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The production of mastitis in cows by the intramammary inoculation of T-mycoplasmas

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SUMMARY

Six milking cows were inoculated with bovine and human T-mycoplasmas and control materials into the udder via the teat canal. Control materials produced only a slight transient cell response in the milk. Bovine T-mycoplasmas produced clinical mastitis in nine out of ten quarters inoculated. Seven developed clinical mastitis together with visible changes in the milk, excretion of T-mycoplasmas and greatly increased cell counts in the milk. In three of these quarters, in two different cows, milk secretion ceased completely. Two quarters in a different cow showed visible milk changes, excretion of T-mycoplasmas and increased cell counts. Two quarters were inoculated with human T-mycoplasmas and neither produced any signs of mastitis.

Infection of the udder with T-mycoplasmas did not stimulate high-titre serum antibody levels as measured by the metabolic inhibition test, but whey samples gave high titres in two of the cows that were able to control and resolve the infection.

INTRODUCTION

T-mycoplasmas were isolated first by Shepard (1954) from the human urogenital tract. Since then they have been isolated from the human oropharynx (Taylor-Robinson & Purcell, 1966), from the urogenital tract of cattle (Taylor-Robinson, Haig & Williams, 1967), from pneumonic calf lungs (Gourlay, 1968), from eyes in cases of infectious bovine keratoconjunctivitis (Gourlay & Thomas, 1969), from the throats of cats (Tan & Markham, 1971), from the genital tract of dogs and the throats of squirrel monkeys (Taylor-Robinson, Martin-Bourgon, Watanabe & Addey, 1971).

The role of T-mycoplasmas in disease is not clear: they have been incriminated in non-specific urethritis in man (Shepard, 1969), abortions and premature births in women (Knudsin, Driscoll & Ming, 1967) and pneumonia in calves (Gourlay & Thomas, 1970). In the last instance pneumonic lesions were produced in calves following the endobronchial inoculation of T-mycoplasmas.

In view, however, of the difficulty of obtaining calves of an appropriate gnotobiotic specification for further experimental work on pneumonia, and the difficulty of monitoring the progress of pneumonia, we decided to investigate whether the bovine mammary gland was an alternative site for studies of T-mycoplasma

infection. An advantage of the bovine udder is that it comprises four separate quarters; all of which are accessible for clinical examination, easy to sample and have a limited and readily determined bacterial flora. This paper reports on the production of mastitis in cows by the intramammary inoculation of T-mycoplasmas and on the potential value of the udder for studying the pathogenesis of bovine T-mycoplasma infections and the immune response of the host.

MATERIALS AND METHODS

Cows

Six Ayrshire or Friesian Cross Ayrshire cows, 3–5 years of age, were used. They were each giving about 3 gal. of milk a day at the time of inoculation, except cow M 153 which was giving only 1½ gal. Total cell counts were performed on samples of milk from each quarter for a few days before inoculation and only quarters that had a cell count of less than $10^{5\cdot2}$ cells/ml. were used. Milk smears were stained by the 'Single Dip' method of Broadhurst & Paley (1939) and cells were counted by the technique described by Pattison & Holman (1951). By cultural methods no large-colony or T-mycoplasmas were demonstrated in the pre-inoculation milk samples.

After the afternoon milking the cows were inoculated in each quarter with 10 ml. of either T-mycoplasma broth culture or control broth material. The inoculum was inserted via the teat canal with a syringe. Sixteen hours after inoculation, and at daily intervals thereafter, milk samples were examined from each quarter for abnormal appearance, T-mycoplasmas, cells and bacteria. The quarters were also examined clinically for signs of mastitis.

Strains of T-mycoplasma

Four strains of T-mycoplasmas were used: the A 417 strain that had been isolated from a pneumonic calf lung (Gourlay & Thomas, 1970), the D 32 strain that had also been isolated from a pneumonic calf lung (calf 24, table 1, Gourlay, Mackenzie & Cooper, 1970), the REOW strain isolated from the human urogenital tract and supplied by Dr D. Taylor-Robinson, and the M 126/68 strain also isolated from the human urogenital tract and supplied by Dr B. E. Andrews. Two different substrains of the A 417 strain were used; the first was at the 10^{-11} dilution from the original lung tissue while the second – 'cloned' – had undergone six subcultures in broth of which three had been from the terminal dilution, as a means of purification, and was at a 10^{-25} dilution from the original lung. The D 32 strain had undergone eight subinoculations, three of them from the terminal dilution, and was at a 10^{-23} dilution from the original lung.

The T-mycoplasmas were grown in U-broth. This was similar to GS broth (Gourlay & Leach, 1970) except that it also contained 0·1% urea, 5% yeast extract (Difco) and 0·05 M Hepes (Sigma). Glucose, lactalbumin hydrolysate and DNA were omitted and the pH adjusted to 6·0. The titre of viable organisms in cultures or milk was estimated by making duplicate serial tenfold dilutions in U-broth. Growth produced an alkaline shift of pH as a result of the metabolism of urea and was indicated by a colour change of phenol red in the medium. Viability was esti-

mated as colour change units (c.c.u.). The titre was the highest dilution that produced a colour change after incubation at 37° C.

Control inocula were either uninoculated broth or broth culture of T-mycoplasmas which had been incubated at 37° C. until viable organisms could no longer be demonstrated (dead organisms). The pH was then adjusted to 7·0.

Histopathology

Representative portions of mammary tissue, taken at autopsy, were fixed in neutral buffered formalin. Paraffin sections were stained by haematoxylin and eosin.

Serology

Sera and whey were examined for antibodies to T-mycoplasma (A 417 strain) by the metabolic inhibition (MI) test of Purcell, Taylor-Robinson, Wong & Chanock (1966). Titres are given as the reciprocal of the highest dilution of serum that inhibited growth.

Sera were also examined for the presence of haemoglobin reactive protein (HRP) as described by Spooner & Miller (1971).

Bacteriology

The number of bacteria was estimated in milk samples by spreading 0·1 ml. of milk over the surface of an ox-blood-agar plate and counting the number of colonies after incubation at 37° C. for 24 hr. The bacterial count in milk was not considered significant if there were fewer than 1000 colony-forming units per ml. and there was an absence of obvious mastitis pathogens.

RESULTS

Udder response

Details of the inocula used for the six cows are given in Table 1. The results obtained from cows A 5, L 629 and L 686 are given in Figs. 1–3. All three cows developed mastitis in the quarters inoculated with 10^6 or more viable A 417 T-mycoplasmas, as indicated by an increase in milk cells, and visible milk and udder abnormalities. T-mycoplasmas were also isolated from these quarters. Plate 1A shows the changes in appearance of milk from the LF quarter of cow L 629. Injection of control materials into the udder produced only a transient cell response in milk. Uninoculated broth gave only a slight cell response but the injection of dead organisms produced a greater though still transient cell response. Cow L 686 reacted slowly and less severely than the others and eventually overcame the infection. The RH and LH quarters of this cow developed only transient cell responses. T-mycoplasmas were not isolated at any time from the milk from the RH quarter even though the control 'dead' inoculum unfortunately contained 10^3 viable organisms. T-mycoplasmas were isolated from milk of the LH quarter (inoculated with REOW organisms) only on the day after inoculation.

Cow L 91 reacted only slightly to the intramammary inoculation of mycoplasmas and only the LF quarter developed more than a transient cell response. It is of

Table 1. *Details of inocula*

Cow	Quarter	Inoculum
A 5	LF	10^6 A 417
	LH	Uninoculated broth
	RF	NI
	RH	NI
L 629	LF	10^7 A 417
	LH	Dead A 417
	RF	10^7 A 417
	RH	Uninoculated broth
L 686	LF	10^7 A 417 'cloned'
	LH	10^7 REOW
	RF	10^6 A 417
	RH	10^3 'dead' A 417 'cloned'
L 91	LF	10^7 A 417 'cloned'
	LH	10^6 M 126/68
	RF	10^6 A 417
	RH	Dead A 417
M 153	LF	10^8 A 417
	LH	NI
	RF	NI
	RH	NI
M 626	LF	NI
	LH	10^6 D 32
	RF	NI
	RH	10^6 A 417 'cloned'

NI = not inoculated.

particular interest that the RF quarter did not develop mastitis, considering the results with the inoculation of A 417 organisms into the first three cows. The milk from the LF quarter had an initial cell response, in which T-mycoplasmas were isolated, but the cell response had returned to normal by the 5th day. On the 6th day, a second response occurred which became moderately severe and persistent. T-mycoplasmas were still being excreted in the milk at a low titre after 41 days. Milk and udder abnormalities were observed at the height of the second response.

Cows M 153 and M 626 were killed before the full extent of their infection could be assessed. The LF quarter of cow M 153 reacted severely and, at slaughter on the 4th day after inoculation, the T-mycoplasma titre was 10^6/ml. and the cell count was $10^{7.7}$ cells/ml. Udder abnormalities were evident, manifest by induration and a drop in milk yield, while the milk became watery and separated. Cow M 626 was killed 9 days after inoculation. The two inoculated quarters both reacted; the RH one more severely than the LH one. T-mycoplasmas were isolated from both quarters up to the time the cow was killed. The maximum titres were 10^6 organisms/ml. in the RH quarter and 10^5 organisms/ml. in the LH quarter. The cell counts in the RH quarter reached a maximum of $10^{8.2}$ cells/ml. and in the LH quarter $10^{7.3}$ cells/ml. Both quarters produced abnormal milk but showed no obvious udder abnormalities. The milk from the RH quarter became watery and separated whereas the milk from the LH quarter contained only clots. Small

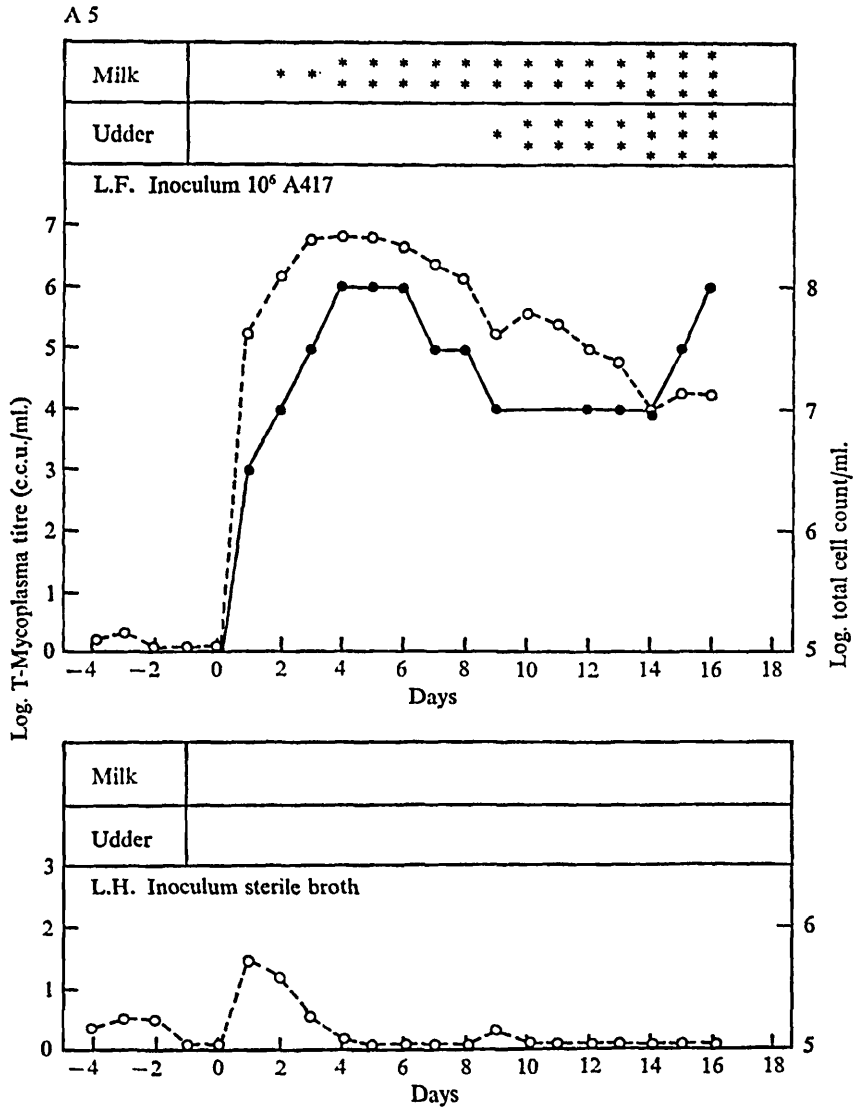

Fig. 1. Cow A 5. Daily milk cell count O--O, T-mycoplasma titre in milk ●——●, and milk and udder abnormalities. Inocula: L.F. quarter, 10^6 A 417; L.H. quarter, uninoculated broth. Milk abnormalities: * dark yellow, ** containing clots, *** separated–clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

pieces of udder tissue were taken from these latter two cows for histopathological examination.

Bacteriology

Bacterial counts in milk were monitored throughout the course of mycoplasma infections, as a guard against the possibility of a concurrent bacterial infection causing the mastitis, coincident with high mycoplasma titres. There was no increase in the bacterial count in any of the experiments reported. In fact, the

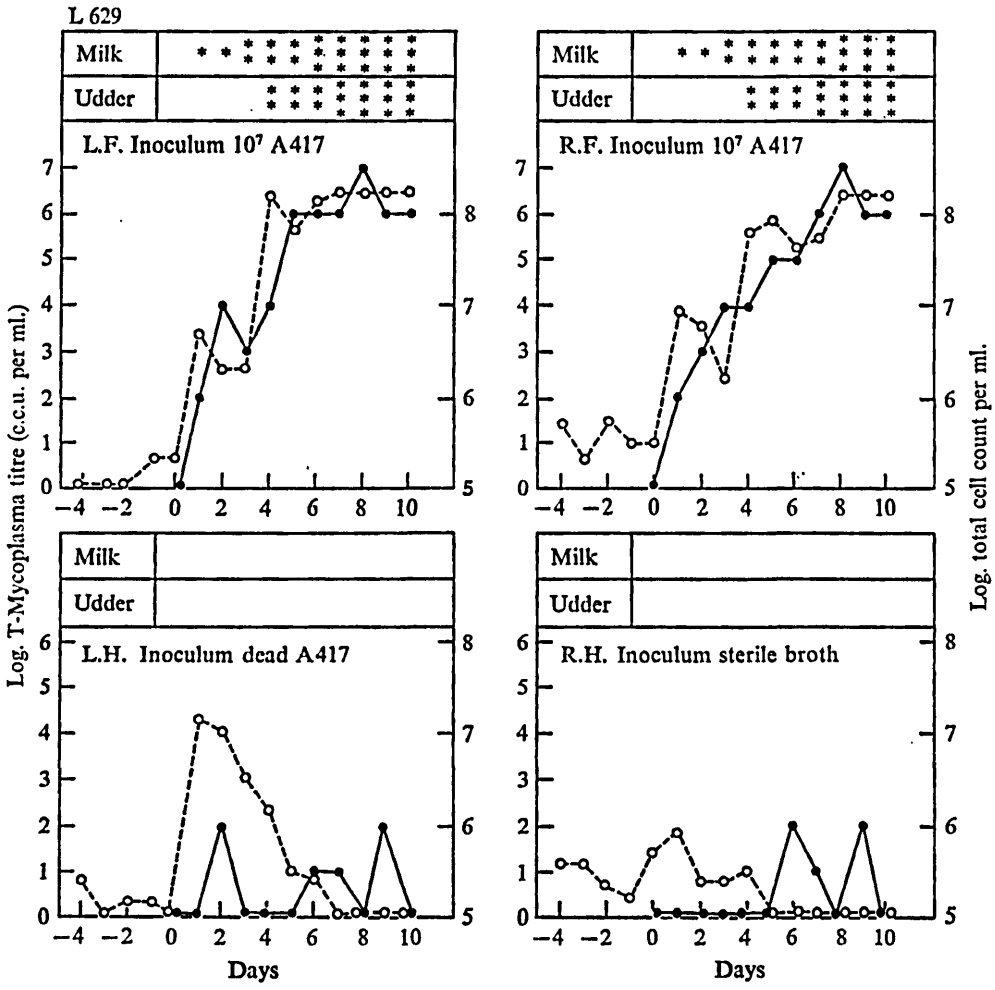

Fig. 2. Cow L 629. Daily milk cell count O--O, T-mycoplasma titre in milk ●—●, and milk and udder abnormalities. Inocula: L.F. and R.F. quarter: 10^7 A 417; L.H. quarter: dead A 417; R.H. quarter: uninoculated broth. Milk abnormalities: * dark yellow, ** containing clots, *** separated – clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

bacterial count noticeably decreased in quarters with high mycoplasma titres and, in many instances, the milk appeared bacteriologically sterile.

Serology

No serum samples were obtained from cow M 153 as it was slaughtered after only 4 days. All the other serum samples tested before inoculation had an antibody titre of < 10. MI antibody titres of 20 were found in the sera of three of the five cows after infection. These were cows A 5, L 629 and L 686.

All whey samples taken before inoculation showed MI antibody titres of < 10. The whey from cow L 91 appeared to stimulate the growth of the T-mycoplasmas. After inoculation, MI antibody (titre > 640) was found in whey samples from all

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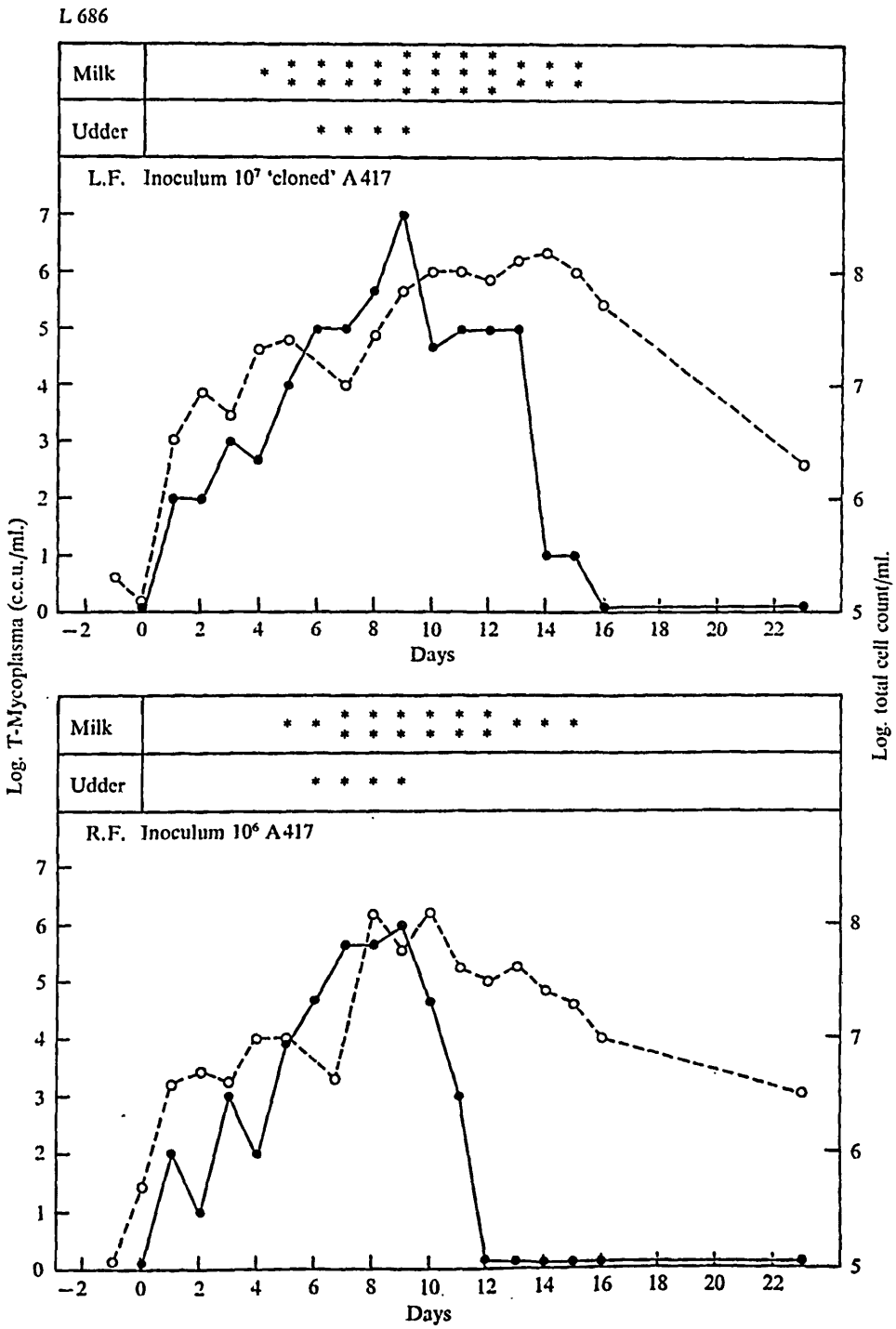

Fig. 3. Cow L 686. Daily milk cell count O– –O, T-mycoplasma titre in milk ●——●, and milk and udder abnormalities. Inocula: L.F. quarter, 10^7 'cloned' A 417; R.F. quarter, 10^6 A 417. Milk abnormalities: * dark yellow, ** containing clots, *** separated – clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

inoculated quarters of cows L 686 and L 626 and in the whey from the LF quarter only of cow L 91. Whey from the three remaining quarters of cow L 91 still stimulated growth of T-mycoplasmas. The whey samples from both quarters of cows A 5 and L 629 had titres of < 10 and 20 respectively.

HRP was not detected in any of the pre-inoculation serum samples except for cow L 91. HRP was detected in the sera of only two of the cows after infection. Cow A 5 possessed HRP in serum taken on day 13, and cow L 629 possessed HRP on day 10 and slight HRP on day 20.

Histopathology

Histopathological examination of udder sections from cow M 153 showed marked infiltration of neutrophils into the alveolar lumina together with interstitial hyperaemia (Plate 1B). Udder sections from cow M 626 showed a similar picture but in addition the RH quarter showed evidence of interstitial infiltration and involution.

DISCUSSION

Certain mycoplasmas have been shown to be responsible for natural outbreaks of mastitis. These are *M. bovigenitalium* (Davidson & Stuart, 1960; Stuart *et al.* 1963), *M. agalactiae var. bovis* or *M. bovimastitidis* (Hale, Helmboldt, Plastridge & Stula, 1962; Jain, Jasper & Dellinger, 1969) and mycoplasma belonging to Leach's (Leach, 1967) serological group 7 (Connole, Laws & Hart, 1967). In addition there is one recorded natural case of mastitis due to *M. bovirhinis* (Langer & Carmichael, 1963). All these mycoplasmas can cause mastitis when experimentally inoculated into the udder. *Acholeplasma laidlawii* failed to produce experimental mastitis (Jain *et al.* 1969).

From our work it is evident that certain T-mycoplasmas isolated from pneumonic calf lungs can produce mastitis when inoculated into the bovine udder. From a total of 10 quarters, in 6 different cows, inoculated with these bovine T-mycoplasmas, 7 developed clinically observable changes in the quarter, milk changes and a greatly increased cell count, and in 3 of these quarters milk secretion ceased completely. Two quarters showed milk changes and greatly increased cell counts, and one did not react at all. Two other quarters, inoculated with human T-mycoplasmas, did not develop mastitis. Five quarters were inoculated with control materials in 4 different cows, 2 with uninoculated broth, 2 with non-viable T-mycoplasmas and 1 with a mixture of non-viable and viable T-mycoplasmas. Uninoculated broth produced only a minimal cell response on the day following inoculation, which rapidly reverted to normal. The non-viable T-mycoplasmas produced a more severe cell response on the day after inoculation; this decreased progressively to revert to normal after about 5 days. The low titre of viable organisms in one of the 'dead' mycoplasma controls had no apparent effect.

T-mycoplasmas were detected in milk from all quarters that were inoculated with the bovine T-mycoplasmas. From one quarter (that which did not develop any signs of mastitis) these organisms were only detected at a very low titre on the day after inoculation. In all the other infected quarters mycoplasmas were

detected daily until either the quarter went dry, the animal was killed or the infection was resolved. However, there was one exception, when T-mycoplasmas were not detected, this being from the LF quarter of cow L 91 on the 5th day after inoculation. There can be no doubt that the T-mycoplasmas actually multiplied in the quarters, considering the large volume of milk produced by the cows throughout the infection period and the consistently high titres of mycoplasmas recorded in the milk. The titre of mycoplasmas excreted in the milk increased to a peak that corresponded roughly to the maximum cell response and also the maximum milk and udder abnormalities. The titre of T-mycoplasmas and the total cell counts in the milk usually corresponded closely and indicated a close relationship between the mycoplasmas and cells. Following the inoculation of human T-mycoplasmas, organisms were detected only on the day following inoculation and this appeared to be due to survival of the inoculum. Except in one animal, T-mycoplasmas were not detected in the milk from quarters inoculated with control materials. In this one animal (L 629) the detection of T-mycoplasmas was not accompanied by a cellular response and we presume contamination of the milk samples occurred at milking. This failure to detect T-mycoplasmas in the control quarters would indicate a lack of spread of infection between quarters, in contrast to what occurs with other mastitis-producing mycoplasmas (Stuart *et al.* 1963; Hale *et al.* 1962; Jain *et al.* 1969).

There was some variation in response of these cows to inoculation of T-mycoplasmas unrelated to inoculum titre. Cows A 5, L 629 and M 153 reacted quickly and severely while the remainder (L 686, L 91 and M 626) reacted more slowly and on the whole less severely. It would appear that these latter three cows possessed some resistance to T-mycoplasma infection, the nature of which is unknown at present.

The failure of the human T-mycoplasmas to produce mastitis may be indicative of a species specificity, but only the REOW strain result can be considered at all significant as the M 126/68 strain was perhaps inoculated at too low a titre in a relatively resistant animal. It is, however, possible that the REOW strain had become attenuated or was an avirulent strain originally.

The evidence from cow M 626 may indicate that the A 417 strain of bovine T-mycoplasma is more pathogenic than the D 32 strain.

Infection of the udder with T-mycoplasmas did not stimulate high titre serum antibody levels. It is interesting to note that in the two cases, cows A 5 and L 629, where high titre MI antibody was not found in whey, the infected quarters eventually ceased to produce milk. In cows L 686, L 91 and M 626 whey samples gave high MI titres as a result of the infection and in the case of L 91 and L 686 this was associated with an ability of the animals to control and resolve the infection respectively. Animal M 626 was killed before the infection had run its course.

The milk and udder abnormalities together with the histopathological findings indicate that T-mycoplasmas produce an inflammatory reaction in the udder and furthermore the demonstration of HRP in the sera of two cows indicates that this may sometimes be of an acute nature.

We have no evidence at all that T-mycoplasmas are responsible for natural cases

of mastitis in cattle, although from the experimental results it is not inconceivable that they might be. From our point of view, however, the main conclusion to be drawn from this work is that the bovine udder appears to be a suitable model for studying the pathogenesis and immunity of bovine T-mycoplasma infections.

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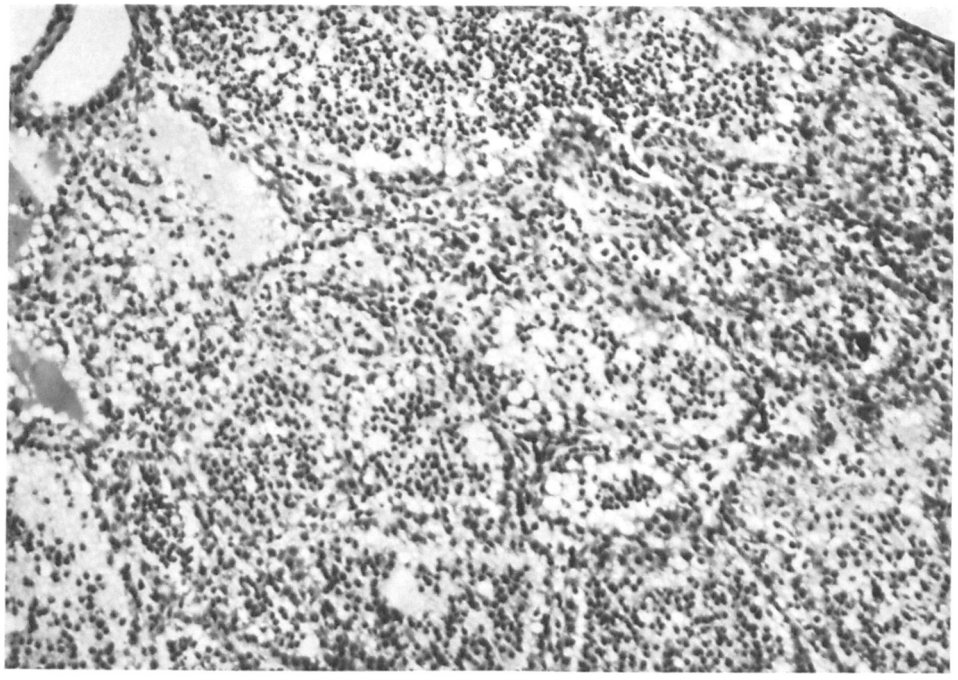

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EXPLANATION OF PLATE

(A) Changes in the appearance of milk from the L.F. quarter of cow L 629 following the intramammary inoculation of 10^7 uncloned A 417 T-mycoplasma culture. Left, day 0; centre, day 5; right, day 7.

(B) Cow M 153. L.F. quarter. Histological section showing neutrophil infiltration of the alveoli, and slight interstitial hyperaemia. H and E. × 147.

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