GROWTH AND PATHOGENICITY STUDIES OF *MYCOPLASMA GALLISEPTICUM* IN CHICKEN TRACHEAL ORGAN CULTURES

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SEVERAL mycoplasma species cause respiratory illnesses in their respective animal hosts (Cottew and Leach, 1969; Fabricant, 1969; Tully, 1969). Although the pathology of these diseases has for the most part been studied extensively, there is relatively little known about their pathogenesis. Recently it has been shown that organ cultures may be valuable for studying the pathogenicity of mycoplasmas (Butler, 1969; Collier, Clyde and Denny, 1969; Cherry and Taylor-Robinson, 1970a). In particular, chicken tracheal organ cultures can be produced in large numbers, so that the effect of mycoplasmas on them is easy to evaluate quantitatively (Cherry and Taylor-Robinson, 1970a). We used this system previously to study the growth and possible virulence factors of *Mycoplasma mycoides* var. *capri* (*M. capri*) (Cherry and Taylor-Robinson, 1970b). In addition, we have shown that several strains of *M. gallisepticum* grow and adversely affect the ciliated epithelium of chicken tracheal organ cultures (Cherry and Taylor-Robinson, 1970a). The present report concerns attempts to elucidate mechanisms of pathogenesis of the J1 strain of *M. gallisepticum* in this organ culture system.

MATERIALS AND METHODS

Organ cultures. The method of preparation has been described in detail elsewhere (Cherry and Taylor-Robinson, 1970a). In brief, tracheas removed from chicken embryos 19–20 days old were cut into ring sections $\frac{1}{2}$ –1 mm thick and placed in screw-capped tubes each containing 1 ml of medium. These organ cultures were incubated at 37°C in a roller drum.

Media. The serum-free medium for organ cultures was Eagle's basal medium containing 0.05M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer, adjusted to pH 7.0 with 0.2N-NaOH. Either 200 units of penicillin G or 500 µg of ampicillin was added per ml of medium. Medium for the growth and titration of *M. gallisepticum* has been described previously (Manchee and Taylor-Robinson, 1968). Thallium acetate was omitted from the medium used to propagate organisms for organ-culture inoculation.

Mycoplasma test strain. Strain J1 of *M. gallisepticum* was received as a field isolate from Dr D. H. Roberts. It was cloned three times on agar medium and then passed once in liquid medium. Pools prepared from the next four thallium-free medium passages and stored at –70°C were used throughout this study.

Titration of mycoplasma. Specimens were diluted in serial 10-fold steps in mycoplasma liquid medium containing phenol red and 0.1 per cent. glucose. The greatest dilution at

Received 9 Nov. 1970; accepted 22 Jan. 1971.

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which a colour change occurred during incubation at 37°C for 7 days was the end-point of the titration and was considered to contain one colour-changing unit (CCU).

Ciliary activity. The observation and grading of ciliary activity have been described in full elsewhere (Cherry and Taylor-Robinson, 1970a). In the present study, assessments of

FIG. 1.—The multiplication and cilia-stopping effect (CSE) of *M. gallisepticum* in chicken tracheal organ cultures after inoculation of a large (●—●) and a smaller (○—○) number of organisms. Observations on uninoculated control organ cultures are plotted thus: ▲—▲.

the extent and vigour of ciliary activity were combined and recorded as percentage ciliary activity. In all experiments, a group of five cultures was used for each factor that was investigated.

Reagents. Purified neuraminidase (from *Vibrio cholerae*; 500 units per ml) was purchased from Koch-Light Laboratories Ltd, Colnbrook, England. Beef liver catalase was obtained from the Boehringer Corporation Ltd, London, as a crystalline suspension in water (20 mg per ml) saturated with thymol; two lots were used.

RESULTS

Mycoplasma growth and its effect on ciliary activity

M. gallisepticum (strain J1) organisms increased in number in the chicken tracheal organ-culture system, attaining viability titres of 10^6 – 10^7 CCU per ml of medium. This growth was about 100-fold less than that in liquid mycoplasma medium. *M. gallisepticum* regularly inhibited ciliary activity, but the rapidity of this occurrence varied from one experiment to another. The cilia-stopping effect (CSE) corresponded relatively closely to histological evidence of damage to the ciliated epithelial cells, although some ciliated cells were still present in cultures in which ciliary activity had ceased.

TABLE I

The lack of relation between the concentration of M. gallisepticum inoculated into chicken tracheal organ cultures and the time taken to produce a 50 per cent. reduction of ciliary activity

Series	Expt no.	Dose (CCU) of <i>Mycoplasma gallisepticum</i> added to culture	Time (days) to 50 per cent. reduction of ciliary activity
Large inoculum	1	10^6	6.3
	2	10^6	3.3
	3	10^6	4.9
			Mean 4.8
Small inoculum	1	10^3	4.2
	2	10^2	4.4
	3	10^4	5.5
			Mean 4.7

After the introduction of a small number of organisms (10^3 CCU per ml medium) into the cultures, it took 5 days for them to attain a titre of 10^6 CCU per ml (fig. 1). However, loss of ciliary activity occurred about as rapidly as after the introduction of a 1000-fold greater concentration of organisms. The lack of relation between the concentration of organisms inoculated and the time required for reduction of ciliary activity is emphasised further by the results presented in table I. Although the initial concentration of organisms in the cultures differed by as much as 100- to 10,000-fold, there was little difference in the time required for 50 per cent. reduction in ciliary activity.

Mycoplasma growth and CSE

Effect of catalase. The haemolysin of several mycoplasmas, including *M. gallisepticum*, is hydrogen peroxide, and it has been suggested that peroxide may be a virulence factor in mycoplasma infections (Somerson, Walls and Chanock, 1965; Cohen and Somerson, 1967; Cole, Ward and Martin, 1968; Sobeslavsky and Chanock, 1968; Sobeslavsky, Prescott and Chanock, 1968). In order to determine whether peroxide was responsible for the CSE of *M. gallisepticum*, catalase was added to the organ-culture medium. It did not affect the ciliary activity of uninfected cultures nor inhibit the multiplication of

organisms in *M. gallisepticum*-infected cultures. Furthermore, catalase did not protect the cilia from the usual adverse effect of *M. gallisepticum*, as the results of three experiments presented in table II indicate. Indeed, catalase in higher concentrations potentiated the CSE.

Effect of glucose. Eagle's basal medium contains 0.1 per cent. glucose. We have noted previously (Cherry and Taylor-Robinson, 1970*b*) that the addition of 0.1–1.0 per cent. glucose to the organ-culture medium protected the ciliated epithelium against damage caused by *M. mycoides* var. *capri*. Because of this we carried out two similar experiments with *M. gallisepticum*-infected cultures. The results are recorded in table III. The addition of 1.0 per cent. glucose delayed the CSE of *M. gallisepticum*, whereas 0.1 per cent. glucose had no appreciable effect.

TABLE II
The effect of catalase on the CSE of M. gallisepticum in chicken tracheal organ cultures

Expt no.	Catalase		Initial concentration of mycoplasmas (CCU per ml)	Time for organ cultures in the stated medium to show 50 per cent. reduction in ciliary activity (days)	
	Lot no.	Concentration (mg per tube)		Eagle's medium only	Eagle's medium with catalase
1	1	0.5	10 ⁴	4.6	5.2
2	2	1.0	10 ⁶	7.5	4.2
3	2	2.0	10 ⁵	6.8	3.3

Effect of neuraminidase. Chicken tracheal epithelial cells adsorb to colonies of *M. gallisepticum* and this cytoadsorption can be prevented by first treating the cells with neuraminidase (Sobeslavsky *et al.*, 1968; Manchee and Taylor-Robinson, 1969). Cytoadsorption could be an important factor in pathogenicity if a close association between mycoplasma and cell were necessary to transmit a toxic factor to the ciliated epithelium. To test this possibility, in three experiments we included neuraminidase in the culture medium in amounts that completely inhibited adsorption of chicken erythrocytes to colonies of *M. gallisepticum*, but, as noted in table IV, the time for 50 per cent. reduction in ciliary activity was not prolonged. In a further experiment both catalase and neuraminidase were added to the same cultures. The result of this experiment was similar to that in which only catalase was added.

Attempts to demonstrate a toxin

In an attempt to determine whether medium in which *M. gallisepticum* had grown contained a toxic substance that would adversely affect ciliary activity two similar experiments were done. Tetracycline (25 µg per ml) was added to

the medium of some organ cultures which were then seeded with 10^6 CCU of *M. gallisepticum*. The culture medium was replaced at 2–3-day intervals by

TABLE III

The CSE of M. gallisepticum in chicken tracheal organ cultures maintained in Eagle's medium compared with that in cultures maintained in medium with added glucose

Expt no.	Time for organ cultures in the stated medium to show 50 per cent. reduction in ciliary activity (days)	
	Eagle's medium	Eagle's medium with added glucose
1	8.4	8.7 (0.1 per cent. glucose)
2	5.7	8.4 (1.0 per cent. glucose)

medium that had been harvested from *M. gallisepticum*-infected tracheal cultures and treated with tetracycline 25 μ g per ml. The results of both experiments were similar and that of one is presented in fig. 2. It appears that culture fluids in which *M. gallisepticum* organisms had grown, but were then suppressed by tetracycline, contained a factor that was mildly toxic to the ciliated epithelium.

TABLE IV

The CSE of M. gallisepticum in chicken tracheal organ cultures maintained in Eagle's medium compared with that in cultures maintained in medium with neuraminidase

Expt no.	Time for organ cultures in the stated medium to show 50 per cent. reduction in ciliary activity (days)	
	Eagle's medium	Eagle's medium with neuraminidase (25 units per ml)
1	4.7	3.7
2	4.8	4.5
3	5.1	5.0

DISCUSSION

M. gallisepticum is a cause of respiratory disease in chickens (Fabricant, 1969). The chicken tracheal organ-culture system is therefore a logical choice for the investigation of the disease process. Previously we showed that several strains of *M. gallisepticum* grew in this organ-culture system and that most of the strains markedly inhibited ciliary activity (Cherry and Taylor-Robinson, 1970a). We have now shown that the speed of inhibition is not directly related to the number of organisms inoculated into the cultures. This finding

contrasts with the results of similar studies in which the CSE of *M. mycoides* var. *capri* was directly related to the size of the inoculum (Cherry and Taylor-Robinson, 1970b).

Many mycoplasmas liberate peroxide *in vitro* and, as pointed out before, it has been suggested that this secretion may be an important virulence factor. Sobeslavsky *et al.* (1968) have proposed also that cytoadsorption might further enhance the damaging effect of peroxide on the ciliated epithelium. In studies similar to those reported here we have previously shown the importance of peroxide, because the addition of catalase to chicken tracheal organ-culture

FIG. 2.—The ciliary activity of chicken tracheal organ cultures: ○—○ = uninoculated controls; ●—● = medium replaced by medium in which *M. gallisepticum* had been grown and then suppressed by tetracycline; ▲—▲ = cultures seeded with viable *M. gallisepticum*.

medium protected the ciliated epithelium from the damaging effect of infection with *M. mycoides* var. *capri* (Cherry and Taylor-Robinson, 1970b). In the light of this work, and because *M. gallisepticum* liberates peroxide *in vitro* (Cole *et al.*, 1968), we expected that the addition of catalase to the *M. gallisepticum*-infected cultures would protect them. That it did not do so is, however, perhaps not so surprising in view of our most recent findings that the amount of peroxide liberated by *M. gallisepticum* in the organ-culture system is less than that liberated by *M. mycoides* var. *capri*; in fact in this system it was difficult to demonstrate that *M. gallisepticum* produced more peroxide than that generated by the culture system itself (Cherry and Taylor-Robinson, 1970c). It is more difficult to explain why the addition of large amounts of catalase actually potentiated the CSE of *M. gallisepticum*. Perhaps the peroxide produced by the tracheal cultures and by the organisms themselves adversely affect organism metabolism and "toxin" production. The addition of catalase would then remove this peroxide and allow greater metabolic activity.

The sparing effect on ciliary activity of added 1.0 per cent. glucose is unexplained. A similar but greater protection of the cilia resulted from the addition of 0.1 per cent. glucose to cultures infected by *M. mycoides* var. *capri*. In the latter case it seemed possible that the protective effect might have been the result of the stimulation of peroxidase-like activity by the glucose. However, since peroxide does not appear to be an important factor in the CSE of *M. gallisepticum*, the same hypothesis cannot be put forward.

Neuraminic acid receptors on avian and mammalian cells are concerned in the close association between such cells and some mycoplasmas, including *M. gallisepticum* (Sobeslavsky *et al.*; Manchee and Taylor-Robinson, 1969). In in-vitro studies, cells treated with neuraminidase do not adsorb to mycoplasma colonies. In the present experiments, the failure of neuraminidase to protect the ciliated epithelium is strong evidence against the proposal that cytoadsorption, with close association between cells and mycoplasmas, is important for the delivery of a damaging substance to the cells.

M. neurolyticum produces a thermolabile protein exotoxin which causes rolling disease in rats and mice (Tully, 1964, 1969; Thomas, Aleu *et al.*, 1966; Thomas and Bitensky, 1966). Thomas, Davidson and McCluskey (1966) showed that the S6 strain of *M. gallisepticum* produced, in turkeys, acute neurotoxic changes that resembled those caused by *M. neurolyticum* in mice. However, they could not demonstrate the presence of a soluble toxin, nor could they liberate a toxin from *M. gallisepticum* organisms by freezing and thawing. Furthermore, pretreatment of birds with tetracycline protected against the toxic manifestations. Their studies suggest that only viable *M. gallisepticum* organisms liberate a toxic substance, which is labile. These findings differ a little from those reported here, because we were able to demonstrate loss of ciliary activity by adding to the cultures a medium in which multiplication of the organisms had been suppressed by tetracycline. Initially, we did several experiments in which fluids from organ cultures containing organisms grown in them were filtered to remove the mycoplasmas and the filtrates were tested for their ability to suppress ciliary activity. However, at some stage of the multiple filtration procedures a few viable organisms passed into the filtrates and so the results of the experiments were not valid. It was for this reason that we began experiments with tetracycline-suppressed mycoplasmas. It is possible that a toxic substance was liberated during multiplication of the organisms before tetracycline treatment or, alternatively, that the organisms themselves might have a toxic effect even though suppressed by tetracycline. The addition to cultures of medium in which *M. gallisepticum* had multiplied was much less damaging than actual infection. This can be explained either on the basis of the postulated toxin being labile or of its being diluted in the medium. In natural *M. gallisepticum* infection the multiplying organisms would liberate toxin close to the ciliated epithelial surface, whereas in our experimental system the postulated toxic product was diluted in the whole medium.

The findings of this study suggest that either (i) a toxin other than peroxide liberated during infection of the organ cultures by *M. gallisepticum*, or (ii) the

organisms themselves, or (iii) both factors, lead to damage of the ciliated epithelium. Further work is necessary to establish which of these possibilities is important. There is good evidence to indicate that neither cytoadsorption nor peroxide production are important factors in the virulence of this mycoplasma.

SUMMARY

The growth and pathogenicity of *Mycoplasma gallisepticum* were studied in chicken embryo tracheal organ cultures. In these cultures *M. gallisepticum* attained titres of 10^6 – 10^7 CCU per ml of Eagle's medium and there was inhibition of ciliary activity. The cilia-stopping effect (CSE) was not closely related to dose, as a 50 per cent. reduction of ciliary activity occurred at about the same time after inoculation of organisms differing in number by as much as 100- to 10,000-fold. The addition of neuraminidase or catalase to the cultures did not inhibit the CSE of *M. gallisepticum*; in fact, the CSE was potentiated in cultures containing catalase. The CSE was present but delayed in organ cultures maintained in medium that contained *M. gallisepticum*, the continued multiplication of which was suppressed by tetracycline. Thus it seems that either the accumulation of toxic products in the medium contributes to tissue damage, or that the organisms themselves do so; but neither cytoadsorption nor peroxide production appears to be important in the pathogenicity of *M. gallisepticum*.

We thank Misses Pamela F. Clayton, Kathleen A. Keast and Elizabeth M. Minton for excellent technical assistance and Mr G. Wardman for processing histological specimens.

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