SHORT ARTICLE

The prolonged persistence of mycoplasmas in culture

D. TAYLOR-ROBINSON and J. BEHNKE

Division of Sexually Transmitted Diseases, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ

Summary. *Mycoplasma fermentans* organisms in medium containing 20% horse serum multiplied to 10^7 – 10^{10} ccu/ml within 5 days at 37°C and were dead usually after 9 days. There was no growth in medium without serum, nor in such medium with the addition of cholesterol or palmitic acid or both, but in some experiments addition of bovine plasma albumin (BPA) increased the number of organisms by up to 1000-fold and some remained viable for up to 84 days. BPA and cholesterol or BPA, palmitic acid and cholesterol more often enhanced growth, in terms of the maximum number of organisms and their survival, than did the addition of BPA alone. The maximum number of organisms in such supplemented serum-free media was usually at least ten-fold less than in medium with horse serum, but some organisms remained viable for up to 131 days. Survival of *Ureaplasma urealyticum* was also longer in the supplemented serum-free medium than in standard horse-serum medium. The possible factors affecting persistence of mycoplasmas in culture are discussed in relation to these observations.

Introduction

Serum globulins in media used to culture mycoplasmas may bind to them and react with antiglobulins when the mycoplasmas are employed in immunological tests, producing non-specific results (Windsor and Thornton, 1973). Therefore, to produce mycoplasmal antigens that were less contaminated than usual by growth-medium components, we attempted to propagate mycoplasmas in medium devoid of horse serum. Razin and Tully (1970) and Edward (1971) had shown that this could be achieved by replacing the serum with a combination of bovine serum albumin, palmitic acid and cholesterol, the latter being especially important. We therefore used these substances, singly or in combination, and because of our interest in producing *Mycoplasma fermentans* antigens, most experiments were conducted with this mycoplasma. We were concerned primarily with the yield of mycoplasma organisms but some of the modified serum-free media not only allowed multiplication but also greatly increased the persistence of viable organisms in the incubated cultures. The details of this observation and other experimental results are presented here.

Materials and methods

Mycoplasmas and inocula

In almost all experiments, the type strain of *M. fermentans* (PG 18) was used. In addition, another strain of this mycoplasma and one of *Ureaplasma urealyticum* were used after their isolation from the human urogenital tract in the standard horse-serum-containing liquid medium described below. In early experiments, the cultures used for inocula were diluted in PPLO broth base

at least 1000-fold so that the concentration of horse serum in the media was negligible (<0.002%) and in later experiments the inocula comprised organisms grown in horse-serum-free medium. The inocula contained approximately 5×10^7 colour-changing units (ccu)/ml of *M. fermentans* and 5×10^6 ccu/ml of *U. urealyticum*.

Medium

Mycoplasma liquid medium has been described before (Manchee and Taylor-Robinson, 1968). In brief, it consisted of 7 parts of PPLO broth (Difco), 1 part of a 25% w/v extract of fresh yeast (Distillers Co. Ltd), 2 parts of unheated horse serum (Wellcome), phenol red 0.002%, thallium acetate 0.05% and penicillin G 1000 units/ml. The medium was supplemented (i) with glucose 1.0% w/v for *M. fermentans* and adjusted to pH 7.8, or (ii) with urea 0.1% for *U. urealyticum* and adjusted to pH 6.5.

Substitutes for horse serum

Cholesterol (Sigma) 0.1 g was added to 1.5 ml of absolute alcohol, which was brought to boiling and then added to 10 ml of boiling distilled water, the boiling then being continued for 10 min. Palmitic acid (Sigma) was dissolved also in boiling alcohol. Each of these substances and globulin-free bovine plasma albumin (BPA; Sigma) was added to horse-serum-free medium in the concentrations given in the text or tables.

Experimental procedure

In each experiment, the multiplication and the persistence of organisms in horse-serum-containing medium were compared with those in media with the various substitutes. Volumes of 50 ml were prepared, inoculated

with organisms (1.0 ml), incubated at 37°C, and 0.2-ml volumes withdrawn at various intervals and titrated for viable organisms as indicated below.

Quantitation of viable mycoplasmas and identification

The number of viable organisms was estimated by making serial ten-fold dilutions in mycoplasmal standard liquid medium containing horse serum. The end-point of the titration was the highest dilution at which the colour of the medium changed (red to yellow for *M. fermentans* and yellow to red for *U. urealyticum*) after incubation at 37°C and this was considered to contain one ccu. Organisms were identified by the disk growth-inhibition technique (Clyde, 1964) with specific rabbit antiserum.

Results

Growth of M. fermentans in medium with and without horse serum

The results of experiments with *M. fermentans* are

shown in table I. In medium containing horse serum 20% v/v, maximum titres of 10^7 – 10^{10} ccu/ml were achieved regularly within about 5 days, but viable organisms were usually not detected after 9 days. In medium containing horse serum 0.2%, the maximum titres tended to be less, 10^5 – 10^8 ccu/ml being achieved within 4–11 days, and viable organisms were not detected after 14 days. In the absence of horse serum, there was no multiplication of inoculated organisms and they disappeared from half of the cultures within one day and from all of them within 5 days.

Growth of M. fermentans in serum-free medium with additives

The addition of either cholesterol (1–20 µg/ml) or palmitic acid (1–5 µg/ml), or both substances, to horse-serum-free medium did not result in any multiplication of *M. fermentans* organisms which, moreover, disappeared from most cultures within 4 days of incubation (table II). However, in five experiments in which the horse serum was replaced by 0.1–0.5% BPA, the organisms multiplied two-, ten-fold and 1000-fold respectively, and persisted

Table I. Growth of *M. fermentans* in medium with and without horse serum

Horse serum	Number of experiments	Number of experiments in which multiplication occurred	Maximum titre (ccu/ml)	Days to achieve maximum titre	Persistence (days)
20%	14	14	10^7 – 10^{10}	4–6	≤9
0.2%	12	12	10^5 – 10^8	4–11	≤14
None	15	0	—	—	≤5

Table II. Growth of *M. fermentans* in horse-serum-free medium with additives

Additives	Number of experiments	Number of experiments in which multiplication occurred	Maximum titre (ccu/ml)	Maximum fold increase	Days to achieve maximum titre	Persistence (days)
Cholesterol 1–20 µg/ml	6	0	—	—	—	0–9
Palmitic acid 1–5 µg/ml	3	0	—	—	—	0–3
Cholesterol and palmitic acid	14	0	—	—	—	0–11
BPA 0.1–0.5%	5	2	10^3 , 10^5	10^1 , 10^3	13, 28	5–84
Cholesterol 1 µg/ml and BPA 0.1%	1	1	10^8	10^6	12	≥25
Palmitic acid 1 µg/ml and BPA 0.1%	1	1	10^5	10^2	28	≥75
Cholesterol 1 µg/ml						
palmitic acid 1 µg/ml BPA 0.5%	7	7	10^5 – 10^9	10^2 – 10^5	6–12	≥17–115
Cholesterol 1 µg/ml						
palmitic acid 1 µg/ml BPA 0.1%	8	8	10^5 – 10^8	10^2 – 10^5	5–13	≥25–131

in four for 20–84 days. Furthermore, the combination of cholesterol 1 µg/ml and BPA 0.1%, or palmitic acid 1 µg/ml and BPA 0.1%, resulted in multiplication of organisms (10⁶-fold and 10²-fold, respectively) and their prolonged persistence in culture (≥25 days and ≥75 days, respectively).

In exploratory experiments, palmitic acid, together with cholesterol 1 µg/ml and BPA 0.1%, was equally growth-promoting at 0.1, 1 or 10 µg/ml, but 100 µg/ml was inhibitory. Palmitic acid at 1 µg/ml was used, therefore, in subsequent combinations. Similarly, in two experiments, cholesterol in combination with palmitic acid 1 µg/ml and BPA 0.1% was equally growth-promoting at 1 and 10 µg/ml, but 100 µg/ml was inhibitory. Cholesterol at 1 µg/ml was used, therefore, in subsequent combinations. As shown in table II, the combination of BPA 0.5% with cholesterol and palmitic acid 1 µg/ml each had an enhancing effect about as great as did BPA 0.1% in similar combination. Although the organisms did not usually reach the same maximum titre in these media as in medium with horse serum, they persisted in a viable form for up to 131 days. These organisms and those from some of the other cultures after prolonged survival were identified and confirmed as *M. fermentans*.

Final pH of *M. fermentans* cultures

The pH of *M. fermentans* cultures containing horse serum 20% diminished usually to a minimal value of 5.6 after about 6–7 days of incubation, with little subsequent persistence of viability (table I). The serum-free medium with the substitution of cholesterol, palmitic acid and BPA exhibited a similar reduction in pH after about 14 days but with prolonged persistence of viability.

Growth of *U. urealyticum* in serum-containing and serum-free medium

In two experiments, the results of which were similar, *U. urealyticum* multiplied only moderately but persisted in medium in which the horse serum was replaced by cholesterol 1 µg/ml, palmitic acid 1 µg/ml and BPA 0.1%. Thus, in one experiment, the organisms attained a maximum titre of 10⁷ ccu/ml after 16 h in medium containing horse serum 20% and no viable organisms remained after 3 days. In the modified serum-free medium, the organisms reached only 10⁵ ccu/ml after 6 days but persisted for at least 8 days.

Discussion

Cholesterol and BPA were first shown to be important for the growth of mycoplasmas by Edward and Fitzgerald (1951) and Edward (1953), and the requirement for cholesterol was again emphasised by Razin and Tully (1970) and Edward (1971). However, the procedures they used to determine growth did not allow them to assess persistence of viable mycoplasmas. Indeed, persistence

for as long as we have observed it to occur in cultures incubated at 37°C is a phenomenon not reported previously. The reasons for some of the organisms remaining viable for much longer, in some cases for more than 4 months, in the supplemented serum-free media than in the standard medium are not clear. Altucci *et al.* (1969) considered that the toxicity of the metabolic by-products of growth was the reason for the relatively short life-span of mycoplasmas in culture. In our experiments, the maximum titres attained in the supplemented media were usually ten-fold less than in medium with horse serum, so that the toxic effect of metabolic products would probably be reduced and this might account for longer survival. However, this factor seems unlikely to be the only one because *M. fermentans* organisms in horse-serum-free medium without additives neither multiplied nor even survived beyond a few days, despite the necessarily minimal metabolic products. A fall in pH of the medium may contribute to the failure of the organisms to survive more than a few days in serum-containing cultures. However, the survival of organisms for months in serum-free medium could not be attributed to greater alkalinity because they were subjected to similar acidic conditions.

The composition of the mycoplasmal membrane is influenced by the growth-medium components and also by the ageing process. For example, in cultures of *M. hominis* it has been shown that there is a decrease in the ratio of lipid to protein in the mycoplasmal membranes as the cultures age, so affecting the activity of membrane-associated enzymes and transport systems (Rottem and Greenberg, 1975). It is suggested that there is a continuous increase in the packing density of protein molecules on the inner surface of the mycoplasmal cell membrane (Amar *et al.*, 1976). It is possible that the components added to the serum-free medium in our experiments were incorporated in the mycoplasmal membrane in a different manner or proportion from those of the serum-containing medium, affecting the membrane so as to make the organisms more thermostable. However, greater stability to thermal decay as an explanation for longer survival has not been assessed by testing the organisms at temperatures greater than 37°C and biochemical analysis of membranes from organisms grown in the horse-serum-containing and chemically defined media are needed to determine whether the composition of membranes differs under these different cultural conditions.

The extent to which the phenomenon of prolonged survival in culture might be seen with other mycoplasmas is not known, although the preliminary results with *U. urealyticum* suggest that it may be more widespread and worth exploring. The ability of mycoplasmas to multiply and then survive for prolonged periods if the conditions are favourable may be one of the factors contributing to the persistence of mycoplasmal infections *in vivo*, some *M. hominis* and ureaplasma infections of the male urogenital tract, for example, persisting for more than 2 years in the absence of sexual contact (Holmes *et al.*, 1974).

REFERENCES

- Altucci P, Varone G L, Catalano G 1969 On a mycoplasma inhibitor obtained from disrupted mycoplasma cells: isolation and some biological properties. *Chemotherapy* **14**:258–263.
- Amar A, Rottem S, Kahane I, Razin S 1976 Characterization of the mycoplasma membrane proteins. VI. Composition and disposition of proteins in membranes from aging *Mycoplasma hominis* cultures. *Biochimica et Biophysica Acta* **426**:258–270.
- Clyde W A 1964 *Mycoplasma* species identification based upon growth inhibition by specific antisera. *Journal of Immunology* **92**:958–965.
- Edward D G ff, Fitzgerald W A 1951 Cholesterol in the growth of organisms of the pleuropneumonia group. *Journal of General Microbiology* **5**:576–586.
- Edward D G ff 1953 A difference in growth requirements between bacteria in the L-phase and organisms of the pleuropneumonia group. *Journal of General Microbiology* **8**:256–262.
- Edward D G ff 1971 Determination of sterol requirement for *Mycoplasmatales*. *Journal of General Microbiology* **69**:205–210.
- Holmes M J, Furr P M, Taylor-Robinson D 1974 The persistence of mycoplasmas in the urogenital tract of men in the Antarctic. *Journal of Hygiene* **72**:355–363.
- Manchee R J, Taylor-Robinson D 1968 Haemadsorption and haemagglutination by mycoplasmas. *Journal of General Microbiology* **50**:465–478.
- Razin S, Tully J G 1970 Cholesterol requirement of mycoplasmas. *Journal of Bacteriology* **102**:306–310.
- Rottem S, Greenberg A S 1975 Changes in composition, biosynthesis, and physical state of membrane lipids occurring upon aging of *Mycoplasma hominis* cultures. *Journal of Bacteriology* **121**:631–639.
- Windsor G D, Thornton G A 1973 Avoidance of non-specific agglutination of *Mycoplasma gallisepticum* by the use of a globulin-free antigen. *Veterinary Record* **92**:147–148.