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Mycoplasma pulmonis infection of the murine oropharynx protects against subsequent vaginal colonization

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SUMMARY

Intranasal inoculation of 12 young adult mice (strain TO) with *Mycoplasma pulmonis* protected all of them against vaginal colonization when they were challenged intravaginally 60 days later with the same mycoplasmal strain. In contrast, all 15 mice without a respiratory infection became colonized vaginally (geometric mean titre [GMT] $4{\cdot}6 \times 10^6$ colour-changing units [c.c.u.]) when challenged in the same way. The GMT of serum antibody, measured by a micro-immunofluorescence technique, prior to challenge was 200 and 8 for the oropharyngeally infected and unexposed mice, respectively. The GMT of antibody in vaginal washings from the two groups was 6 and 3, respectively. All four nude BALB/c mice were susceptible to vaginal colonization (GMT $5{\cdot}6 \times 10^6$ c.c.u.) after oropharyngeal infection (GMT $5{\cdot}1 \times 10^4$ c.c.u.) resulting from intranasal inoculation, as were all six nude mice (vaginal GMT $1{\cdot}4 \times 10^7$ c.c.u.) that had not been inoculated intranasally. In contrast, all ten of their immunocompetent counterparts were resistant to vaginal colonization after oropharyngeal infection (GMT $1{\cdot}3 \times 10^3$ c.c.u.), whereas all nine such mice that had not been infected oropharyngeally were susceptible to vaginal colonization (GMT $7{\cdot}6 \times 10^6$ c.c.u.). These results show the important role that a respiratory infection has in protecting the vagina against colonization and that protection is dependent on a functioning T-lymphocyte system.

INTRODUCTION

Mycoplasma pulmonis is a murine respiratory pathogen which may disseminate to other anatomical sites, including the spleen and kidney [1]. In addition, some strains are arthritogenic [2, 3]. We showed previously that vaginal colonization with *M. pulmonis* occurred consistently and was prolonged if the mice were first treated with progesterone and then inoculated intravaginally [4]. Circulating and local antibodies were detected after vaginal colonization and mice from which the organisms had been eliminated were resistant to recolonization after intravaginal challenge. Live *M. pulmonis* organisms given intravenously stimulated antibody production and partly protected against vaginal colonization, but killed organisms given likewise or intravaginally did not confer protection, despite stimulating

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antibody production [5]. Hence, the role of antibody in protection was equivocal. Furthermore, there was some evidence that respiratory infection which ensued as a consequence of vaginal colonization contributed, at least partly, to vaginal immunity. We have, therefore, pursued the issue of whether a respiratory infection with *M. pulmonis* confers protection against colonization of the murine vagina by this mycoplasma. The conclusive results form the substance of this report.

MATERIALS AND METHODS

Mice

Female mice of the TO, BALB/c or BALB/c-*nu* strains, 6–8 weeks old, were used. They were bred in the Specific Pathogen-free unit at the Clinical Research Centre and were screened for mycoplasmas before use.

Progesterone administration

Depo-provera (Upjohn Ltd, Crawley, Sussex) was administered in a dose of 2·5 mg/0·2 ml, subcutaneously, on four occasions at weekly intervals to enhance vaginal colonization with *M. pulmonis*.

Mycoplasma medium

Glucose-containing broth medium used for growth and isolation of *M. pulmonis* has been described previously [6].

M. pulmonis *inoculum*

M. pulmonis, strain JB, was obtained originally from J. G. Tully (National Institute of Allergy and Infectious Diseases, Frederick, MD, USA) and had been passed five times in medium in this laboratory before use. The organisms that constituted the mouse inoculum were grown in broth medium at 37 °C for 3 days. Their number was estimated by making serial tenfold dilutions in medium to 10^{-10}, incubating at 37 °C and observing the medium for a colour change from red to yellow. The final dilution at which a change occurred was deemed to contain one colour-changing unit (c.c.u.).

Intranasal inoculation

Mice, not treated previously with progesterone, were anaesthetized with a mixture of one part of Hypnorm (Janssen Pharmaceutical Ltd, Oxford), two parts of water and one part of Hypnovel (Roche Products Ltd, Welwyn Garden City, Hertfordshire) which was administered intraperitoneally (0·3 ml/30 g body weight). The inoculum of *M. pulmonis*, containing $2{\cdot}5 \times 10^8$ c.c.u., was introduced intranasally in a 50 μl aliquot with an Eppendorf pipette. A group of mice serving as a control was anaesthetized as indicated but received 50 μl of broth medium only.

Intravaginal inoculation

Intravaginal inoculation of mice with *M. pulmonis* coincided with the second injection of progesterone, a 50 μl aliquot containing $2{\cdot}5 \times 10^6$ c.c.u. being introduced into the vagina with an Eppendorf pipette.

Collection of specimens

Throat specimens were obtained by inserting a plain cotton-wool nasopharyngeal swab (MW 142, Medical Wire and Equipment Co Ltd, Corsham, Wiltshire) into the throat of the mouse and expressing the content into 1·8 ml of mycoplasmal medium. This was regarded as a 10^{-1} dilution and further dilutions were prepared as described previously.

Vaginal specimens were procured with a nasopharyngeal swab which was inserted into the vagina, rotated and then rolled along a microscope slide (vaginal smear). The remaining content of the swab was expressed into 1·8 ml of mycoplasmal medium and dilutions were prepared as indicated previously.

Cytological examination

Vaginal smears were air dried, fixed in methanol for 30 min, stained with Giemsa for 30 min and finally rinsed in 30% alcohol followed by buffered distilled water, pH 6·8, and de-ionized water. Slides were examined microscopically to determine the stage of the murine reproductive cycle as defined by Rugh [7].

Collection of blood and vaginal washings

Samples were collected after intranasal inoculation of organisms and 7 days before vaginal challenge. Mice were bled from the tail vein and vaginal washings were collected by introducing 50 μl of sterile phosphate-buffered saline (PBS) into the vagina, removing it and repeating the instillation twice more with the same PBS. The washings and sera were stored at -20 °C.

Antibody measurement

Sera and vaginal washings were examined for antibody to *M. pulmonis* (strain JB) by means of a quantitative indirect micro-immunofluorescence technique. A rabbit polyvalent (IgM, IgG) antiserum or a rabbit anti-IgA, followed by a fluorescein-labelled goat anti-rabbit serum, was used as described previously [8]. IgA antibody was not detected in any of the specimens tested.

RESULTS

Effect of respiratory infection by M. pulmonis *on the response to intravaginal challenge*

Response of immunocompetent mice

Recovery of M. pulmonis. Fifteen mice were inoculated intranasally with *M. pulmonis* and 15 were kept as controls (Table 1). All the inoculated mice became infected in the oropharynx. However, three of these mice died within 30 days after inoculation and another within 60 days. At this time, the 11 remaining mice that were infected in the oropharynx and the 15 uninfected mice, all of which were in the dioestrous stage of the reproductive cycle following progesterone treatment, were inoculated intravaginally with *M. pulmonis*. As shown in Table 1, none of the 11 mice infected previously in the oropharynx became colonized in the vagina, whereas all the 15 mice without an oropharyngeal infection became colonized in the vagina, large numbers of organisms being recovered (geometric mean titre [GMT] $4{\cdot}6 \times 10^6$ c.c.u.). It is noteworthy that 11 of these 15 mice had also become

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Table 1. *Effect of respiratory infection by* M. pulmonis *on the response of immunocompetent TO mice to intravaginal challenge*

	Number of days after			
	Intranasal inoculation		Intravaginal challenge	
	30	60	7	15
Mice inoculated intranasally ($n = 12$)				
No. with organisms in oropharynx	12	11†	NT	11
GMT* of organisms in oropharynx	$1{\cdot}8 \times 10^4$	$3{\cdot}5 \times 10^4$		$2{\cdot}8 \times 10^4$
No. with organisms in vagina	0	0	0	0
Mice not inoculated intranasally ($n = 15$)				
No. with organisms in oropharynx	0	0	NT	11
GMT* of organisms in oropharynx				$6{\cdot}6 \times 10^3$
No. with organisms in vagina	0	0	15	15
GMT* of organisms in vagina			$4{\cdot}6 \times 10^6$	$4{\cdot}6 \times 10^6$

* Geometric mean titre expressed as c.c.u.
† One mouse in group died.
NT, not tested.

Table 2. *Effect of respiratory infection by* M. pulmonis *on the response of BALB/c nude and immunocompetent mice to intravaginal challenge*

Status of mice and whether inoculated intranasally	No. of mice in group	No. mycoplasma-positive (GMT*) in	
		Oropharynx 28 days after intranasal inoculation	Vagina 7 days after intravaginal inoculation
Nude inoculated	4	4 ($5{\cdot}1 \times 10^4$)	4 ($5{\cdot}6 \times 10^6$)
Nude not inoculated	6	—	6 ($1{\cdot}4 \times 10^7$)
Immunocompetent inoculated	10	10 ($1{\cdot}0 \times 10^3$)	0
Immunocompetent not inoculated	10	—	10 ($7{\cdot}6 \times 10^6$)

* Geometric mean titre expressed as c.c.u.

colonized in the oropharynx 2 weeks after intravaginal challenge, presumably as a consequence of it.

Development of antibody. Seven days before intravaginal challenge, all the 11 mice that had been inoculated intranasally had developed high titres of circulating antibody (titre range 64–512; GMT 200) and low titres of local vaginal antibody (titre range < 2–16; GMT 4). Essentially, the 15 mice that had not been inoculated intranasally were devoid of both circulating antibody (titre range $<$ 2–8) and local antibody (titre range < 2–2).

Response of nude mice compared to that of immunocompetent mice

Recovery of M. pulmonis. Seven nude mice and ten immunocompetent mice were inoculated intranasally with *M. pulmonis* and ten of each were kept as controls (Table 2). Twenty-eight days later, when all the inoculated mice were shown to be infected in the oropharynx, they and the uninoculated control mice were treated

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with progesterone and inoculated intravaginally with *M. pulmonis*. Of the nude mice that were infected or were uninfected oropharyngeally, four in each group died. All of the remaining four mice infected in the oropharynx became colonized in the vagina (GMT $5{\cdot}6 \times 10^6$ c.c.u.), as did the six nude mice (vaginal organism GMT $1{\cdot}4 \times 10^7$ c.c.u.) that had not been infected in the oropharynx. In contrast, none of the ten immunocompetent mice that had been infected in the oropharynx became colonized in the vagina, whereas all the ten mice uninfected in the oropharynx became colonized in the vagina (GMT $7{\cdot}6 \times 10^6$ c.c.u.).

DISCUSSION

The results of previous work [4] showed that *M. pulmonis*, which usually produces respiratory disease in mice, would infect the genital tract of female mice if they were first treated with progesterone. An opportunity was provided, therefore, to determine whether initial vaginal colonization resulted in resistance to recolonization and, if so, the mechanism involved. The development of solid immunity to vaginal recolonization was demonstrated [9] and this was shown subsequently [5] to be associated only partly with circulating and/or local vaginal antibody. Although there was a little evidence that genital tract infection *per se* resulted in vaginal immunity, many of the mice developed a concurrent mycoplasmal respiratory infection which continued after vaginal colonization was no longer demonstrable. There was some evidence that the respiratory infection contributed to genital tract immunity, but the exact role that pulmonary exposure might have in preventing colonization of the genital tract required clarification. In contrast to the rapid transmission of organisms from the genital tract to the upper respiratory tract with colonization of the oropharynx, shown previously [5] and currently, *M. pulmonis* does not easily infect the genital tract unless the mice are in the dioestrous phase of the reproductive cycle. Indeed, in the current experiments, the organisms were not transmitted naturally from the respiratory tract to the genital tract of the mice despite exposure for more than 60 days. After progesterone treatment, however, cytological examination showed all mice to be in the dioestrous stage of the cycle and, therefore, potentially susceptible to vaginal colonisation. Even so, those mice which had been previously exposed intranasally to *M. pulmonis* and were colonized in the oropharynx were refractory when challenged vaginally with a large number of the organisms. Thus, resistance of the genital tract to infection by *M. pulmonis* is twofold. First, natural resistance breached when the reproductive cycle of the mice is brought into the dioestrous phase, either naturally or artificially and second, resistance bestowed by infection of the respiratory tract. It is likely in many cases that infection extended beyond the oropharynx to the lower respiratory tract. Although we did not examine the mice at necropsy to establish whether infection of the lower tract had occurred, the results of previous studies would suggest that this was so [10].

Although it is possible that hormone administration may suppress immune responses [11] and, therefore, predispose to genital-tract infection or infection at other sites, clearly this was insufficient to jeopardize the development of resistance in the genital tract. Most of the mice developed high titres of circulating antibody to *M. pulmonis* prior to genital challenge, although the titres in the vaginal

washings were low or insufficient to measure. The latter finding does not suggest that antibody in the vagina is a major factor in protection, although whether it has any role is not clear. It is clear, however, that nude mice with an *M. pulmonis* respiratory infection are as susceptible to vaginal colonization as immunocompetent mice without such an infection. In other words, the respiratory infection in them does not confer protection, inferring that a functioning T-lymphocyte system is essential for immunity in the genital tract. Such immunity presumably involves the mucosal associated lymphoid tissue, the mechanism being the migration of sensitized lymphocytes from the submucosa of the respiratory tract to that of the genital tract [12].

The observations made on the murine model may have relevance for human disease. *Mycoplasma genitalium* has been shown to occur in the urethra, particularly of men with non-gonococcal urethritis [13]. It is feasible that a respiratory infection with *M. pneumoniae* could influence infection of the human genital tract with *M. genitalium*, particularly as these mycoplasmas are known to have antigenic similarities [14, 15]. It may prove difficult to evaluate this idea epidemiologically, but evaluation of the notion is possible experimentally since *M. pneumoniae* is capable of infecting the murine respiratory tract (P. M. Furr, unpublished) and *M. genitalium* has been shown to colonize the vagina of progesterone-treated mice [16].

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