THE ANTI-CHLAMYDIAL EFFECT OF EXPERIMENTAL *MYCOPLASMA PULMONIS* INFECTION IN THE MURINE GENITAL TRACT

MAUREEN A. TUFFREY, PATRICIA M. FURR, P. FALDER AND D. TAYLOR-ROBINSON

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

SUMMARY. Experimental *Chlamydia trachomatis* infection of the genital tract of female CBA and TO mice was greatly curtailed by a concurrent genital infection with *Mycoplasma pulmonis*. TO mice in which chlamydial infection had been suppressed by the mycoplasma infection were treated with the anti-mycoplasma agent, gentamicin. This did not cause a reappearance of the chlamydiae, suggesting that these organisms had been eliminated completely. The *M. pulmonis* infection stimulated a striking and persistent polymorphonuclear leukocyte response, which may have been the cause of the curtailment of the chlamydial infection.

INTRODUCTION

Mixed infections of the human genital tract are common, but the effect of one micro-organism on another may be difficult to determine in patients because of the need for repeated microbiological examinations without treatment. Investigation of mixed infections in animal models, however, is not subject to these limitations. Tuffrey and Taylor-Robinson (1981) produced a chlamydial genital-tract infection of female CBA mice by pre-treating the animals with progesterone and flooding the entire reproductive tract with a human strain of *Chlamydia trachomatis*. In this communication we describe the modification of such an infection by an intercurrent infection with *Mycoplasma pulmonis*. The results may have implications for chlamydial and mycoplasmal infections, which occur frequently in the human genital tract.

MATERIALS AND METHODS

Mice. Ten-week-old inbred females (specific pathogen free) of the CBA/Ca/CRC sub-line and outbred TO mice maintained by the minimal inbreeding system were used.

Micro-organisms. The "fast egg-killing" human strain of *C. trachomatis*, designated SA-2f, was used. It was known to be serologically identical to serovar LGV₂ (Wang and Grayston, 1971). *M. pulmonis* strain JB was received from J.G. Tully (National Institutes of Health, USA). Since the isolation, cloning and specific typing of this mycoplasma (Barden and Tully, 1969), it had been subcultured several times but was still capable of producing arthritis and respiratory disease in mice.

Infection of mice. All the mice were treated with progesterone (Depo-Provera; Upjohn), each being given 2.5 mg by subcutaneous injection at approximately weekly intervals to prevent the onset of oestrous. Such treatment enhances genital infections of mice with *C. trachomatis* (Tuffrey and Taylor-Robinson, 1981) and with *M. pulmonis* (Furr and Taylor-Robinson, in press).

The chlamydiae and mycoplasmas were given by the intrauterine (i.u.) route (Tuffrey and Taylor-Robinson, 1981). The mice were anaesthetised with pentobarbitone (Sagatal; May and Baker Ltd) given intraperitoneally (0.01 ml of a 1 in 10 dilution per g body weight) and the uterus was exposed through a small lateral incision over the ovarian fat pad. The inoculum (0.1 ml) was introduced through a 30-gauge needle into the uterine cavity immediately below the utero-tubal junction, and was seen to issue from the vagina. The peritoneum was closed with a

Received 19 Sep. 1983; accepted 30 Nov. 1983.

fine silk suture and the skin with a surgical clip. The chlamydial and mycoplasmal inocula were introduced into different uterine horns.

Detection of organisms. Vaginal samples were taken with sterile cotton-wool nasopharyngeal swabs (Medical Wire and Equipment Co. Ltd). Swabs for the detection of each micro-organism were taken weekly on different days.

For the isolation of C. trachomatis, each swab was expressed in 1 ml of cold sucrose-phosphate transport medium (2SP) containing heat-inactivated fetal calf serum (10%). The liquid specimens were then stored in liquid nitrogen until inoculated into cycloheximide-treated McCoy cell cultures as described by Thomas *et al.* (1977). Two monolayers of cells were used for each specimen. The results are shown as the number of chlamydial inclusions per monolayer. The numbers of chlamydiae in specimens are expressed as inclusion-forming units (ifu).

For the isolation of M. pulmonis, each swab was expressed in 1.8 ml of glucose-containing mycoplasma medium (Manchee and Taylor-Robinson, 1968). The resulting liquid specimen was deemed to be a 1 in 10 dilution; it was diluted further in a series of 10-fold dilutions (0.2 ml in 1.8 ml) up to 10^{-8} . The last dilution at which a change in colour of the medium from red to yellow occurred during incubation at 37°C was considered to contain one colour-changing unit (ccu).

Detection of inflammatory cells. In experiment II (see below), at approximately weekly intervals, cotton-wool naso-pharyngeal swabs were moistened with saline, inserted into the vaginas of the mice, and smeared on glass slides. This sampling did not coincide with the microbiological sampling. The smears were fixed with methanol, stained by Giemsa's method, and examined microscopically for polymorphonuclear (PMN) leukocytes.

Experimental design. Two in-vivo experiments are described. Experiment I comprised four groups of CBA mice treated as follows: (1) *C. trachomatis* alone (2×10^6 ifu), (2) *M. pulmonis* alone (5×10^5 ccu), (3) *M. pulmonis* (5×10^6 ccu) 1 week before the chlamydiae, and (4) *M. pulmonis* (5×10^5 ccu) 1 week after the chlamydiae. Experiment II was of similar design except that TO mice were used, and that the numbers of mycoplasma ccu in groups (3) and (4) were 5×10^6 and 5×10^7 , respectively. In addition, half of the mice were treated with gentamicin (Gentovet; Arnolds Veterinary Products Ltd, Reading, Berks.) to eliminate the mycoplasmas. It was given daily for 5 days in doses of 500 µg/mouse from day 35 after inoculation of the chlamydiae. When treatment began the mice given chlamydiae only still gave positive chlamydial culture results, and those given both micro-organisms were chlamydia-negative.

RESULTS

The results of experiments I and II are summarised in tables I and II, respectively.

Recovery of micro-organisms from the genital tract

Isolation of M. pulmonis. Mycoplasmas were not isolated from the genital tract of any of the mice before inoculation. All the mice given *M. pulmonis* became infected with mycoplasmas and large numbers of organisms were recovered, as indicated by the geometric mean titres shown in tables I and II. The numbers of mycoplasmas isolated from mice given these organisms only (not shown in the tables) were similar to those isolated from mice which had received chlamydiae also. Most mice remained infected with mycoplasmas throughout the course of the experiments, but the number of organisms recovered usually began to diminish from about the fortieth to fiftieth day after inoculation of the chlamydiae. A similar decline in numbers occurred also in the mice given mycoplasmas only. There was, therefore, no evidence that the persistence of the mycoplasmas or the number recovered was influenced by the presence of chlamydiae.

Inhibition of C. trachomatis by M. pulmonis. Chlamydiae were isolated from the *M. pulmonis*-free CBA mice for at least 42 days after inoculation (table I). In contrast, chlamydial infection persisted for a much shorter time in both groups of mice given *M. pulmonis*. The most obvious effect was seen when *M. pulmonis* was given 1 week before *C. trachomatis*: one mouse was chlamydia-negative throughout the experiment, and four of the remaining five mice were negative by the fourteenth day, and remained so. When *M. pulmonis* was inoculated 1 week

TABLE I

Recovery of chlamydiae and mycoplasmas from progesterone-treated CBA mice after intrauterine inoculation of Chlamydia trachomatis with or without Mycoplasma pulmonis

Inocula	Number of mice	Numbers of mice from which chlamydiae or mycoplasmas (numbers in italics) were isolated on indicated day after inoculation of chlamydiae						
		8	14	21	28	35	42	62
<i>C. trachomatis</i> only	6	4 (1-4)*	6 (2-39)	6 (16-340)	6 (1-88)	4 (1-14)	5 (1-43)	0
<i>M. pulmonis</i> 1 week before <i>C. trachomatis</i>	6	5 (9-141)*	0	1 (3)	0	...	0	...
		$10^{6.8}$ †	$10^{6.5}$	$10^{5.5}$	$10^{5.3}$	$10^{6.0}$	$10^{5.5}$	$10^{4.8}$
<i>M. pulmonis</i> 1 week after <i>C. trachomatis</i>	6	5 (1-3)*	3 (1-26)	3 (1-50)	2 (1;1)	0	0	0
			$10^{6.7}$ †	$10^{6.6}$	$10^{6.3}$	$10^{7.0}$	$10^{5.5}$	$10^{4.0}$

* Range of number of inclusions per McCoy-cell monolayer (for *C. trachomatis*).

† Geometric mean titre (ccu) of *M. pulmonis*.

after *C. trachomatis*, there was also a considerable curtailment of the chlamydial infection. Only two of six mice were chlamydia-positive 1 month after inoculation and chlamydiae were not recovered from samples taken thereafter.

The curtailment of the chlamydial infection by the concurrent mycoplasma infection was demonstrated also in TO mice (table II). In these animals, chlamydial infection in the mycoplasma-free group persisted for up to 77 days—i.e., longer than that seen in the CBA mice. In contrast, chlamydiae were isolated for a much shorter time from both groups of mice with concurrent mycoplasma infections.

Effect of gentamicin on the recovery of micro-organisms. Gentamicin, which inhibits the growth of *M. pulmonis* but not chlamydiae, was given for 5 days to half of the mice in each group in experiment II from the thirty-fifth day after inoculation of the chlamydiae. Thereafter, *M. pulmonis* was not recovered from any of these mice. Chlamydiae did not reappear, however, despite elimination of the mycoplasmas.

The inflammatory response

Vaginal smears were made from mice in experiment II. A striking PMN leukocyte response occurred 5 days after inoculation of *M. pulmonis*. Mice given chlamydiae alone had a later response, beginning on about day 12; it was also less severe and less protracted. For example, 44 days after inoculation of *C. trachomatis* alone, none of nine mice exhibited a PMN leukocyte response (fig. 1), whereas nine of ten mice given *M. pulmonis* a week before the chlamydiae had severe responses (fig. 2), as did eight of nine mice given *M. pulmonis* a week after the chlamydiae. Chlamydiae were isolated at about the same time (day 40) from five of the mice given chlamydiae alone but from none of the mice in the groups with the mixed infections.

The effect of M. pulmonis on C. trachomatis in tissue culture

M. pulmonis in vaginal specimens could conceivably have inhibited the development of chlamydial inclusions in the McCoy-cell cultures. To investigate this point, cell monolayers were inoculated with sufficient chlamydiae to produce 20–30 inclusions per monolayer, and with the same chlamydial inoculum mixed with different numbers of *M. pulmonis*, ranging from 10^2 to 10^7 ccu. The cultures were incubated for 48 h at 37°C and processed as described for the

TABLE II
Recovery of chlamydiae and mycoplasmas from progesterone-treated TO mice after intrauterine inoculation of C. trachomatis with or without M. pulmonis

Inocula	Number of mice	Numbers of mice from which chlamydiae or mycoplasmas (numbers in italics) were isolated on indicated day after inoculation of chlamydiae											
		6	12	19	26	33	40	47	54	68	77	83	
<i>C. trachomatis</i> only	9	9 (1->250)*	9 (1-93)	7 (1-72)	7 (1-173)	5 (2-96)	5 (1-206)	4 (2-81)	2 (2; 169)	2 (9; 41)	1 (60)	0	
	10	9 (2-282)*	1 (8)	2 (1; 3)	2 (1; 9)	1 (32)	0	0	0	...	...	...	
<i>M. pulmonis</i> 1 week before <i>C. trachomatis</i>	10	10 (10 ^{7.5})†	9 (10 ^{7.2})	10 (10 ^{6.0})	10 (10 ^{5.4})	9 (10 ^{5.3})	3§ (10 ^{2.8})	2 (10 ^{2.0})	2 (10 ^{1.6})	1 (10 ^{0.8})	1 (10 ^{0.8})	1 (10 ^{1.2})	
	10	10 (1-2000)*	6 (1-49)	0	0	0	0	0	0	...	...	...	
<i>M. pulmonis</i> 1 week after <i>C. trachomatis</i>	10	10 (10 ^{7.6})†	10 (10 ^{8.0})	10 (10 ^{7.3})	10 (10 ^{7.3})	10 (10 ^{7.3})	5§ (10 ^{2.4})	4 (10 ^{6.0})	4 (10 ^{4.8})	4 (10 ^{4.2})	...	4 (10 ^{4.2})	

* Range of number of inclusions per McCoy cell monolayer (for *C. trachomatis*).

† Geometric mean titre (ccu) of *M. pulmonis*.

§ Of five animals because remaining five given gentamicin.

FIG. 1.—Absence of vaginal PMN leukocyte response 44 days after inoculation of *C. trachomatis* only.

FIG. 2.—Severe vaginal PMN leukocyte response 44 days after inoculation of *C. trachomatis* in mice given *M. pulmonis* 1 week earlier.

isolation of chlamydiae. There was, however, no evidence that *M. pulmonis* caused a reduction in the number of chlamydial inclusions formed.

DISCUSSION

These experiments show that chlamydial infection of the mouse genital tract was curtailed by a concurrent genital infection with *M. pulmonis*. Two observations suggest that the effect was genuine, and not due to mycoplasmal inhibition of chlamydial inclusions in the McCoy-cell cultures used in the examination of vaginal samples. First, chlamydiae were isolated in the early phase of the experiments from specimens which also contained large numbers of mycoplasmas; and second, *M. pulmonis* did not inhibit the formation of chlamydial inclusions in mixed infection experiments *in vitro*.

The disappearance of mycoplasmas after gentamicin treatment of mice given both micro-organisms, at a time when chlamydiae were still present in mice given chlamydiae only, did not result in the re-emergence of chlamydiae. It would seem, therefore, that the chlamydiae were eliminated rather than merely suppressed. The exact reason for elimination has not been established. The pH of the vaginal mucosa during the *M. pulmonis* infection would have been about 6.5 (P.M. Furr and D. Taylor-Robinson, unpublished observation); this is unlikely to have influenced the chlamydial infection. Because the chlamydiae were not affected by mycoplasmas in tissue culture, competition for receptors or nutrients would not seem to have played a part. Interferon has been induced weakly and inconsistently in the serum of mice by intraperitoneal inoculation of *M. pulmonis* (Rinaldo *et al.*, 1974); whether it can be stimulated by intrauterine inoculation and cause inhibition of chlamydial multiplication is debatable. A more likely explanation for the curtailed chlamydial infection is that the chlamydiae were ingested and killed by the PMN leukocytes, the presence of which was stimulated by the *M. pulmonis* infection. In this regard, it would be interesting to determine the effect of an infection by *M. pulmonis* which did not stimulate a PMN leukocyte response and to assess the effect of a PMN leukocyte response induced by other means.

The question arises as to whether the mouse experiments described are likely to be applicable to man. Chlamydiae, *M. hominis*, ureaplasmas, gonococci, trichomonads and other micro-organisms may all be found together in the human genital tract; it is obvious, therefore, that one is not necessarily inimical to the others. However, how one may affect another under all conditions and in protracted multiple infections is unknown. It is possible that mycoplasmas could inhibit a chlamydial infection, although it seems unlikely that ureaplasmas or *M. hominis* could produce a cellular response as severe as that stimulated by *M. pulmonis* in mice. Whether some of the other micro-organisms, such as the trichomonads, could influence a human chlamydial infection remains to be investigated.

REFERENCES

- Barden J A, Tully J G 1969 Experimental arthritis in mice with *Mycoplasma pulmonis*. *Journal of Bacteriology* 100:5-10.
- Furr P M, Taylor-Robinson D Enhancement of experimental *Mycoplasma pulmonis* infection of the mouse genital tract by progesterone treatment. *Journal of Hygiene*. In press.
- Manchee R J, Taylor-Robinson D 1968 Haemadsorption and haemagglutination by mycoplasmas. *Journal of General Microbiology* 50:465-478.
- Rinaldo C R, Cole B C, Overall J C, Glasgow L A 1974 Induction of interferon in mice by mycoplasmas. *Infection and Immunity* 10:1296-1301.
- Thomas B J, Evans R T, Hutchinson G R, Taylor-Robinson D 1977 Early detection of chlamydial inclusions combining the use of cycloheximide-treated McCoy cells and immunofluorescence staining. *Journal of Clinical Microbiology* 6:285-292.
- Tuffrey M, Taylor-Robinson D 1981 Progesterone as a key factor in the development of a mouse model for genital tract infection with *Chlamydia trachomatis*. *FEMS Microbiology Letters* 12:111-115.
- Wang S-P, Grayston J T 1971 Studies on the identity of the "fast" egg-killing chlamydia strains. In: Nichols R L *Trachoma and related disorders caused by chlamydial agents*, Excerpta Medica International Congress Series pp 322-336.