Observations on the occurrence of mycoplasmas in the central nervous system of some laboratory animals

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Summary

*Mycoplasma pulmonis* was isolated from the brains of 6 (23%) of 26 mice which had a naturally-occurring respiratory infection with this mycoplasma, and from the brains of 6 (8%) of 71 mice which had been inoculated intranasally or intravenously. The incidence of natural infection was greater in older mice, but there was no obvious mouse strain difference except for higher incidence in athymic nude mice. There was no evidence that the organisms passed the blood-brain barrier. Some isolations, especially from nude mice, may have been extraneous contaminants, as these were fewer when the mouse skulls were sterilized with ignited methanol. *M. pneumoniae* was not isolated from the brains of 14 hamsters which had a respiratory infection after intranasal inoculation nor were ureaplasmas isolated from the cerebrospinal fluids of 12 marmosets with a natural oropharyngeal infection. The aetiology of *M. pneumoniae* encephalitis in man is discussed.

*Mycoplasma neurolyticum* occurs in the brains of some mice. It often remains latent but may multiply under conditions of stress to produce an exotoxin which causes 'rolling' disease (Thomas & Bitensky, 1966). In mice and rats, *M. pulmonis* is found mainly in the respiratory and genital tracts, but occasionally disseminates to joints and other organs including the brain (Cassell & Hill, 1979). Recently, Hill (1979) reported the isolation of *M. pulmonis* from the brains of 98% of naturally- and experimentally-infected mice and rats, and that mycoplasmas occurred very frequently in the brains of marmosets and Chinese hamsters. These high recovery rates contrast sharply with our findings that *M. pulmonis* was isolated infrequently from mouse brain (Taylor & Taylor-Robinson, 1975). In view of this and the possible significance of mycoplasmal infection of brain in man and other animals, we were stimulated to reexamine critically the frequency with which mycoplasmas occurred in the brains of laboratory animals, particularly *M. pulmonis* in mice infected both naturally and experimentally.

Materials and methods

Laboratory animals

Mice of 5 strains with naturally-occurring *M. pulmonis* respiratory infections were examined; 4 of these strains were a year or more old (Table 1). CBA mice, 8-12 weeks old, and nude mice of the same strain and age were used for experimental inoculation of *M. pulmonis*. Also examined were Syrian hamsters (*Mesocricetus auratus*), 12-16 weeks old, inoculated intranasally with *M. pneumoniae*, and adult marmosets (*Callithrix jacchus*), naturally infected in the oropharynx with ureaplasmas (Furr, Taylor-Robinson & Hetherington, 1976).

Mycoplasmas

Some mice were inoculated with a strain (JB) of *M. pulmonis* known to produce pneumonia and arthritis, obtained from Dr J. G. Tully (National Institutes of Health, Bethesda, USA), while others were given a variant of the same strain rendered non-pathogenic for the lungs by multiple passes through liquid medium. Hamsters were given a strain of *M. pneumoniae* which had been isolated from the sputum of a patient with pneumonia and which had been subcultured 4 times.

Media and isolation procedure

The composition of media for the isolation and growth of mycoplasmas, including ureaplasmas, has been described previously (Manchee & Taylor-Robinson, 1968; Taylor-Robinson, Martin-Bourgon, Watanabe & Addey, 1971). The number of organisms in a specimen was estimated by making serial 10-fold dilutions in medium and incubating at 37°C until colour changes ceased to occur. The highest dilution at which a colour change was observed was considered to contain 1 colour-changing unit (ccu) and the number of organisms in a suspension is expressed as ccu/0.2 ml.

Experimental procedures

Mice were inoculated with 2.5 x 10⁶ ccu of *M. pulmonis*, some intranasally while anaesthetized with pentobarbitone (Taylor & Taylor-Robinson, 1976) and others intravenously. Hamsters were also...
anaesthetized and inoculated intranasally with *M. pneumoniae*, one group with $10^3$ and another group with $10^5$ ccu. Experimentally- and naturally-infected animals were killed by intraperitoneal administration of sodium pentobarbitone. Attempts were made to isolate the organisms from the throat, blood, brain and lungs in this sequence. A calcium alginate nasopharyngeal swab (A. R. Horwell Ltd, London, UK) was used to obtain a specimen from the throat, and blood was collected from the axilla. Recovery of mycoplasmas from the lungs was achieved by perfusion (Taylor-Robinson, Denny, Thompson, Allison & Mardh, 1972). The procedure used to isolate mycoplasmas from the brain was as follows. Methanol was applied to the fur on the dorsum of the head and back and the tissues were then incised to expose the cranium. Methanol was applied again, ignited, and after 5-10 s the flames were extinguished. The skull was opened with sterile scissors, the whole brain removed and homogenized in mycoplasma medium in a tissue grinder to produce a 10% suspension from which serial 10-fold dilutions were made immediately. In a few instances (see Results) brain tissue was rubbed over agar medium and some tissue, rather than being homogenized, was chopped into 3 or 4 pieces and placed in liquid medium. All media in which a colour change was observed after incubation at 37°C were subcultured to fresh medium. Organisms were identified by agar growth-inhibition (Clyde, 1964).

**Results**

*Evaluation of the isolation technique*

Brains were examined from 10 mice of strain B10AQR which were infected naturally in the throat with *M. pulmonis*. The skulls of 4 of them were treated with methanol and ignited, the other 6 being untreated. In each case, half of the brain tissue was rubbed over agar medium and then placed in liquid medium. The other half was homogenized and the suspension diluted in liquid medium (see Materials and methods). *M. pulmonis* was isolated from the brains of 1 of the 6 mice not treated with methanol, but from none of the others; isolation was achieved by all 3 procedures. It was particularly noteworthy that a 10% homogenate of all brain specimens caused a reduction in the pH of the liquid medium usually within 24 h of incubation. This colour change mimicked that produced by metabolism of *M. pulmonis* in glucose-containing medium. However, it was not inhibited when 1-0 µg/ml doxycycline was incorporated in the medium, a concentration of antibiotic which inhibited the multiplication of *M. pulmonis*. Furthermore, the colour change was barely perceptible or was not seen when the homogenate was diluted a further 10-fold.

To assess whether methanol flaming of mouse skulls and homogenization of brain tissue inhibited isolation of *M. pulmonis*, $10^4$ ccu of the organisms were inoculated through the skull of each of 4 mycoplasma-free animals before isolation was attempted; in 1 case *M. pulmonis* was added to homogenized tissue before it was diluted further. In all cases, mycoplasmas were recovered. In further isolation attempts, therefore, brains were homogenized and the suspensions diluted as described previously in order to assess the numbers of organisms present.

Isolation from mice naturally infected with *M. pulmonis*

*M. pulmonis* was isolated from the oropharynx of all 26 mice examined (range $10^4$-$10^6$ ccu; geometric mean titre, GMT, $10^{5.3}$), but it was found (GMT $10^{4.5}$) in the lungs of only 4 mice (Table 1). The mycoplasma was isolated from the brains of 2 of 9 mice (22%) which had their skulls flamed and 4 of 17 (23.5%) which did not.

### Table 1. Isolation of *Mycoplasma pulmonis* from various anatomical sites of mice

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Sex</th>
<th>Age (months)</th>
<th>Mycoplasmal infection</th>
<th>Mice with mycoplasma in:</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>throat</td>
<td>lung</td>
</tr>
<tr>
<td>NZW</td>
<td>♂</td>
<td>≥12</td>
<td>+</td>
<td>5/5</td>
<td>1/5</td>
</tr>
<tr>
<td>NZW B10G</td>
<td>♂</td>
<td>≥12</td>
<td>+</td>
<td>3/3</td>
<td>1/3</td>
</tr>
<tr>
<td>B6K2</td>
<td>♂</td>
<td>≥12</td>
<td>+</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>B10S</td>
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<td>≥12</td>
<td>+</td>
<td>3/3</td>
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</tr>
<tr>
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<td>+</td>
<td>10/10</td>
<td>2/10</td>
</tr>
<tr>
<td>CBA</td>
<td>♂</td>
<td>2-3</td>
<td>+</td>
<td>46/52*</td>
<td>42/52*</td>
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<tr>
<td>CBA nude</td>
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<td>2-3</td>
<td>+</td>
<td>19/19</td>
<td>19/19</td>
</tr>
<tr>
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<td>+</td>
<td>3/10</td>
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<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>94/107</td>
<td>65/97</td>
</tr>
</tbody>
</table>

* 10 of these mice were inoculated with *M. pulmonis* which had received 100 passes in medium.
† Not tested.
**Mycoplasmas in the CNS**

*Isolation from mice experimentally inoculated with M. pulmonis*

*Intranasal inoculation.* M. *pulmonis* was isolated from the oropharynx of 65 of 71 mice (range $10^1$-$10^3$ ccu; GMT $10^{4.5}$) 7-14 days after inoculation. The brains of 61 of these mice, 56 of which had a respiratory infection (oropharynx and/or lung), were examined (Table 1). The mycoplasma was isolated from the brains of 2 of 49 mice (4%) with flamed skulls and from 4 of 12 mice (33%) with unflamed skulls. Those mice from which *M. pulmonis* was isolated from the brain also had a mycoplasmal respiratory infection.

*Intravenous inoculation.* To determine the extent to which haematogenous spread might lead to infection of the brain, 10 mice were inoculated intravenously with $2.5 \times 10^6$ ccu *M. pulmonis*. At autopsy 9 weeks later the mycoplasma was isolated from the oropharynx of 3 of the mice but it was not recovered from the brain of any mouse, the skull having been flamed in each instance (Table 1). In all, the brains of 97 mice were examined and *M. pulmonis* was isolated from 12 of them (12%). The organisms came from 4 (6%) of 68 animals which had flamed skulls and from 8 (27%) of 29 animals with unflamed skulls. In 6 instances the number of organisms isolated from the brain was less than the number isolated from the throat. In the other cases, however, the number of organisms recovered from the brain exceeded the number from the throat, suggesting that they were derived from the brain and were not extraneous contaminants acquired at autopsy.

**Influence of strain, sex, age and immunological status of mice on isolation of M. pulmonis**

There was no clear indication that *M. pulmonis* was isolated more frequently from the brains of mice of a particular strain than from any other (Table 1). It was isolated from the brains of 2 (15%) of 13 female mice and from those of 10 (12%) of 84 male mice. All of 81 experimentally-inoculated mice were young adults and *M. pulmonis* was recovered from 6 (7%) of the 71 brains tested; it was isolated from the brain of 1 (10%) of 10 young adult naturally-infected mice, and from the brains of 5 (31%) of 16 naturally-infected mice which were 12 months of age or older. The mycoplasma was isolated from 1 (2.4%) of 42 brains from immunologically-competent CBA mice but from 5 (26%) of 19 brains from nude CBA mice. However, 4 of the latter isolates were from animals in which the skulls had not been flamed.

**Attempted isolation of M. pneumoniae from hamsters**

16 hamsters were inoculated intranasally with *M. pneumoniae* and examined 2 weeks later. The mycoplasma was recovered from the oropharynx of 11 animals (range $10^1$-$10^3$ ccu; GMT $10^{3.5}$) and from the lungs of 14 (range $10^2$-$10^8$ ccu; GMT $10^{4.5}$), but not from the brain of any animal.

**Attempted isolation of ureaplasmas from the cerebrospinal fluids of marmosets**

Fluids from 13 marmosets were examined, 12 being naturally infected with ureaplasmas in their oropharynx (range $10^1$-$10^6$ ccu; GMT $10^{4.8}$). Ureaplasmas were not recovered from any of the specimens.

**Discussion**

Brain tissue was homogenized to produce a 10% suspension and this was diluted serially in liquid medium to determine the number of *M. pulmonis* organisms which might be present. The change in colour of the medium from pink to yellow produced by the organisms was used as a means of detecting them. However, a 10% suspension of brain tissue always produced a colour change. Evidence that this colour change was due to continued metabolism of brain tissue was that it occurred rapidly, was almost always confined to medium containing the 10% suspension and not to dilutions thereof, could not be subcultured, and was not inhibited by a concentration of a tetracycline which inhibited the colour change produced by *M. pulmonis*. We were confident, therefore, of our ability to distinguish between this spurious colour change and the specific one caused by the presence of mycoplasmas and also, by serial dilution, to estimate the number of organisms present.

Since there are so many mycoplasmas in the respiratory tract which may contaminate the fur and lead to contamination of the skull during dissection, there is a particular opportunity for them to gain access to the brain when it is removed from the skull. Of course, organisms in the ear may also enter brain tissue before or during dissection. The technique of flaming the tissues before opening the skull was undertaken to reduce this possibility of contamination. Inoculating *M. pulmonis* organisms through the skull and recovering them from the brain after flaming showed that heating the skull and mincing tissue did not kill the organisms. In other words, if present, mycoplasmas within the brain should have been recoverable by the technique we used. Overall, the organisms were isolated from the brains of fewer mice when the flaming technique was used and we regard this as vindication for using the technique to minimize mycoplasmal contamination. The isolation of *M. pulmonis* organisms from brain tissue after flaming and sometimes in numbers exceeding those in the oropharynx we consider to be unequivocal evidence of their presence in the brain and that, in this circumstance, they were present just superficially as contaminants. Nevertheless, it is interesting that we
obtained *M. pulmonis* organisms from only a few of the brains, irrespective of whether the mice were naturally or experimentally infected. This is in accord with our previous findings (Taylor & Taylor-Robinson, 1975) and in contrast to those of Hill (1979). Moreover, we have not been able to recover *M. pneumoniae* from the brains of experimentally-infected hamsters nor isolate ureaplasmas from the cerebrospinal fluids of naturally-infected marmosets. Our finding of mycoplasmas in a few brains only was not due to our overlooking them in part of the brain substance because we tested the whole brain. Furthermore, it is not arguable that the medium we used was inadequate to isolate the mycoplasmas from the brains because it supported growth of *M. pulmonis* taken from the respiratory tract of nearly all the mice and as many as 10^6 organisms were detected in the lungs of some of them.

In those few mice where we believe *M. pulmonis* organisms were present in the brain before autopsy, the question must arise as to how they gained access. The more frequent isolation of *M. pulmonis* organisms from the brains of nude CBA mice than from normal mice suggests, at first sight, that they may have spread haematogenously. However, this is probably not so because the organisms were isolated infrequently from the brains of nude mice when the flaming technique was used. Furthermore, the failure to recover mycoplasmas from the brains of mice after inoculation of a large number of organisms intravenously suggests that haematogenous spread from the respiratory tract of immunocompetent mice is unlikely to be the usual means by which the organisms enter the brain. It seems more likely that organisms in the respiratory tract, particularly the oropharynx, gain access more directly, possibly via the ear. The finding of mycoplasmas more often in the brains of old rather than young mice naturally infected in the oropharynx is in keeping with the greater opportunity there has been for this to occur.

Although we failed to isolate *M. pneumoniae* from hamster or ureaplasmas from marmoset cerebrospinal fluid, the recovery of *M. pulmonis* from the brains of a few mice leaves open the question of whether the neurological complications which have been seen after *M. pneumoniae* infections of man (Lind, Zoffmann, Larsen & Jessen, 1979) are due to direct invasion of the central nervous system by this mycoplasma or to an immunopathological mechanism. Of course, the latter is attractive as an explanation in view of the autoimmune phenomena associated with *M. pneumoniae*.

References


Beobachtungen zum Auftreten von Mykoplasmen im Zentralnervensystem einiger Versuchstiere

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Zusammenfassung

*Mycoplasma pulmonis* wurde aus 6 Gehirnen von 26 Mäusen (23%) isoliert, die natürliche Atemwegsinfektionen mit diesen Mykoplasmen hatten, sowie aus 6 Gehirnen von 71 Mäusen (8%), die intranasal oder intravenös infiziert
Mycoplasmas in the eNS
