures.

**INTRODUCTION**

Hayflick & Stinebring (1955) first reported the growth of human and avian strains of mycoplasmas in tissue-culture cells. It has since been reported that growth of mycoplasmas occurs: (a) Without production of visible cytopathic effect (c.p.e.)—(Robinson, Wichelhausen & Roizman, 1956; Rothblat, 1960; Pollock, Kenny & Syverton, 1960; Carski & Shepard, 1961), although it may result in depression of the growth of the tissue-culture cells (Kenny & Pollock, 1963); or (b) with the production of a visible c.p.e. of varying type and degree (Hayflick & Stinebring, 1960; Nelson, 1960; Casterjon-Diez, Fisher & Fisher, 1963; Rovozzo, Luginbuhl & Helmboldt, 1963; Grumbles, Hall & Cummings, 1964; Butler & Leach, 1964). During the course of investigation of outbreaks of bovine infertility strain M 120 of *Mycoplasma bovigenitalium* was isolated from vaginal swabs obtained from a heifer showing lesions of granular vulvovaginitis (Afshar, Stuart & Huck, 1966). This paper gives a detailed account of the growth and cytopathic effect of this strain in tissue-culture systems. The effect of concurrent infection of the tissue culture with a strain of I.B.R. virus and mycoplasmas is also described.

**METHODS**

**Organism.** The M 120 strain of *Mycoplasma bovigenitalium* (Afshar et al. 1966) was used throughout the experiments, the strain had previously been passaged twice in calf-kidney cell cultures (M 120 CK2). A stock suspension of M 120 was prepared by collecting the supernatant after freezing and thawing and light centrifugation (174g for 5 min.). The stock suspension contained $10^{8.3}-10^{6.7}$ organisms/ml.

**Preparation of mycoplasma 'toxins'**

(a) M 120 CK2 was filtrated through a gradocol membrane (19 µ a.p.d.) using the technique described by Elford (1931).

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(b) M 120CK2 was centrifuged at 120,000g for 2 hr. The supernatant was collected
and treated with Kanamycin as described by Pollock et al. (1960). Both the filtrate
and the supernatant preparations were shown to be free from mycoplasmas by culturing
on Edward agar medium (Edward & Fitzgerald, 1952).

Cell cultures. Primary calf-kidney and pig-kidney cell monolayers were prepared
in 2 oz. medical flats by the trypsinization method of Dulbecco & Vogt (1954) modified
by Youngner (1954). The cells were grown in Hanks saline containing 10 % (v/v)
inactivated calf serum, 0-5 % (w/v) lactalbumin hydrolysate, 0-01 % (w/v) yeast
extract to which had been added 100 units penicillin, 100 μg streptomycin, 100 units
polymixin B and 25 units mycostatin per ml. Primary monkey-kidney cell mono-
layers were obtained from the Wellcome Research Laboratories, Beckenham, Kent.
Secondary calf-kidney cell monolayers were prepared on glass coverslips (⅛ × ⅜ in.).
All cultures were shown to be free from any contaminant mycoplasmas by culturing
the fluids on Edward agar medium.

Infection of cell cultures. The primary calf-, pig-, and monkey-kidney cell monolayers
were washed with phosphate buffered saline and inoculated with 0-5 ml. of a stock
suspension of M 120 grown in Edward liquid medium. The organism was allowed to
adsorb on to the cells by incubation of the cultures at 37° for 30 min. The cultures were
then overlaid with Earle saline containing 2 % (v/v) inactivated horse serum, 0-5 %
(w/v) yeast extract and 100 units penicillin per ml. (E.Y.L.). Cultures were examined
daily for c.p.e. and harvested 10–14 days after inoculation. For cytological examina-
tion secondary calf-kidney cell monolayers were inoculated with 0-1, 0-2, 0-5 or 1-2 ml.
of the stock suspension of M 120CK2. Following adsorption the cultures were overlaid
with E.Y.L. to give a final volume of 1-2 ml. per culture. Cultures were examined daily
and the coverslips removed at varying intervals, fixed in Bouin fluid and stained with
haematoxylin and eosin. Calf-kidney cell monolayers grown on coverslips were also
inoculated with similar volumes of the ‘toxins’ preparations of M 120CK2.

Growth-curve studies. Primary calf-kidney cell monolayers grown in Thompson
bottles (each consisting of approximately 2-5×10^7 cells) were inoculated with 10 ml.
of the stock suspension of M 120CK2. This gave a mycoplasma:cell ratio in one experi-
ment of 1:8890 and in two further experiments of 1:56. After adsorption each culture
was overlaid with 170 ml. of E.Y.L. In order to study the growth of the mycoplasma in
tissue-culture medium alone, similar bottles, each containing 170 ml. of E.Y.L. without
penicillin were inoculated with 1-0 ml. of the stock suspension of M 120CK2. All
bottles were incubated at 37° and at varying intervals 0-5 ml. samples of the super-
natants were withdrawn, diluted in buffered saline and titrated by the drop method
described by Miles & Misra (1938) using Edward agar medium. After 48 hr incubation
at 37° in a moist atmosphere the colonies were counted and the number of organisms
per 1-0 ml. of the supernatant was calculated.

The growth of i.b.r. virus in calf-kidney cell cultures previously infected with mycoplasma.

A primary calf-kidney cell monolayer grown in a Thompson bottle was infected with
1-0 ml. of the stock suspension of M 120CK2 as described above. Forty hr later, the
supernatant was removed and the cell monolayer was washed with buffered saline.
Control (mycoplasma-free) and infected cultures were inoculated with 1-0 ml. of the
Oxford strain of i.b.r. virus (Dawson et al. 1962). The virus suspension had a titre of
10^6.8 tissue culture infecting doses (t.c.i.d. 50) per ml. Cultures were adsorbed and over-
laid as described above. At varying intervals 0-5 ml. of the supernatant from each
bottle was removed and assayed for virus content as described by Dawson et al. (1962). Samples from the cultures infected with mycoplasmas were first treated with Kanamycin (see above) in order to inhibit further growth of the mycoplasmas. The endpoints of virus titration were calculated using the method described by Kärber (1931).

**RESULTS**

Cytopathology of *Mycoplasma bovigenitalium* and mycoplasma 'toxins'. Calf-kidney cell monolayers grown on coverslips infected with 0·1 ml. of M120CK2 did not show any changes until 48 hr after infection when the cytoplasm of the cells became granular. The granules were perinuclear and their number had increased by 52 hr (Pl. 1, fig. 2). Compared with the uninfected controls (Pl. 1, fig. 1) there was a marked enlargement of the cells which became accentuated by 148 hr (Pl. 1, fig. 3) when the surviving cells were about 6 times as large as the controls. Most of the infected cells had become detached from the glass by this time. However, the detachment of the cell sheet was not complete even at 240 hr after inoculation when about 20–30% of the cells, all showing granulation of the cytoplasm, still remained attached on to the glass surface. Examination of the fixed and stained preparations 72 hr after infection showed numerous eosinophilic intracytoplasmic inclusions, each one surrounded by a halo (Pl. 2, fig. 5). The inclusions were irregular in size and shape with a sponge-like structure. The stained control calf-kidney cell monolayers (Pl. 2, fig. 4) showed very few cells bearing homogeneous cytoplasmic inclusions after an incubation period of 148 hr. The development of the complete cycle of c.p.e., i.e. granulation, enlargement and detachment of cells, was found to be related to the volume of inoculum as shown in Table 1.

<table>
<thead>
<tr>
<th>Time after inoculation (hr)</th>
<th><em>M. bovigenitalium</em> (10⁶ org./ml.) (ml.)</th>
<th>'Toxin' preparations (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0·1</td>
<td>0·2</td>
</tr>
<tr>
<td>24</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>G.E.</td>
<td>G.E.D.</td>
</tr>
<tr>
<td>72</td>
<td>G.E.D.</td>
<td>+</td>
</tr>
<tr>
<td>120</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>148</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>192</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>240</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

G = granulation; E = enlargement; D = detachment; + = complete cytopathic effect; — = no cytopathic effect.

Complete c.p.e. occurred 24 hr after inoculation of calf-kidney cell cultures only when a large volume (1·2 ml.) of mycoplasma 'toxins' was used (Pl. 2, fig. 6). The c.p.e. was incomplete when smaller volumes of 'toxins' were used (Table 1). When showing c.p.e. these cultures were found to be free of any mycoplasma by the culture of the supernatants on Edward medium. The addition of arginine (4·0 m./ml.) did not prevent the appearance of c.p.e. in cultures inoculated with either M120CK2 or mycoplasma 'toxins'.
The changes in infected pig- and monkey-kidney cell cultures produced by M120 CK~ were similar to those described for calf-kidney cells, except that the c.p.e. developed more rapidly in monkey-kidney cell cultures and led to total destruction of the cells within 120 hrs.

_Growth curve of Mycoplasma bovigenitalium in calf-kidney cell culture and tissue culture medium_

The growth curves of _Mycoplasma bovigenitalium_, strain M120 CK~, in the supernatant of calf-kidney cell cultures obtained by the colony count method (Fig. 1) resembled a bacterial growth curve. The doubling time during the logarithmic phase was about 2-2 hr. The maximum titres were reached 32–48 hr after inoculation by which time the cell-culture medium had become turbid. The turbidity, due both to the detachment of the cells and to the increase in the number of organisms, increased during the stationary and the decline phase. During the period of the experiments the pH, estimated using standard paper indicators, varied from 7-6–6-8.

In cell-free medium the growth of mycoplasma was irregular—frequently no viable organisms could be detected in undiluted samples (Table 2). However, in all the experiments the number of organisms increased, ranging from 63 to 158,500-fold. In one experiment (Table 2, Expt. 1) the growth in cell-free medium was similar to that in cell cultures. The pH of the medium increased from 7-6 to 8-8 during the course of the experiments.

_Growth of I.B.R. virus in mycoplasma-infected calf-kidney cell cultures._ In cultures previously infected with _Mycoplasma bovigenitalium_ the growth rate of I.B.R. virus was initially delayed although the final titre of virus produced did not differ significantly
Mycoplasmas in cell cultures

from that produced in mycoplasma-free cultures (Table 3). A delay of about 25 hr in the appearance of c.p.e. in the cultures infected with mycoplasma was also noted. Examination of stained cultures showed that the number of I.B.R. virus intranuclear inclusions was decreased in mycoplasma-infected cultures and they did not appear until somewhat later after inoculation. In cultures infected with both the mycoplasma and I.B.R. virus there were more enlarged cells with 3–6 nuclei per cell than in control virus cultures.

Table 2. Growth of Mycoplasma bovigenitalium in cell-free tissue-culture medium

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
<th>Expt. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.7</td>
<td>1.8</td>
<td>2.4</td>
<td>1.7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>3.6</td>
<td>1.8</td>
<td>3.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>6.8</td>
<td>0</td>
<td>2.8</td>
<td>3.0</td>
<td>2.3</td>
</tr>
<tr>
<td>32</td>
<td>8.0</td>
<td>0</td>
<td>0</td>
<td>3.9</td>
<td>n.t.</td>
</tr>
<tr>
<td>48</td>
<td>6.8</td>
<td>0</td>
<td>2.0</td>
<td>2.7</td>
<td>3.1</td>
</tr>
<tr>
<td>56</td>
<td>6.4</td>
<td>0</td>
<td>2.1</td>
<td>4.8</td>
<td>n.t.</td>
</tr>
<tr>
<td>72</td>
<td>5.9</td>
<td>0</td>
<td>3.0</td>
<td>4.9</td>
<td>2.0</td>
</tr>
<tr>
<td>80</td>
<td>6.1</td>
<td>0</td>
<td>0</td>
<td>4.8</td>
<td>3.1</td>
</tr>
<tr>
<td>96</td>
<td>5.4</td>
<td>0</td>
<td>2.8</td>
<td>n.t.</td>
<td>3.7</td>
</tr>
<tr>
<td>120</td>
<td>5.1</td>
<td>4.6</td>
<td>0</td>
<td>n.t.</td>
<td>4.3</td>
</tr>
<tr>
<td>144</td>
<td>4.7</td>
<td>7.3</td>
<td>0</td>
<td>n.t.</td>
<td>5.9</td>
</tr>
<tr>
<td>168</td>
<td>5.3</td>
<td>7.0</td>
<td>0</td>
<td>n.t.</td>
<td>5.65</td>
</tr>
<tr>
<td>176</td>
<td>5.9</td>
<td>7.1</td>
<td>3.0</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>192</td>
<td>5.9</td>
<td>n.t.</td>
<td>4.9</td>
<td>n.t.</td>
<td>5.4</td>
</tr>
<tr>
<td>200</td>
<td>6.0</td>
<td>5.8</td>
<td>4.9</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>216</td>
<td>5.6</td>
<td>n.t.</td>
<td>4.9</td>
<td>n.t.</td>
<td>5.6</td>
</tr>
<tr>
<td>240</td>
<td>n.t.</td>
<td>4.7</td>
<td>4.5</td>
<td>n.t.</td>
<td>5.4</td>
</tr>
</tbody>
</table>

n.t. = not tested.

Table 3. Growth of I.B.R. virus in mycoplasma-infected and normal calf-kidney cell cultures

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Infected</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log. tissue culture infecting dose 50 I.B.R. virus per ml.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.5</td>
<td>3.25</td>
</tr>
<tr>
<td>8</td>
<td>3.25</td>
<td>1.75</td>
</tr>
<tr>
<td>24</td>
<td>3.25</td>
<td>4.75*</td>
</tr>
<tr>
<td>34</td>
<td>3.25</td>
<td>5.5</td>
</tr>
<tr>
<td>49</td>
<td>4.5*</td>
<td>5.2</td>
</tr>
<tr>
<td>60</td>
<td>4.5</td>
<td>5.5</td>
</tr>
<tr>
<td>72</td>
<td>5.0</td>
<td>5.5</td>
</tr>
<tr>
<td>80</td>
<td>5.5</td>
<td>5.75</td>
</tr>
</tbody>
</table>

* Appearance of I.B.R. virus c.p.e.

DISCUSSION

The only previously recorded attempt on the growth of Mycoplasma bovigenitalium in cell cultures is that of Butler & Leach (1964) who reported that this organism failed to grow in a human cell line (HEp. 2). It has been shown that a related strain, M 120, grew and produced a marked c.p.e. in primary calf-, pig-, and monkey-kidney cell
cultures. The c.p.e. was characterized by enlargement of the cells, appearance of intra-
cytoplasmic inclusions and partial destruction of the cell monolayers. These charac-
teristics resemble those described for other types of mycoplasma. The increase in the
granularity of cell monolayers and appearance of the cytoplasmic inclusion bodies
have been observed by Hayflick & Stonebring (1955, 1960), Shepard (1958) and
Rovozzo et al. (1963). Based on their histological observations, these workers sug-
gested that the inclusions and granulations were intracellular forms of the organism.
Barile, Malizia & Riggs (1962) and Clyde (1963) using fluorescent antibody techniques
showed that the cytoplasmic granulations and inclusions, produced by contaminant
mycoplasmas or Eaton agents, were aggregates of the organisms. Similar intracyto-
plasmic inclusions to those described by Shepard (1958) have been observed both in
cells infected with M. bovigenitalium and the mycoplasma ‘toxins’, suggesting that
from the appearance of cytoplasmic inclusions or granules the growth of mycoplasmas
in cell cultures does not necessarily imply intracellular existence. From the cytological
and growth studies of M. bovigenitalium, M120 strain, both in calf-kidney cell cultures
and cell-free tissue-culture medium, it is reasonable to assume that this organism grew
extracellularly and caused c.p.e. when sufficient toxic substances had been produced
or when the medium had been depleted of substances necessary for tissue-culture cell
maintenance. These suggestions are supported by Powelson (1961), Kenny & Pollock
(1963) and Rouse, Bonifas & Schlesinger (1963) who showed that the multiplication of
contaminant mycoplasmas in tissue culture medium depleted the medium of L-
arginine which subsequently resulted in changes in the tissue-culture cells. Kraemer
(1964) showed that the depletion of L-arginine was in fact due to the production of the
mycoplasma ‘toxins’. Whether or not the toxin produced results in enzymic degrada-
tion of L-arginine is not yet clear. In contrast to Kraemer’s findings that the addition
of L-arginine to the cell-culture medium prevented the cytotoxic effects, the c.p.e.
caused by M. bovigenitalium or the ‘toxins’ was not prevented by the addition of 4
times the normal concentration of L-arginine to the medium.

The growth of Mycoplasma bovigenitalium in cell-free tissue-culture medium was
irregular compared to the growth in the presence of calf-kidney cells, suggesting that
the cells or cell residues were utilized by mycoplasmas and resulted in more regular
growth. The regular growth in the presence of cells is comparable with the growth of
other mycoplasmas studied in synthetic media (Kelton, 1960; Butler & Knight, 1960).
The growth of mycoplasmas in cell-free tissue-culture medium has been reported by
Nelson (1960) and Fabricant, Fabricant & van Demark (1964). Fabricant et al. (1964)
found that the addition of ribonucleotides and deoxyribonucleotides to ‘199’ medium
improved the growth of M. bovigenitalium. This supports our suggestion that the
regular growth of a related strain (M 120) in calf-kidney cell cultures was due to the
utilization of cell residues.

According to Rouse et al. (1963) and Butler & Leach (1964) the growth of adeno-
virus and myxovirus in cell cultures contaminated with mycoplasmas was inhibited.
Rouse and his colleagues demonstrated that inhibition of virus multiplication was
directly caused by the depletion of arginine from the medium by the mycoplasmas, and
the addition of arginine increased the virus yield. According to Schulze & Schlesinger
(1963) the growth of the arbovirus—Dengue-type 2—was not affected in cell cultures
contaminated with mycoplasma. It has been shown that growth of I.B.R. virus, a
herpesvirus (Armstrong, Pereira & Andrews, 1961), proceeded initially at a slower rate
and that the onset of the visible c.p.e. was delayed in the presence of concurrent infection with Mycoplasma bovigenitalium. This finding is of particular interest as I.B.R. virus has been shown to be identical to infectious pustular vulvovaginitis (I.P.V.) virus (McKercher, Straub, Saito & Wada, 1959; Gillespie, McEntee, Kendrick & Wagner, 1959) which is a natural pathogen of the genitalia of cattle as is M. bovigenitalium (Edward & Fitzgerald, 1952). Kendrick, Gillespie & McEntee (1958) showed that I.P.V. virus caused lesions in a similar situation to that described for M. bovigenitalium (Afshar et al. 1966).

I am indebted to Dr A. B. Paterson, Messrs R. A. Huck and P. S. Dawson for valuable critical discussion. I am grateful to Miss B. V. Cook and Mr P. Napthine for their technical assistance.

I thank the Wellcome Trust and the Pahlavi Fund for financial support.

REFERENCES


EXPLANATION OF PLATES

PLATE 1

Fig. 1. Uninfected calf-kidney cell monolayer, unfixed. × 140.

Fig. 2. Mycoplasma bovigenitalium-infected calf-kidney cell monolayer (0·1 ml.), unfixed, 52 hr after infection, showing perinuclear granules. × 140.

Fig. 3. M. bovigenitalium-infected calf-kidney cell monolayer (0·1 ml.), unfixed, 148 hr after infection, showing enlarged cells. × 140.

PLATE 2

Fig. 4. Uninfected calf-kidney cell monolayer, fixed, and stained with haematoxylin and eosin. × 552.

Fig. 5. Mycoplasma bovigenitalium-infected calf-kidney cell monolayer, 72 hr after infection, fixed and stained with haematoxylin and eosin, showing cytoplasmic inclusions surrounded by haloes. × 552.

Fig. 6. Calf-kidney cell monolayer inoculated with mycoplasma ‘toxins’, unfixed, 24 hr after inoculation. × 140.
Plate 1

A. AFSHAR

(Facing p. 110)