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A study of F38-type and related mycoplasmas by mycoplasmaemia and cross-immunization tests in mice

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SUMMARY

In vivo methods were used to study the F38-type mycoplasma in parallel with related mycoplasmas.

Three of five strains of 'bovine serogroup 7' with an unknown history of subculture produced mycoplasmaemia in mice inoculated intraperitoneally. A strain of 'bovine serogroup L' also produced mycoplasmaemia, but no evidence of similar ability could be found for single strains of *Mycoplasma capricolum*, *M. equigenitalium* and *M. primatum*, or for two strains of the F38-type mycoplasma.

In cross-immunization tests a bovine serogroup 7 strain (NCTC 10133) and a strain ('Blenheim') of the SC (small colony) type of *M. mycoides* subsp. *mycoides* were used for the purpose of challenge. Cross-protection was described as 'complete' or 'partial', depending on whether it was as great as, or less than, that produced by homologous vaccine. Although strain NCTC 10133 protected strongly, possibly completely, against Blenheim, and Blenheim gave partial protection against NCTC 10133, challenge with NCTC 10133 and Blenheim gave strikingly different results. Thus (1) F38-type strains, *M equigenitalium* and *M. primatum* all gave partial cross-protection against NCTC 10133 but not against Blenheim, (2) NCTC 10133, unlike Blenheim, was seldom susceptible to partial cross-protection by LC (large colony) strains of *M. mycoides* subsp. *mycoides*, and (3) three SC strains – which would have protected completely against Blenheim – protected only partially against NCTC 10133. NCTC 10133 and Blenheim were similar, however, in that *M. capricolum* and *M. mycoides* subsp. *capri* failed to cross-protect against them both.

INTRODUCTION

The classical causative agent of contagious caprine pleuropneumonia (CCPP) has long been accepted as *Mycoplasma mycoides* subsp. *capri* (Edward, 1953), an organism with robust growth properties. In recent years, however, other mycoplasmas have been isolated from CCPP (see Smith, 1984). Of these, the 'F38' mycoplasma (MacOwan & Minette, 1976), an organism with delicate growth properties, is now realized to be the common cause of CCPP in Kenya. F38 also

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occurs in the Sudan (Harbi *et al.* 1981) and North Africa (Perreau, 1981), and further studies are needed to determine its prevalence in other countries. CCPP has been reproduced experimentally with both *M. mycoides* subsp. *capri* (Watson *et al.* 1968) and F38.

The taxonomic position of F38 has been the subject of much recent study and, by means of *in vitro* techniques such as gel double diffusion, complement fixation, growth precipitation, growth inhibition, immunofluorescence, two-dimensional polyacrylamide gel electrophoresis, and DNA hybridization, the organism has variously been shown to be related to the so-called SC (small colony) and LC (large colony) types of *M. mycoides* subsp. *mycoides*, the 'bovine serogroup 7' mycoplasma, *M. capricolum*, *M. equigenitalium* and *M. primatum* (MacOwan & Minette, 1976; Ernø & Salih, 1980; Christiansen & Ernø, 1982; Rodwell, 1982; Ernø, 1983; Ernø *et al.* 1983).

In studying *M. mycoides* subsp. *mycoides* (the cause of contagious bovine pleuropneumonia; CBPP) and *M. mycoides* subsp. *capri* Smith (1967*a*, *b*, 1968, 1969*a*, *b*) devised a method for producing mycoplasmaemia in mice by intraperitoneal inoculation. The mycoplasmaemia, which could readily be demonstrated by tail-blood culture in a selective medium, did not develop in immunized mice. This observation formed the basis of a mouse-protection test for the study of CBPP (Smith, 1971; Dyson & Smith, 1975). Subsequently these methods were modified to produce a mycoplasmaemia test and an active cross-immunization test capable of distinguishing between the SC and LC strains of *M. mycoides* subsp. *mycoides* (Hooker, Smith & Milligan, 1979; Smith, Hooker & Milligan, 1980; Smith & Oliphant, 1981*a*, *b*, 1982, 1983*a*).

The application of similar *in vivo* tests to the study of F38 and related mycoplasmas is reported in this paper.

MATERIALS AND METHODS

Mycoplasma strains

The small colony (SC) type of *M. mycoides* subsp. *mycoides* was represented by strains Blenheim (from CBPP), O goat and P goat; and the large colony (LC) type by strains Y goat, Mankefår 2833, F 30, 74/2488, Ojo I, and Cov 2. *M. mycoides* subsp. *capri* was represented by strain Smith 1423. Information on the source of these strains, their history of laboratory subculture, and method of cloning has been given by Hooker, Smith & Milligan (1979), Smith, Hooker & Milligan (1980) and Smith & Oliphant (1981*a*, 1983*a*).

The mycoplasma commonly responsible for CCPP in Kenya was represented by strain F38 (MacOwan & Minette, 1976), which had undergone many laboratory subcultures, and strains G183/82 and G275/82, which had undergone few; each strain had been cloned three times by the filtration method (Report, 1979).

No information on history of laboratory subculture was available in respect of the remaining mycoplasmas. The following were obtained from the National Collection of Type Cultures: *M. capricolum*, NCTC 10154; *M. equigenitalium*, NCTC 10176; *M. primatum*, NCTC 10163; bovine serogroup 7 (Leach, 1967), NCTC 10133. The remainder, all of which had been cloned three times by the filtration method, comprised: the serogroup 7 strains L2917 and QR1 from bovine arthritis in

Australia and M717/74 and M718/74 from bovine milk and semen respectively in Canada – all supplied by Dr R. H. Leach; and the 'bovine serogroup L' strain B144P of Al-Aubaidi & Fabricant (1971), isolated in California by Moulton, Boidin & Rhode (1956) from the joints, kidneys and spleen of a calf with arthritis and bronchopneumonia – supplied by Dr H. Ernø.

Mice

Female Swiss white mice weighing 16–18 g were obtained from two outbred closed colonies. The numbers of mice in each experiment, and their distribution in various groups of 6–25 animals, are shown in Tables 1–4.

Culture media

Unless stated otherwise, BVF-OS broth medium (Turner, Campbell & Dick, 1935) contained 10% of serum (Calf Serum No. 1, Wellcome Reagents) or, for mycoplasmas of the F38 type, 50%. ONB-OS medium (Hooker, Smith & Milligan, 1979) contained calf serum 20%. Newing's tryptose medium as modified by Brown, Gourlay & McLeod (1965) contained calf serum 20% or, for mycoplasmas of the F38 type, 50%.

Blood agar, used as a solid medium for all mycoplasmas except those of the F38 type, consisted of Blood Agar Base No. 2 (Oxoid CM 271) with Defibrinated Horse Blood (Oxoid SR50) 15%. Solid medium for F38-type mycoplasmas (BVF-OS agar) consisted of BVF broth (Turner, Campbell & Dick, 1935) containing Agar Noble (Difco) 1·7% and calf serum 30%.

Where stated, PTA (penicillin 100 units/ml and thallium acetate 0·05%) was incorporated in the medium to inhibit the growth of organisms other than mycoplasmas.

Viable counts

These were assessed by a modification of the method of Miles, Misra & Irwin (1938), decimal dilutions in BVF-OS medium being sampled (0·02 ml drops) on solid medium containing PTA. The mean of two or three counts was taken as the true value and expressed as colony-forming units (c.f.u.).

Mycoplasmaemia tests

BVF-OS medium was seeded by the addition of a small piece of solid medium bearing colonies aged 4 days (5–6 days for F38-type mycoplasmas). The liquid cultures were incubated for 4 days and used either undiluted or diluted in BVF-OS medium, groups of mice being inoculated intraperitoneally with 0·5 ml doses. Blood cultures (see below) were made from the mice 1, 2 and 3 days after inoculation. The infections were invariably symptomless.

Cross-protection tests with living vaccines

Two experiments were carried out. In the first the mice were challenged with the SC strain Blenheim of *M. mycoides* subsp. *mycoides*, in the second with the bovine serogroup 7 strain NCTC 10133. Immunity was detected by the absence of mycoplasmaemia 1 day or 1, 2 and 3 days after challenge. It was described as 'complete' or 'partial' according to whether it was as great as, or significantly less than, that produced by homologous vaccine.

In the first experiment six groups of mice were immunized by intravenous injections (0·25 ml/mouse) of undiluted living BVF-OS cultures prepared as described under *Mycoplasmaemia tests* (above). The cultures used and their relative turbidities (indicated by plus signs) were: *M. capricolum* (+ + + +); bovine serogroup 7, strain NCTC 10133 (+ +); *M. equigenitalium* (+ + +); *M. primatum* (+); strain F38 (+); and the homologous strain Blenheim (+ +). Mice in a seventh group (controls) each received 0·25 ml sterile BVF-OS intravenously. All cultures were checked for viability and purity. Five mice from each of the six vaccinated groups were killed 15 days after inoculation and their spleens and livers examined culturally; the results were uniformly negative except that one mouse given the bovine serogroup 7 culture still harboured viable mycoplasmas in the spleen. Three weeks after vaccination all mice were challenged intraperitoneally with 0·5 ml of a 4-day BVF-OS culture of strain Blenheim, diluted 1 in 100 with BVF-OS (dose *ca.* 5×10^6 c.f.u.). Blood cultures (see below) were made 1, 2 and 3 days later.

The second experiment comprised three similar tests carried out on different occasions. In tests 1 and 2, groups of mice were immunized as above with living BVF-OS cultures of the 17 mycoplasma strains shown in Table 3; The cultures of the three F38-type mycoplasmas were grown in BVF-OS containing 50 % instead of 10 % serum. In test 3 of the experiment all the vaccine cultures were grown in BVF-OS containing 50 % serum. The turbidity of the vaccine cultures varied, being least with mycoplasmas of the F38 type and greatest with the LC strains of *M. mycoides* subsp. *mycoides*. Three weeks after vaccination all mice were challenged intraperitoneally with 0·5 ml of undiluted 4-day BVF-OS culture of bovine serogroup-7 strain NCTC 10133. Blood cultures (see below) were made 1 day later.

Detection of mycoplasmaemia by blood culture

A drop of tail-blood from each mouse was cultured in 5 ml of ONB-OS medium containing PTA. After incubating for 7 days, subcultures were made on sectored plates of appropriate solid medium (see above). The subcultures were incubated for at least 4 days and usually longer before the results were read.

Where stated (Table 1) the blood cultures in liquid medium containing PTA were made in Newing's tryptose broth and BVF-OS, as well as in ONB-OS.

RESULTS

Mycoplasmaemia tests

It is seen from Table 1 that a bovine serogroup 7 strain (NCTC 10133) showed a tendency to produce mycoplasmaemia. No such tendency could be demonstrated in one strain each of *M. capricolum*, *M. equigenitalium*, *M. primatum* and the F38-type mycoplasma, despite the use of no less than three types of blood culture medium. In a further experiment in which undiluted culture of a second strain of the F38 type – this time a fresh field strain (G275/82) – was injected into groups of eight mice, blood cultures in BVF-OS broth 1, 2 and 3 days later failed once again to demonstrate mycoplasmaemia.

Table 2 shows that, unlike two Canadian strains (M717/74 and M718/74) of bovine serogroup 7, the Australian strains L2917 and QR1 and a bovine serogroup-L strain (B144P) produced mycoplasmaemia readily.

Table 1. *Mycoplasmaemia tests: various mycoplasmas including a bovine serogroup 7 strain*

Organism	Dilution of mycoplasma culture	Dose of mycoplasma (c.f.u. × 10^6) per mouse	Mice with mycoplasmaemia† in groups of six at the stated intervals (days) after inoculation		
			1	2	3
Bovine serogroup 7 (NCTC 10133)	1/1	440	5	1	2
	10^{-1}	44	3	1	1
	10^{-2}	4·4	2	1	0
	10^{-3}	0·44	0	0	0
M. capricolum (NCTC 10154)	1/1	220	0	0	0
M. equigenitalium (NCTC 10176)	1/1	115	0	0	0
M. primatum (NCTC 10163)	1/1	50	0	0	0
F38 (MacOwan & Minette, 1976)	1/1	—*	0	0	0

* Viable count not available, but mycoplasmas readily seen by dark ground microscopy.

† Blood culture medium was Newing's tryptose broth; in two further tests similar results were obtained by culturing blood in ONB-OS and BVF-OS respectively.

Table 2. *Mycoplasmaemia tests: four strains of bovine serogroup 7 and one of bovine serogroup L*

Mycoplasma strain	'Bovine serogroup'	Dose of mycoplasma (c.f.u. × 10^6) per mouse	Mice with mycoplasmaemia in groups of six at the stated intervals (days) after inoculation		
			1	2	3
L2917	7	213	6	6	4
		21·3	5	3	3
		2·13	3	4	1
QR1	7	350	6	6	5
		35	5	5	2
		3·5	5	4	1
		0·35	2	2	0
M717/74	7	575	2	0	0
		57·5	0	0	0
M718/74	7	1300	4	2	1
		130	0	0	0
B144P	L	600	5	4	2
		60	3	1	1
		6	4	0	0
		0·6	0	0	0

Table 3. *Cross-immunization against challenge with* M. mycoides *subsp.* mycoides (SC strain Blenheim)

Vaccine	Mycoplasmaemia in groups of mice at the stated intervals (days) after challenge*		
	1	2	3
Bovine serogroup 7 (NCTC 10133)	1/13	0/13	0/13
M. capricolum (NCTC 10154)	13/14	7/14	5/14
M. equigenitalium (NCTC 10176)	13/15	8/15	8/15
M. primatum (NCTC 10163)	14/15	10/15	9/15
F38 (MacOwan & Minette, 1976)	14/15	12/15	11/15
M. mycoides subsp. *mycoides* (Blenheim)	0/21	0/21	0/21
None (controls)	20/25	13/25	7/25

* Challenge dose *ca* 5×10^6 c.f.u.

Cross-immunization against challenge with Mycoides *subsp.* mycoides (*SC strain Blenheim*)

Table 3 shows that a bovine serogroup 7 strain (NCTC 10133) gave highly significant cross-protection against strain Blenheim. The challenge dose revealed no difference between the protection given by NCTC 10133 vaccine and homologous vaccine.

One strain each of *M. capricolum*, *M. equigenitalium*, *M. primatum* and F38-type mycoplasma failed completely to cross-protect against challenge with the Blenheim strain.

Cross-immunization against challenge with bovine serogroup 7 (*strain NCTC* 10133)

In three tests carried out on different occasions (Table 4), 16 mycoplasma strains belonging to seven species or types were examined for their ability to cross-protect against bovine serogroup 7 (NCTC 10133).

As may be seen from the results marked thus * in Table 4, nine heterologous strains afforded cross-protection, in each instance 'partial', i.e. significantly less than that given by the homologous strain. The nine protective strains comprised: each of three strains of the SC type of *M. mycoides* supsp. *mycoides*; one of seven strains of the LC type; each of three F38-type strains; and one strain each of *M. equigenitalium* and *M. primatum*. One strain of *M. capricolum*, six of seven LC-type strains, and one strain of *M. mycoides* subsp. *capri* failed to produce cross-protection.

DISCUSSION

Two F38-type strains, one of them a fresh field isolate, failed to produce mycoplasmaemia in mice inoculated intraperitoneally. Unfortunately, therefore, F38 challenge in immunization and cross-immunization experiments was ruled out. Several bovine serogroup 7 strains produced mycoplasmaemia, some apparently

Table 4. *Cross-immunization against challenge with bovine serogroup 7 (strain NCTC 10133)*

Vaccine prepared from mycoplasma of		Mycoplasmaemia in groups of vaccinated mice 1 day after challenge with stated dose (c.f.u. $\times 10^6$) in test no.			
Species or type	Strain	1 (242)	2 (2125)	3 (1550)	1–3 (aggregated results)
M. mycoides subsp. *mycoides* (SC type)	Blenheim	5/11	3/8	6/7	14/26*
	O goat	3/12	5/8	1/7	8/27*
	P goat	3/11	3/8	3/7	9/26*
M. mycoides subsp. *mycoides* (LC type)	Y goat	9/11	7/8	4/6	20/25
	Mankefår 2833	8/11	7/8	6/7	21/26
	F30	9/12	6/8	5/5	21/28
	74/2488	8/12	6/8	6/8	19/25
	Ojo I	4/12	5/8	8/8	17/28*
	Cov 2	10/12	8/8	6/8	24/28
M. mycoides subsp. *capri*	Smith 1423	9/12	8/8	6/7	23/27
F38-type mycoplasma	F38	3/12	4/8	5/8	12/28*
	G183/82	3/12	4/8	3/8	10/28*
	G275/82	6/12	2/8	4/8	12/28*
M. capricolum	NCTC 10154	7/12	8/8	8/8	23/28
M. equigenitalium	NCTC 10176	8/11	4/8*	...	...
M. primatum	NCTC 10163	1/12	3/8	1/8	5/28*
Bovine serogroup 7	NCTC 10133	0/25	0/25	3/25	3/75
None (controls)	...	19/25	25/25	23/25	67/75

* Cross-protection occurred as seen by comparison with the untreated controls ($P < 0{\cdot}001$; Wilson & Miles, 1975) but was 'partial' as seen by comparison with mice that received homologous vaccine ($P < 0{\cdot}022$).

more easily than others, but two did not. The number of laboratory subcultures undergone by these strains was, however, unknown; and it should be borne in mind that mycoplasmaemic ability may be affected adversely by repeated subculture (Smith, 1968; Dyson & Smith, 1976). A bovine serogroup L strain also produced mycoplasmaemia. *M. capricolum*, *M. equigenitalium* and *M. primatum* failed to do so, but the number of subcultures undergone by the strains examined was again unknown.

In the cross-immunization tests that followed, two challenge strains were used – Blenheim (*M. mycoides* subsp. *mycoides*, SC type) and NCTC 10133 (bovine serogroup 7). The range of immunizing strains used against NCTC 10133 challenge included a number of representatives of the SC and LC types of *M. mycoides* subsp. *mycoides*, and a strain of *M. mycoides* subsp. *capri*. These were omitted when Blenheim was used as the challenge strain because their inclusion would merely have repeated a part of studies made earlier and reviewed by Smith (1983).

M. capricolum, *M. equigenitalium*, *M. primatum* and F38 all failed to cross-protect against Blenheim, but serogroup 7 (strain NCTC 10133) cross-protected strongly and possibly 'completely' (as defined above). It should be noted, however, that

the challenge was comparatively small, and that a larger dose might have revealed the cross-protection to be 'partial'.

Despite the apparently close relation between Blenheim and NCTC 10133, strikingly different results were obtained in cross-protection tests in which these two strains were used for the purpose of challenge. Thus (1) F38-type strains, *M. equigenitalium* and *M. primatum* all gave partial cross-protection against NCTC 10133 but not against Blenheim, (2) of seven LC strains only one gave any cross-protection against NCTC 10133, whereas earlier work (Hooker, Smith & Milligan, 1979; Smith, Hooker & Milligan, 1980) suggested that all LC strains gave partial cross-protection against Blenheim, and (3) three SC strains gave only partial cross-protection against NCTC 10133, whereas earlier work (Smith, 1969*b*; Hooker *et al.* 1979; Smith, Hooker & Milligan, 1980) suggested that all SC strains gave complete cross-protection against heterologous SC strains. Strains NCTC 10133 and Blenheim were similar, however, in that *M. capricolum* and *M. mycoides* subsp. *capri* (Smith, 1969*b*; Smith & Oliphant, 1983*a*, *b*) failed to cross-protect against them both.

The experiments described show that in analysing the complex relation between F38 and allied mycoplasmas, as in a study of the subspecies and types of *M. mycoides* (see Smith, 1983), mycoplasmaemia and cross-protection tests in mice provide a valuable complement to serological and other *in vitro* methods.

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