The Growth of *Mycoplasma bovis* in Cell Cultures

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SUMMARY

A strain of *Mycoplasma bovis*, 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 mycoplasma 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 bovis* 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 bovis* (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 $10^{3.3}$ – $10^{6.7}$ 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 monolayers were obtained from the Wellcome Research Laboratories, Beckenham, Kent. Secondary calf-kidney cell monolayers were prepared on glass coverslips ($\frac{1}{4} \times \frac{7}{8}$ 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 examination secondary calf-kidney cell monolayers were inoculated with 0.1, 0.2, 0.5 or 1.2 ml. of the stock suspension of M120CK2. 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 M120CK2.

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 M120CK2. This gave a mycoplasma:cell ratio in one experiment 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 M120CK2. All bottles were incubated at 37° and at varying intervals 0.5 ml. samples of the supernatants 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 M120CK2 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 $10^{5.5}$ tissue culture infecting doses (t.c.i.d. 50) per ml. Cultures were adsorbed and overlaid 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 Kana-mycin (see above) in order to inhibit further growth of the mycoplasmas. The end-points of virus titration were calculated using the method described by Kärber (1931).

RESULTS

Cytopathology of Mycoplasma bovis genitalium 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 1. *Cytopathology of Mycoplasma bovis genitalium and mycoplasma 'toxins' in calf-kidney cell cultures grown on coverslips*

Time after inoculation (hr)	<i>M. bovis genitalium</i> (10 ⁶ org./ml.) (ml.)				'Toxin' preparations (ml.)			
	0.1	0.2	0.5	1.2	0.1	0.2	0.5	1.2
24	—	G.E.	G.E.	G.E.D.	—	E	G.E.	G.E.D.
48	G.E.	G.E.	G.E.D.	+	—	E	G.E.	+
52	G.E.	G.E.D.	+	+	—	E	G.E.	+
72	G.E.D.	+	+	+	—	E	G.E.	+
120	+	+	+	+	—	E	G.E.	+
148	+	+	+	+	—	E	G.E.	+
192	+	+	+	+	—	E	G.E.	+
240	+	+	+	+	—	E	G.E.	+

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 CK2 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 bovisgenitalium in calf-kidney cell culture and tissue culture medium

The growth curves of *Mycoplasma bovisgenitalium*, strain M120CK2, 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

Fig. 1. The growth of *Mycoplasma bovisgenitalium* in calf-kidney cell cultures. All cultures measured by colony count of viable particles in the supernatants, ●, ■, ▲ are 3 experiments.

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 bovisgenitalium* the growth rate of I.B.R. virus was initially delayed although the final titre of virus produced did not differ significantly

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 bovis genitalium in cell-free tissue-culture medium*

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

n.t. = not tested.

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

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

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

DISCUSSION

The only previously recorded attempt on the growth of *Mycoplasma bovis genitalium* 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 intracytoplasmic inclusions and partial destruction of the cell monolayers. These characteristics 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 & Stinebring (1955, 1960), Shepard (1958) and Rovozzo *et al.* (1963). Based on their histological observations, these workers suggested 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 intracytoplasmic inclusions to those described by Shepard (1958) have been observed both in cells infected with *M. bovis genitalium* 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. bovis genitalium*, 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 degradation 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. bovis genitalium* 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 bovis genitalium* 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. bovis genitalium*. 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 adenovirus 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 bovis genitalium*. 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. bovis genitalium* (Edward & Fitzgerald, 1952). Kendrick, Gillespie & McEntee (1958) showed that I.P.V. virus caused lesions in a similar situation to that described for *M. bovis genitalium* (Afshar *et al.* 1966).

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Uninfected calf-kidney cell monolayer, unfixed. $\times 140$.

Fig. 2. *Mycoplasma bovis genitalium*-infected calf-kidney cell monolayer (0.1 ml.), unfixed, 52 hr after infection, showing perinuclear granules. $\times 140$.

Fig. 3. *M. bovis genitalium*-infected calf-kidney cell monolayer (0.1 ml.), unfixed, 148 hr after infection, showing enlarged cells. $\times 140$.

PLATE 2

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

Fig. 5. *Mycoplasma bovis genitalium*-infected calf-kidney cell monolayer, 72 hr after infection, fixed and stained with haematoxylin and eosin, showing cytoplasmic inclusions surrounded by haloes. $\times 552$.

Fig. 6. Calf-kidney cell monolayer inoculated with mycoplasma 'toxins', unfixed, 24 hr after inoculation. $\times 140$.