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The ability of *Mycoplasma mycoides* subspecies *mycoides* and closely related strains from goats and sheep to immunize mice against subspecies *capri*

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SUMMARY

Small colony (SC) strains of *Mycoplasma mycoides* subsp. *mycoides* from contagious bovine pleuropneumonia (CBPP) and from goats were compared with large colony (LC) strains of so-called *M. mycoides* subsp. *mycoides* from goats and sheep by means of a cross-protection test in which mice were challenged with *M. mycoides* subsp. *capri*.

Of 13 LC strains, all gave partial cross-protection, and 11 were shown to be more closely related than four SC strains to subspecies *capri*. In a further experiment, six SC strains – three from CBPP and three from goats – all gave weak partial cross-protection against subspecies *capri*.

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) – one of the great cattle plagues – is now confined to a number of African countries and to parts of Asia. It is caused by *Mycoplasma mycoides* subsp. *mycoides*.

In recent years numerous caprine and ovine mycoplasmas said to be indistinguishable from CBPP strains by *in vitro* serological tests have been isolated in CBPP-free countries (see Hooker, Smith & Milligan, 1979). Such strains are often openly referred to by the name *M. mycoides* subsp. *mycoides*. They are of two types – small colony (SC) and large colony (LC). The SC strains appear to be indistinguishable in all respects from CBPP strains (Cottew & Yeats, 1978; Hooker *et al.* 1979). The LC strains differ from SC strains by certain *in vitro* characteristics (Cottew & Yeats, 1978); they also differ in protecting mice partially instead of completely against challenge with SC strains, and in being unable – with the single exception of the Mankefår 2833 strain – to produce mycoplasmaemia readily in mice (Hooker *et al.* 1979; Smith, Hooker & Milligan, 1980). Two-dimensional gel electrophoresis of cell proteins led Rodwell & Rodwell (1978) to suggest that the LC strains were more closely related to *M. mycoides* subsp. *capri* than to the SC strains.

By means of active and passive cross-protection tests in mice Smith (1969) found that a CBPP strain (Blenheim) immunized slightly but significantly against a strain of subspecies *capri* (now known as strain 'Smith 1423'). The present paper

is concerned with a comparison of the ability of the SC and LC types of genuine or so-called *M. mycoides* subsp. *mycoides* to cross-protect mice against subspecies *capri*.

MATERIALS AND METHODS

Mycoplasma strains

Strains of *M. mycoides* subsp. *mycoides* from CBPP comprised the virulent organisms Blenheim and Gladysdale, and the attenuated vaccine strain KH_3J. All CBPP strains are small colony (SC) producers.

SC caprine strains, indistinguishable from *M. mycoides* subsp. *mycoides*, comprised strains O goat, P goat, and Vom/Parkville.

Large colony (LC) strains of so-called *M. mycoides* subsp. *mycoides* from goats and sheep comprised strains Y goat, Ojo I, Ojo II, Cov 2, 74/2488, 143-A66 Conn, 2605-Razi, Vom/Plum Island, 222-69 N.Y., F 30, Mankefår 2833, Ghaleh Morghi-16, and S-5-64. The three last-named strains were of ovine origin; the other ten were from goats.

A single strain of *M. mycoides* subsp. *capri* – 'Smith (1423)' – was used.

The strains and their source were described by Hooker *et al.* (1979) and Smith *et al.* (1980); strain Gladysdale was described by Smith (1968). The majority came from either Dr A. H. Dardiri or Dr G. S. Cottew, both of whom have used them in taxonomic studies (Al-Aubaidi, Dardiri & Fabricant, 1972; Cottew & Yeats, 1978). Before use the strains were subcultured from single colonies on at least one, and usually on three occasions, but not by passage through filters.

Viable counts

The method was as described by Hooker *et al.* (1979) except that the means of triplicate counts of colony-forming units (c.f.u.) were taken as the true values.

Mice

Female Swiss white mice from an outbred closed colony were used. They weighed 18–20 g at the beginning of each experiment except that concerned with living vaccines, in which they weighed 16–18 g.

Active cross-protection tests with killed vaccines

The methods were based on those described by Smith (1967, 1969, 1971) and Hooker *et al.* (1979). Groups of mice were immunized intravenously with 0·25 ml volumes of mycoplasma culture grown for 3 or 4 days in BVF-OS medium (Turner, Campbell & Dick, 1935) and killed by heating at 56 °C for 30 min in a waterbath. Control mice received 0·25 ml of sterile BVF-OS intravenously. Each mouse was challenged 21 days later by the intraperitoneal injection of *M. mycoides* subsp. *capri*. The tests were assessed by the presence or absence of mycoplasmaemia 1, 2 and 3 days after challenge.

Three such tests were made with the 18 vaccines shown in Table 1. A fresh batch of vaccines was prepared for each test and used either after storage at 4 °C for not more than 24 h, or after storage for a longer period at −20°C. In the first test the four vaccines prepared from SC strains (see Table 1) had an optical density lower than that of the 14 vaccines prepared from LC strains. In the second and third

tests this state of affairs was deliberately reversed; the mycoplasmas in each of the four SC-strain vaccines were deposited by centrifugation and resuspended in one-fifth of the total volume of supernate.

Passive cross-protection test

For the preparation of each immune antiserum 60 mice were inoculated intraperitoneally with mycoplasmas grown in BVF-OS for 4 days, each mouse receiving 0·5 ml of a 1 in 5 dilution of culture. Four such groups of mice received strain Blenheim, O goat, P goat, or Vom/Parkville in doses (c.f.u., 10^6) of 429, 297, 420 and 271, respectively. A further group received 0·5 ml of sterile BVF-OS. After 28 days the mice were bled by the method of Evans & Perkins (1954) and five pools of serum – four immune and one control – were prepared, sterilized by filtration through cellulose acetate membranes, and stored at −20 °C. Cultures from the liver and spleen of six mice from each of the four infected groups showed that no living mycoplasmas remained 28 days after inoculation.

The protection test was made by injecting each of the five pools of undiluted serum subcutaneously into a group of mice (0·25 ml per mouse). Each group was then subdivided to allow for intraperitoneal challenge, 2–3 h later, with a large and a smaller dose of *M. mycoides* subsp. *capri*. The test was assessed by the presence or absence of mycoplasmaemia 1 and 2 days after challenge.

Active cross-protection test with living vaccines

Seven groups of mice received intravenous injections (0·25 ml per mouse) of 3-day cultures in BVF-OS of strains Blenheim, Gladysdale, KH_3J, O goat, P goat, Vom/Parkville, and Smith (1423). The doses of mycoplasmas per mouse were respectively (c.f.u., 10^6) 1118, 1276, 484, 1010, > 118, and 322. An eighth group (controls) received sterile BVF-OS (0·25 ml per mouse) intravenously. Blood cultures made 24 h later showed that all mice had mycoplasmaemia, except those in the control group. This mycoplasmaemia would have lasted no more than a few days. Twenty-eight days after infection each group was subdivided to allow for intraperitoneal challenge with a large and a smaller dose of *M. mycoides* subsp. *capri*. The test was assessed on the presence or absence of mycoplasmaemia 24 h after challenge.

Challenge procedure

Each mouse received an intraperitoneal injection of strain Smith (1423) of *M. mycoides* subsp. *capri* in a dose of 0·1 ml of a 3-day BVF-OS culture – either undiluted or diluted in BVF-OS – mixed with 0·4 ml of a 5 % suspension of mucin. The mucin came from an old batch of 1701-W Granular Mucin (Wilson Laboratories, Chicago, U.S.A.); the suspension was prepared, autoclaved and neutralized according to the manufacturer's instructions. The infections were less severe than those produced earlier by comparable inocula (Smith, 1967), possibly owing to the age of the mucin preparation; the mice did not become obviously ill, and the number of animals with mycoplasmaemia declined appreciably within 3 days.

Detection of mycoplasmaemia in mice

A drop of tail-blood from each mouse was cultured in 5 ml of ONB-OS medium (Hooker *et al.* 1979) containing selective substances (penicillin, 100 I.U./ml; thallous acetate, 0·05%). After incubation for 7 days, subcultures were made on Blood Agar Base No. 2 (Oxoid) containing defibrinated horse blood 15%, and selective substances. The subcultures were incubated for not less than 4 days.

RESULTS

Active cross-protection tests with killed vaccines

The 18 vaccines used were prepared from four SC and 13 LC strains of genuine or so-called *M. mycoides* subsp. *mycoides*, and from the Smith (1423) strain of *M. mycoides* subsp. *capri*. Table 1 shows the results of blood cultures made 1 and 3 days after challenge with Smith (1423) in three different experiments; it also shows the aggregated results of the three experiments. Four conclusions can be drawn.

(1) Vaccines prepared from each of the 13 LC strains of so-called *M. mycoides* subsp. *mycoides* gave significant protection against challenge with subspecies *capri*. This is shown by the blood cultures made on day 1 in experiment 3 ($P < 0·005$); except for Ghaleh Morghi-16 vaccine it is also shown by the aggregated results of blood cultures made on day 1 ($P < 0·03$).

(2) The protection given by the 13 LC-strain vaccines against subspecies *capri* was 'partial', i.e. significantly less than that given by homologous vaccine. This is shown for all vaccines except Ojo I by the aggregated results of blood cultures made on day 1 ($P < 0·03$). It is shown for Ojo I vaccine by data not included in Table 1, namely the aggregated results of blood cultures made on day 2; for the Ojo I and Smith (1423) vaccines these results were, respectively, 6/24 and 1/24 ($P < 0·05$).

(3) Two of four SC-strain vaccines (Blenheim and O goat) gave slight but significant protection against subspecies *capri*. This is shown by the aggregated results of blood cultures made on day 3 ($P < 0·001$).

(4) The protection given by all except two (143-A66 Conn and Ghaleh Morghi-16) of the 13 LC-strain vaccines was significantly greater ($P < 0·03$) than that given by any SC-strain vaccine. This is shown for 10 strains by the aggregated results of blood cultures made on day 1 ($P < 0·025$), and for strain 2605-Razi by the blood cultures made on day 1 in experiment 3 ($P < 0·013$).

The data given in Table 1 show that, where partially protective vaccines are concerned, the results of a single cross-protection test may be misleading – hence the need to repeat such tests on different occasions, with different challenge doses (see also Hooker *et al.* 1979; Smith *et al.* 1980).

Passive cross-protection test

Smith (1969) found that the CBPP strain Blenheim protected very slightly but significantly against subspecies *capri*. The experiments just described confirmed this finding and showed that a second SC strain (O goat) behaved similarly. The apparent failure of two further SC strains to cross-protect seemed to warrant

Table 1. *The ability of* M. mycoides *subsp.* mycoides *and closely related strains from goats and sheep to immunize mice against challenge* 21 *days later with subspecies* capri (*strain Smith* 1423)

Vaccine	Colonial type of vaccine strain	Mycoplasmaemia in groups of mice: 1 day after challenge in experiment no. 1	1 day after challenge in experiment no. 2	1 day after challenge in experiment no. 3	1 day after challenge in experiments no. 1–3 (aggregated)	3 days after challenge in experiments no. 1–3 (aggregated)
Blenheim	SC	7/7	8/8	7/8	22/23	6/23
O goat	SC	7/8	8/8	6/8	21/24	5/24
P goat	SC	8/8	8/8	6/8	22/24	14/24
Vom/Parkville	SC	8/8	8/8	6/7	22/23	12/23
Y goat	LC	4/8	8/8	2/8	14/24	2/24
Ojo I	LC	4/8	3/8	0/8	7/24	1/24
Ojo II	LC	4/7	2/8	2/8	8/23	1/23
Cov 2	LC	5/7	6/8	0/8	11/23	1/23
74/2488	LC	7/8	4/8	2/8	13/24	3/24
2605-Razi	LC	8/8	8/8	1/8	17/24	7/24
Vom/Plum Island	LC	6/8	2/8	1/8	9/24	0/24
222-69 N.Y.	LC	6/8	5/8	2/8	13/24	3/24
143-A66 Conn	LC	8/8	8/8	3/8	19/24	12/24
Mankefår 2833	LC	7/8	4/8	1/8	12/24	7/23
F 30	LC	7/8	4/8	0/8	11/24	2/24
Ghaleh Morghi-16	LC	8/8	8/8	4/8	20/24	10/24
S-5-64	LC	7/7	4/8	2/8	13/23	4/23
Smith (1423)	(LC)	1/8	1/8	0/8	2/24	0/24
None (controls)	—	19/20	19/20	19/20	57/60	35/60

SC = small colony; LC = large colony.
The challenge doses (c.f.u., 10^6) in experiments 1, 2 and 3 were 318, 229 and 111, respectively.

Table 2. *Attempted passive immunization of mice against* M. mycoides *subsp.* capri (*strain Smith* 1423) *with antisera against SC strains* (*one bovine, three caprine*) *of subspecies* mycoides

Interval after challenge (days)	Mice pretreated with serum against	Mycoplasmaemia in groups of 20 mice challenged 24 h earlier with subspecies *capri* in doses (c.f.u., 10^6) of	
		120	24
1	Blenheim	19	9*
	O goat	19	14
	P goat	18	15
	Vom/Parkville	19	15†
	Broth (controls)	18	18
2	Blenheim	13	1*
	O goat	11	6
	P goat	11	2
	Vom/Parkville	17	2†
	Broth (controls)	10	7

* Significantly different from controls ($P < 0{\cdot}02$).
† Nineteen mice in group.

Table 3. *Active immunization of mice against* M. mycoides *subsp.* capri (*strain Smith* 1423) *with living cultures of SC strains* (*three bovine and three caprine*) *of subspecies* mycoides

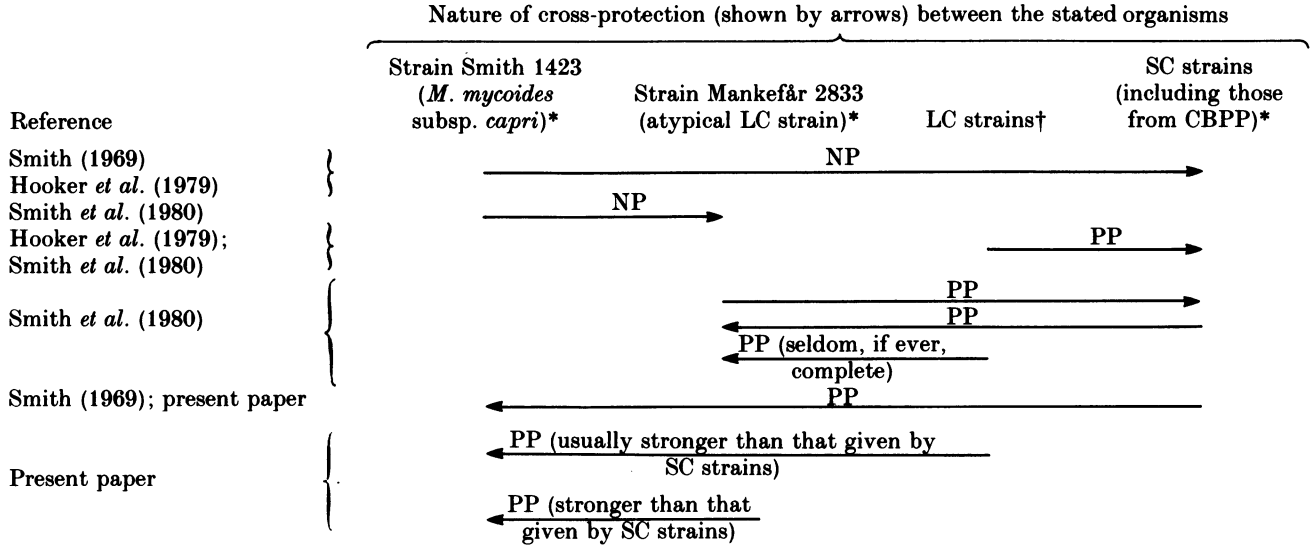
Vaccine	Source of vaccine strain	Mycoplasmaemia in groups of mice 24 h after challenge with *M. mycoides* subsp. *capri* in doses (c.f.u., 10^6) of	
		169	34
Blenheim	CBPP	8/12*	2/12†
Gladysdale	CBPP	7/12*	0/12†
KH_3J‡	CBPP	10/12	3/12†
O goat	Goat	8/12*	1/12†
P goat	Goat	6/11*	5/12†
Vom/Parkville	Goat	8/12*	6/11†
Smith (1423)	Goat	0/12	0/11
Broth (control)	—	19/20	18/20

* Protection significant ($P < 0{\cdot}04$) but less than that given by homologous vaccine ($P < 0{\cdot}005$).
† Protection significant ($P < 0{\cdot}025$).
‡ Attenuated CBPP-vaccine strain. CBPP = contagious bovine pleuropneumonia.

re-investigation. The method chosen was one that Smith (1969) found more satisfactory than an active protection test with killed vaccine.

Table 2 shows the results of a passive protection test in which groups of mice were challenged with subspecies *capri* after pretreatment with antisera produced by infecting mice with living organisms. The smaller of two challenge doses readily demonstrated cross-protection by strain Blenheim. It failed, however, to show such protection by strains O goat, P goat, and Vom/Parkville; this failure led to the next experiment.

Table 4. *Summary of information given by cross-protection tests on the relation between SC strains, LC strains, and* M. mycoides *subsp.* capri



* Used as challenge strain in protection tests.
† Not yet used as challenge strains.
NP = no protection; PP = partial protection (i.e. significantly less than that given by homologous vaccine).

Active cross-protection test with living vaccines

Groups of mice were immunized with live cultures of the four SC strains used in the preceding experiment, the two SC strains Gladysdale and KH_3J, and the challenge strain (Smith 1423) of subspecies *capri*. Table 3 shows the results of blood cultures made 24 h after challenge.

The method readily demonstrated cross-protection by the three SC strains from CBPP, including the highly attenuated strain KH_3J, and by the three SC strains from goats. The protection was invariably partial, i.e. significantly less that that given by the homologous strain.

DISCUSSION

This study showed that of 13 LC strains of so-called *M. mycoides* susp. *mycoides*, 11 were more closely related than four SC strains to *M. mycoides* subsp. *capri*. The cross-protection given by SC strains was so slight that it could sometimes only be demonstrated by a particularly potent immunizing procedure, namely, infection with living organisms. This procedure was successful not only for caprine SC strains and virulent SC strains from CBPP, but also for a highly attenuated CBPP-vaccine strain (KH_3J).

The various relationships between SC strains, LC strains, and subspecies *capri* shown in this study and in earlier reports (Smith, 1969; Hooker *et al.* 1979; Smith *et al.* 1980) are brought together in Table 4. The cross-protection between subspecies *capri* on the one hand, and both the LC-strain Mankefår 2833 and the SC strains on the other, is seen to be uni-directional. Smith *et al.* (1980) suggested, with certain reservations, that the LC strains might be more closely related to the SC strains than to subspecies capri. The present study, in which the LC strains gave some protection against subspecies *capri*, is not inconsistent with an opposite view, expressed earlier by Rodwell & Rodwell (1978).

No differences were demonstrated between SC strains from goats and those from CBPP. Such strains remain indistinguishable from each other by all known methods.

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