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Immunogenic variation among the so-called LC strains of *Mycoplasma mycoides* subspecies *mycoides*

By G. R. SMITH and JANET C. OLIPHANT

Nuffield Laboratories of Comparative Medicine, Institute of Zoology, The Zoological Society of London, Regent's Park, London NW1 4RY

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SUMMARY

Much evidence of immunogenic heterogeneity among the LC strains of *Mycoplasma mycoides* ssp. *mycoides* emerged from cross-immunization and -hyperimmunization experiments in mice in which three LC strains (Vom/Plum Island, 74/2488, and Mankefår 2833) were used for challenge purposes. All heterologous LC-strain vaccines cross-immunized against the three challenge strains, but protection was usually only 'partial', i.e. significantly less than that given by homologous vaccine. Cross-hyperimmunization with all heterologous LC but not SC strains produced protection against challenge with Vom/Plum Island that was virtually 'complete', i.e. similar to that produced by homologous vaccine. Challenge with 74/2488 gave generally similar results; but against Mankefår 2833 six heterologous LC vaccines gave complete protection and six did not.

Vaccines prepared from the Smith (1423) strain of *M. mycoides* ssp. *capri* gave some protection against Vom/Plum Island but none against 74/2488 or Mankefår 2833. The cross-immunizing ability of three further *M. mycoides* ssp. *capri* strains appeared to resemble that of Smith (1423).

In a cross-hyperimmunization experiment, vaccines prepared from SC strains of *M. mycoides* ssp. *mycoides* varied greatly in their ability to protect against challenge with strains 74/2488 and Mankefår 2833.

INTRODUCTION

Mycoplasma mycoides subspecies (ssp.) *mycoides* and ssp. *capri* are, respectively, the causative agents of contagious bovine pleuropneumonia (CBPP) and of one form of contagious caprine pleuropneumonia (CCPP). In recent years strains of the so-called SC (small colony) and LC (large colony) types of *M. mycoides* ssp. *mycoides* – designations that are open to criticism (Valdivieso-Garcia & Rosendal, 1982) but nonetheless widely used – have been isolated from goats and occasionally other species including sheep. The caprine SC strains, of which few have been isolated, are thought to be identical with CBPP strains. The caprine LC isolates, of which there are many, are indistinguishable by *in vitro* serological tests from SC (including CBPP) strains; they are, however, distinguishable on the basis of proteolysis and heat-resistance (Cottew & Yeats, 1978), sorbitol fermentation and streptomycin sensitivity (Smith & Oliphant, 1982), and mycoplasmaemia and

cross-immunity tests in mice (Hooker, Smith & Milligan, 1979; Smith, Hooker & Milligan, 1980; Smith & Oliphant, 1981*a*, *b*).

On intraperitoneal inoculation a symptomless mycoplasmaemia is produced readily in mice by SC strains but much less readily by the LC variety – with the single known exception of Mankefår 2833 (Smith *et al.* 1980), a strain that has unusually robust growth properties (Smith & Oliphant, 1982). Cross-immunity tests are based on the ability of heterologous-strain vaccines to prevent the development of mycoplasmaemia after intraperitoneal challenge.

Cross-immunization and -hyperimmunization against challenge with SC strains suggest that, in respect of mouse-protective antigens, the SC (including CBPP) strains are all identical; but that SC strains, although sharing one or more antigens with LC strains, possess at least one unshared antigen of major importance (Smith, 1969*b*; Hooker *et al.* 1979; Smith *et al.* 1980; Smith & Oliphant, 1981*b*).

Cross-immunization against challenge with *M. mycoides* ssp. *capri* in mucin suggests that the LC and SC strains, though not identical with *M. mycoides* ssp. *capri*, are related to it, the former more closely than the latter (Smith & Oliphant, 1981*a*). Despite this relationship, *M. mycoides* ssp. *capri* vaccine failed to protect against challenge with two SC strains, or with the LC strain Mankefår 2833 (Smith, 1969*b*; Hooker *et al.* 1979; Smith *et al.* 1980).

Cross-immunization against LC strains has so far been limited to challenge with Mankefår 2833 by the absence of any other LC isolate capable of producing mycoplasmaemia with ease. Challenge with Mankefår 2833 suggests that this strain is related to all SC and other LC strains but that few if any of the latter are identical with it (Smith *et al.* 1980); to regard the LC strains as a homogeneous group would therefore be unwise. This is supported by the unusual mycoplasmaemic and cultural characters of Mankefår 2833, and by the slightly unusual properties of another LC strain, 143-A66 Conn (Smith *et al.* 1980; Smith & Oliphant, 1982).

The aims of the work described here were (1) to devise methods by means of which LC strains other than Mankefår 2833 could be used for challenge purposes in cross-immunization and -hyperimmunization experiments, and (2) to use such methods in studying further the possible heterogeneity of the LC strains, and their relationship to the SC strains. The rationale underlying the use of cross-hyperimmunization in addition to cross-immunization was, as already stated by Smith & Oliphant (1981*b*), that this procedure might be expected to obliterate the effects of quantitative – but not qualitative – differences between the protective-antigen content of different strains.

MATERIALS AND METHODS

Swiss white mice of one sex, usually female, were obtained from an outbred closed colony; those used in hyperimmunization experiments weighed 16–18 g, and the remainder 18–20 g. The mouse-immunization and -hyperimmunization tests were based on methods described by Smith (1967, 1969*a*, *b*, 1971), Hooker *et al.* (1979), and Smith & Oliphant (1981*a*, *b*). Viable counts of colony-forming units (c.f.u.) were made as described by Smith & Oliphant (1981*a*).

Mycoplasma strains

The three *M. mycoides* ssp. *capri* strains BQT, ZZ, and 74/5907A were described by Smith & Oliphant (1982), and the remaining 19 mycoplasma strains (5 SC, 13 LC, and 1 *M. mycoides* ssp. *capri;* see Table 3) by Smith & Oliphant (1981*a*). The minimum method of cloning was as stated by Smith & Oliphant (1981*a*), but many of the cultures were in addition cloned three times by the filtration method (Report, 1979).

Mycoplasmaemia tests of LC strains, with and without mucin

Three-day BVF-OS (Turner, Campbell & Dick, 1935) cultures of 12 LC strains were tested in dilutions of 1 in 1, 10, and 100, with and without mucin. The inoculum for each mouse contained 0·1 ml of an appropriate dilution of culture mixed with either 0·4 ml of 5 % mucin suspension (1701-W Granular Mucin; Wilson Laboratories, Chicago, USA) or 0·4 ml of BVF-OS broth. The mice were tested for mycoplasmaemia (see below) 1, 2, and 3 days after infection.

Immunization and hyperimmunization

For the purpose of this paper these terms are defined as follows.

'Immunization' means the intravenous injection, 21 days before challenge, of 0·25 ml of a 3-day BVF-OS culture killed by heating at 56 °C for 30 min in a waterbath. The SC-strain vaccines were usually somewhat less turbid than the LC- or *M. mycoides* ssp. *capri*-strain vaccines, except in one experiment (strain 74/2488 challenge; experiment no. 2), in which this state of affairs was deliberately reversed by five-fold concentration of the SC vaccines – without noticeable effect.

'Hyperimmunization' means the intravenous injection of two 0·25 ml doses of living BVF-OS culture; of a total of 198 vaccine cultures, 194 had been incubated for 3 days and four for 4–6 days. The interval between the two hyperimmunizing doses was 21 days except in one experiment (strain 74/2488 challenge), in which it was 10 days. The interval between the second dose and challenge was always 21 days. Smith & Oliphant (1981*a*) showed the value of a single intravenous injection of living mycoplasma culture as an exceptionally potent immunizing procedure. The use of two doses was thought likely to provide a near-maximal antigenic stimulus.

Unimmunized control mice received 0·25 ml doses of BVF-OS broth intravenously.

Challenge

Mouse intraperitoneal inocula consisted of either (1) 0·5 ml of 3-day BVF-OS culture, undiluted or diluted 1 in 2 in BVF-OS, or (2) 0·1 ml of a similar material mixed with 0·4 ml of 5 % mucin suspension. Information as to whether mucin was or was not used in the various experiments is given in the Tables. The tests were assessed by the presence or absence of mycoplasmaemia 1 day, and often 2 and 3 days also, after challenge.

Detection of mycoplasmaemia

The method of tail-blood culture in selective medium was as described by Smith & Oliphant (1981*a*).

RESULTS

Mycoplasmaemia tests of 12 LC strains, with and without mucin*

In general the enhancing effect of mucin on mycoplasmaemia was undramatic – less than that seen with *M. mycoides* ssp. *capri* (Smith & Oliphant, 1981*a*) – and in a few instances absent altogether. For challenge purposes in cross-immunity experiments the two most suitable strains appeared to be 74/2488 (with or without mucin) and Vom/Plum Island (with mucin). In large doses these strains produced mycoplasmaemia of at least 3 days' duration in most mice. At least eight other strains might also have been suitable; admittedly, however, some of them produced mycoplasmaemia of only short duration, even with very large infecting doses.

Cross-immunization against challenge with the LC strain Vom/Plum Island

Vaccines prepared from 18 mycoplasma strains (4 SC, 12 heterologous LC, the homologous LC, and 1 *M. mycoides* ssp. *capri*) were used. The blood cultures made three days after challenge (Table 1) showed that all heterologous vaccines, including that prepared from *M. mycoides* ssp. *capri* strain Smith (1423), were protective ($P < 0{\cdot}004$). The blood cultures made 1 day after challenge showed, for all heterologous vaccines except Cov 2, that this protection was 'partial' ($P < 0{\cdot}013$), i.e. significantly less than that given by homologous vaccine. Blood cultures made on day 2 (not recorded in Table 1) showed that the same was true of Cov 2 vaccine; for the Cov 2 and Vom/Plum Island vaccines the results were, respectively, 3/8 and 0/20 ($P < 0{\cdot}004$).

Cross-immunization against challenge with the LC strain 74/2488

The vaccines used were similar to those in the previous experiment. The results of three separate experiments are shown in Table 2. In contrast to its behaviour in the previous experiment, vaccine prepared from *M. mycoides* ssp. *capri*, strain Smith (1423) gave no protection. This is clear from the data in Table 2; and also from data not shown, namely, the aggregated results (experiments 1–3) of blood cultures made 3 days after challenge – 21/60 (unvaccinated controls) and 10/24 (Smith 1423 vaccine). Vaccines prepared from all the SC and LC strains of *M. mycoides* ssp. *mycoides* were protective; this is shown by the blood cultures made in experiments 1 and 2 ($P < 0{\cdot}004$) and by the aggregated results of experiments 1–3 ($P < 0{\cdot}001$). The aggregated results show, however, that of the 16 heterologous-strain vaccines that gave protection, 12 protected only partially ($P < 0{\cdot}0025$, 10 strains; $P < 0{\cdot}025$, two strains); the protection given by the remaining four vaccines (Y goat, Ojo I, F 30, and S-5-64) did not differ significantly from that given by homologous vaccine ($P > 0{\cdot}05$).

Cross-immunization against challenge with the LC strain Mankefår 2833

For the sake of completeness this already published experiment (Smith *et al.* 1980) is briefly mentioned. Vaccine prepared from the *M. mycoides* ssp. *capri* strain Smith (1423) gave no protection. SC vaccines and all except three of 12 heterologous

* A Table describing these tests may be obtained from the Library of The Zoological Society of London.

Table 1. *Cross-immunization against challenge with the LC strain Vom/Plum Island*

Vaccine (killed, single dose)	Colonial type of vaccine strain	Mycoplasmaemia in groups of mice at the stated intervals (days) after challenge*	
		1	3
Blenheim	SC	7/8	1/8
O goat	SC	8/8	4/8
P goat	SC	8/8	4/8
Vom/Parkville	SC	6/8	3/8
Y goat	LC	7/8	2/8
Ojo I	LC	5/8	0/8
Ojo II	LC	5/8	1/8
Cov 2	LC	4/8	1/8
2605-Razi	LC	8/8	3/8
74/2488	LC	6/8	3/8
222-69 N.Y.	LC	5/8	2/8
143-A66 Conn	LC	7/7	2/7
Mankefår 2833	LC	7/8	1/8
F 30	LC	6/8	3/8
Ghaleh Morghi-16	LC	8/8	5/8
S-5-64	LC	7/8	1/8
Smith 1423 (ssp. *capri*)	(LC)	8/8	3/8
Vom/Plum Island	LC	3/20	0/20
None (controls)	—	20/20	20/20

* Challenge dose (c.f.u., 10^6), 258 (with mucin); SC, small colony; LC, large colony.

LC vaccines gave partial protection. The three apparently exceptional LC vaccines (F 30, S-5-64, and Ghaleh Morghi-16) gave protection that was complete, i.e. it resembled the protection given by homologous vaccine.

Cross-hyperimmunization against challenge with the LC strains Vom/Plum Island, 74/2488, and Mankefår 2833

Of the 19 vaccine strains used, all except one (*M. mycoides* ssp. *capri*, strain Smith 1423) had been shown by the cross-immunization experiments just described to be capable of protecting, incompletely as a rule, against heterologous challenge with strains Vom/Plum Island, 74/2488, and Mankefår 2833. Strain Smith (1423) protected only against Vom/Plum Island. The purpose of the present experiment was to test, in respect of each vaccine strain, the ability of hyperimmunization to produce 'complete' cross-protection, i.e. protection not differing significantly from that produced by homologous vaccine. As in similar experiments reported by Smith & Oliphant (1981*b*) a small proportion of the mice died from anaphylactic shock shortly after challenge; most, however, were unaffected.

Against Vom/Plum Island challenge, each of three SC strains failed ($P < 0{\cdot}006$) to give complete protection, but 12 heterologous LC strains, and *M. mycoides* ssp. *capri* strain Smith (1423), succeeded in doing so (Table 3).

Against 72/2488 challenge, each of four SC strains protected only partially

Table 2. *Cross-immunization against challenge with the LC strain* 74/2488

Vaccine (killed, single dose)	Colonial type of vaccine strain	Mycoplasmaemia in groups of mice 1 day after challenge* in experiment no: 1	2	3	1–3 (aggregated)
Blenheim	SC	4/8	4/8	5/8	13/24
O goat	SC	5/8	5/8	4/8	14/24
P goat	SC	3/7	3/8	6/8	12/23
Vom/Parkville	SC	2/8	1/8	5/8	8/24
Y goat	LC	3/8	1/8	3/8	7/24
Ojo I	LC	1/8	1/7	2/7	4/22
Ojo II	LC	3/8	4/7	4/8	11/23
Cov 2	LC	2/8	2/8	5/8	9/24
2605-Razi	LC	4/8	2/8	7/8	13/24
Vom/Plum Island	LC	3/8	1/6	8/8	12/22
222-69 N.Y.	LC	2/8	2/8	7/8	11/24
143-A66 Conn	LC	5/8	4/8	3/8	12/24
Mankefår 2833	LC	4/8	3/8	6/8	13/24
F 30	LC	1/8	0/8	2/8	3/24
Ghaleh Morghi-16	LC	2/8	0/7	8/8	10/23
S-5-64	LC	0/8	2/8	4/8	6/24
Smith 1423 (ssp. *capri*)	(LC)	8/8	7/8	8/8	23/24
74/2488	LC	2/19	1/19	4/20	7/58
None (controls)	—	20/20	20/20	20/20	60/60

* Challenge doses (c.f.u., 10^6) in experiments 1, 2 and 3 were, respectively, 144 and 116 (with mucin), and 982 (without mucin).

SC, small colony; LC, large colony.

($P < 0{\cdot}01$ to $< 0{\cdot}001$), but 10 of 12 heterologous LC strains protected completely. Two caprine SC strains (O goat and P goat) were more immunogenic than two bovine SC strains (Blenheim and Gladysdale) ($P < 0{\cdot}04$ to $< 0{\cdot}001$). The *M. mycoides* ssp. *capri* strain Smith (1423) gave no protection.

Against Mankefår 2833, four of five SC strains failed to give complete protection ($P < 0{\cdot}02$ to $< 0{\cdot}001$); the fifth (P goat) succeeded, and in doing so differed strikingly from the other four strains ($P < 0{\cdot}014$ to $< 0{\cdot}001$). Of 12 heterologous LC strains, six gave complete protection and six failed to do so ($P < 0{\cdot}04$ to $< 0{\cdot}001$); *M. mycoides* ssp. *capri* strain Smith (1423) failed completely to protect.

The cross-hyperimmunizing ability of three additional strains of M. mycoides *ssp.* capri

The experiments already described had shown that the *M. mycoides* ssp. *capri* strain Smith (1423) cross-protected against the LC strain Vom/Plum Island but not against the LC strains 74/2488 and Mankefår 2833. As mentioned earlier, it failed to protect against two SC strains, one of which was strain Blenheim (Smith, 1969*b*; Hooker *et al.* 1979).

Vaccines prepared from *M. mycoides* ssp. *capri* strains BQT, ZZ, and 74/5907A were tested for their ability to protect against the three strains Blenheim,

Table 3. *Cross-hyperimmunization against challenge with the LC strains Vom/Plum Island*, 74/2488, *and Mankefår* 2833

Vaccine (living, two doses)	Colonial type of vaccine strain	Mycoplasmaemia in groups of mice one day after challenge* with strain		
		Vom/Plum Island	74/2488	Mankefår 2833
Blenheim	SC	6/11	10/12	9/11
Gladysdale	SC	N	12/12	7/12
O goat	SC	4/12	3/12	10/11
P goat	SC	6/12	5/12	0/12
Vom/Parkville	SC	N	N	5/12
Y goat	LC	1/12	1/11	4/11
Ojo I	LC	0/12	0/10	2/12
Ojo II	LC	0/12	0/7	1/10
Cov 2	LC	2/12	3/12	4/10
2605-Razi	LC	0/12	2/11	3/11
222-69 N.Y.	LC	0/12	3/11	6/9
143-A66 Conn	LC	0/12	2/12	9/11
F 30	LC	0/12	0/9	0/11
Ghaleh Morghi-16	LC	0/12	1/11	5/10
S-5-64	LC	0/11	0/12	0/10
Vom/Plum Island	LC	**0/20**	1/11	8/12
74/2488	LC	0/12	**0/17**	2/10
Mankefår 2833	LC	0/12	1/22	**1/18**
Smith 1423 (ssp. *capri*)	(LC)	1/12	12/12	10/11
None (controls)	—	18/19	19/19	19/20

* Challenge doses (c.f.u., 10^6) of Vom/Plum Island, 74/2488, and Mankefår 2833 were, respectively, 367 (with mucin), and 425 and 1045 (without mucin).

SC, small colony; LC, large colony; N, not done.

The results for groups (not shown) that received heat-killed Vom/Plum Island, 74/2488, and Mankefår 2833 vaccines were, respectively, 0/20, 1/17, and 1/20.

Vom/Plum Island, and Mankefår 2833 (Table 4). Like strain Smith (1423) they protected against Vom/Plum Island ($P < 0{\cdot}001$) but not against the other two strains.

DISCUSSION

In-vitro tests have demonstrated a close similarity between different strains of the LC type; no differences have emerged apart from the unusual growth curves of strain Mankefår 2833 and 143-A66 Conn (Smith & Oliphant, 1982). As mentioned earlier, however, mycoplasmaemia and cross-immunization experiments have already suggested that the LC strains are not entirely homogeneous. The present study has given much support to this view. (1) Like strain Mankefår 2833 (Smith *et al.* 1980), strains Vom/Plum Island (Table 1) and 74/2488 (Table 2) showed in cross-immunization experiments that they were related to all other LC (and SC) strains but, because the immunity produced was only partial, were rarely identical with them. (2) Cross-hyperimmunization (Table 3) with all heterologous LC but not SC strains produced virtually complete protection against challenge with strain Vom/Plum Island; challenge with strain 74/2488 gave results that were

Table 4. *The cross-hyperimmunizing ability of M. mycoides ssp. capri strains BQT, ZZ, and 74/5907A*

Vaccine (living, two doses)	Mycoplasmaemia in groups of mice at the stated intervals after challenge* with strain					
	Blenheim		Vom/Plum Island		Mankefår 2833	
	1 day	3 days	1 day	3 days	1 day	3 days
BQT	8/8	5/8	3/8	0/8	7/7	7/7
ZZ	8/8	6/8	2/8	0/8	8/8	8/8
74/5907A	7/7	4/7	2/7	0/7	8/8	8/8
Blenheim	0/8	0/8	—	—	—	—
Vom/Plum Island	—	—	0/8	0/8	—	—
Mankefår 2833	—	—	—	—	0/7	0/7
None (controls)	20/20	14/20	19/19	15/19	19/20	19/20

* Challenge doses (c.f.u., 10^6) of Blenheim, Mankefår 28333, and Vom/Plum Island were, respectively, 114 and 774 (without mucin) and 141 (with mucin).

generally similar though slightly less clear cut. Challenge with strain Mankefår 2833, however, gave results of a completely different kind: six heterologous LC vaccines gave complete protection and six did not. (3) Cross-immunization and -hyperimmunization with vaccine prepared from the Smith (1423) strain of *M. mycoides* ssp. *capri* clearly gave some protection against strain Vom/Plum Island but none against strains 74/2488 or Mankefår 2833 (Tables 1–3). Results that accorded completely with this observation were obtained when vaccines prepared from three further *M. mycoides* ssp. *capri* strains were used to cross-immunize against challenge with strains Vom/Plum Island and Mankefår 2833 (Table 4).

CBPP strains are thought to belong to a single antigenic type and to be indistinguishable from SC strains from goats. It seemed surprising, therefore, that in a cross-hyperimmunization experiment (Table 3) vaccines prepared from different SC strains varied greatly in their ability to protect against challenge with strains 74/2488 and Mankefår 2833: challenge produced mycoplasmaemia in 25–100 % and 0–90·9 % of mice, respectively, according to the vaccine strain used. These possibly important observations require careful confirmation.

The immunogenic relationship between *M. mycoides* ssp. *capri* and *M. mycoides* ssp. *mycoides* (SC and LC strains), and its sometimes unidirectional nature, require further study. The SC strains Blenheim, Gladysdale, KH_3J, O goat, P goat, and Vom/Parkville all protect to some degree against the Smith (1423) strain of *M. mycoides* ssp. *capri* (Smith, 1969*b*; Smith & Oliphant, 1981*a*). Strain Smith (1423), however, is completely unable to protect against Blenheim or O goat (Smith, 1969*b*; Hooker *et al.* 1979); even four consecutive doses of dead, or two consecutive doses of living, strain Smith (1423) are without protective effect against Blenheim (G. R. Smith, unpublished). Again, all LC strains protect to some degree against the Smith (1423) strain of *M. mycoides* ssp. *capri* (Smith & Oliphant, 1981*a*); the latter, however, does not protect against the LC strains Mankefår 2833 (Smith *et al.* 1980; and Table 3 of this paper) and 74/2488 but does so against the LC strain Vom/Plum Island (Tables 1–3). The behaviour of three further *M. mycoides* ssp.

capri strains shown in Table 4 is consistent with the foregoing observations. The mechanisms concerned in unidirectional protection would seem an intriguing topic for further study.

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