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Localized Immunity in Experimental Bovine Mastitis Caused by *Mycoplasma dispar*

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Development of immunity to reinfection with *Mycoplasma dispar* occurred in the bovine mammary gland and appeared to be confined to the quarter or quarters previously infected with this mycoplasma; it was not generalized throughout the gland. Immunity followed the inoculation of virulent strains of *M. dispar* but not of an avirulent strain. The immunity engendered protected against both homologous and heterologous strains of *M. dispar*. Challenge of immune quarters with virulent *M. dispar* resulted in a transient polymorphonuclear cell response greater than that observed in previously uninoculated quarters.

Mycoplasma dispar is considered to be involved in the etiology of calf pneumonia (4, 5, 9). The use of the bovine mammary gland to examine pathogenicity of *M. dispar* in bovine infections has proved of value, and in a recent paper it was reported that six out of seven strains of *M. dispar* were capable of producing clinical mastitis when inoculated into the lactating bovine mammary gland via the teat canal (2).

The purpose of the experiments reported here was to use the bovine mammary gland to study immunity in *M. dispar* infections and to determine particularly whether previous infection resulted in immunity, whether this immunity, if it occurred, was strain or species specific, and whether it was restricted to the previously infected quarter or was generalized throughout the mammary gland. The results indicate that immunity to *M. dispar* does occur but differs in some important respects from the immunity to the ureaplasmas (T-mycoplasmas) previously reported, using the bovine mammary gland system (6).

MATERIALS AND METHODS

M. dispar strains. Details of the strains of *M. dispar* and the medium used to grow them have been published (7).

Inoculation of cows. Four cows were inoculated in one or two quarters of the mammary gland with virulent strains of *M. dispar* (Gri226, F370, and 462/2), and two cows were inoculated with an avirulent strain (Vic12). The infections produced by the virulent strains were monitored as described previously for studies on the immunity to ureaplasma mastitis (6). The criteria used to determine whether mastitis was produced were persistent increase in the number of milk inflammatory cells, increased conductivity of the milk compared with preinoculation samples, and persistent isolation of *M. dispar* from the milk. The animals were challenged with a virulent strain of *M. dispar* by the intramammary route when the initial infection had resolved, that is, when mycoplasmas were no longer detectable in the milk and the milk cells and conductivity had returned to normal, which was usually within a month of the primary inoculation. In the case of the cows primarily inoculated with the avirulent strain the cows were challenged 3 to 4 weeks after the primary inoculation.

Antibody measurement. Serum and whey taken before and after inoculation were subsequently examined for antibody to *M. dispar* by the indirect hemagglutination test, using tanned formalinized sheep erythrocytes (8) and strain 462/2 (NCTC 10125) grown in glucose-calf serum broth (7) and not sonicated as antigen. Titers were expressed as the reciprocal of the highest dilution of serum that caused agglutination.

BioGel chromatography. Serum and whey were fractionated on BioGel 1A as previously described (1).

RESULTS

Effect of previous infection on the susceptibility of the mammary gland to reinfection with the same and different strains of M. dispar. The results of the challenge experiments are given in Table 1. Six quarters (in the mammary glands of four cows) previously infected with virulent *M. dispar* strains were challenged; three quarters were challenged with the homologous strain and three with heterologous strains of *M. dispar*. At the same time quarters in the same cows not previously exposed were also challenged. All previously exposed quarters proved to be immune to reinfection, but in these cows previously uninoculated quarters possessed no immunity to chal-

TABLE 1. *Susceptibility of previously infected and noninfected quarters to challenge with Mycoplasma dispar*

Animal no.	Quarters[a]	Primary infection strain	Challenge		
			Strain	Dose[b]	Mastitis
C197	LF	Nil	F370	10^9	+
	LH	Gri226	F370	10^9	−
	RF	Nil	Gri226	10^8	+
	RH	Gri226	Gri226	10^8	−
B89	LF	Nil	F370	10^{10}	+
	LH	Gri226	F370	10^{10}	−
C182	LF	Gri226	Gri226	$10^{7.7}$	−
	LH	Nil	Gri226	$10^{7.7}$	+
M106	LH	Nil	F370	10^9	+
	RF	462/2	F370	10^9	−
	RH	F370	F370	10^9	−
D657	LF	Vic12[c]	462/2	10^9	+
	LH	Vic12	Gri226	$10^{8.7}$	+
	RF	Nil	462/2	10^9	+
	RH	Nil	Gri226	$10^{8.7}$	+
C60	RF	Vic12[c]	Gri226	$10^{8.7}$	+
	RH	Nil	Gri226	$10^{8.7}$	+

[a] LF, Left fore quarter; LH, left hind quarter; RF, right fore quarter; RH, right hind quarter.
[b] Total *M. dispar* (color-change units) inoculated.
[c] No infection resulted.

lenge with either homologous or heterologous strains of *M. dispar*.

The Vic12 strain of *M. dispar*, which appeared to be avirulent to the bovine mammary gland in previous experiments (2), was inoculated into two quarters of one cow (D657) and one quarter of another cow (C60). No infection resulted, and when these quarters and the uninoculated quarters were challenged no immunity was observed (Table 1).

Antibody response. The antibody titers in the sera of two cows, B89 and C197, and whey samples of all six cows, measured by the indirect hemagglutination test, are shown in Table 2. A rise in antibody titer was detected in the sera and some whey samples by the metabolism inhibition test (10). This test proved to be less sensitive than the indirect hemagglutination test, but essentially the results were the same with both tests. In general the quarters in which primary infections were produced possessed higher titers of antibody than the uninoculated quarters. In the case of cow B89 this was particularly marked, the inoculated quarter possessing a titer of 512 whereas the uninoculated quarter had a titer of only 16.

Prechallenge serum and whey from cow B89 were fractionated on a BioGel column. The peak of antibody activity tested by the indirect hemagglutination test was found in identical fractions of serum and whey and corresponded to the immunoglobulin G peak in previous experiments (1). Thus no evidence was obtained for the antibody present in serum and whey being of different classes.

Isolation of M. dispar from the milk. In all the challenged quarters *M. dispar* was isolated from the milk on the day after inoculation. In the quarters that were subsequently shown to be immune the organisms could no longer be detected on the 2nd day after challenge or thereafter. On the other hand, in the nonimmune quarters *M. dispar* was isolated daily throughout the period of infection.

Cell response in the milk. In all the challenged quarters an increased cell response was observed on the day after challenge. In those quarters that were subsequently shown to be immune this response returned to normal within 4 to 6 days. In 5 of the 11 quarters that were not immune the initial increased cell response remained high as the quarters became infected. In the remaining six quarters the cell response returned to normal temporarily before increasing again as infection developed.

The total number of cells and the conductivity readings in the milk on the day before and

TABLE 2. *Reciprocal of the antibody titers in serum and whey samples measured by indirect hemagglutination*

Animal no.	Sample[a]	Preinoculation titer	Prechallenge titer
C197	Serum	256	≥2,048
	Whey LF	ND[b]	16
	LH[c]	ND	64
	RF	ND	8
	RH[c]	5	32
B89	Serum	2	1,024
	Whey LF	2	16
	LH[c]	2	512
C182	Whey LF[c]	2	2
	LH	2	<2
M106	Whey LH	<2	<2
	RF[c]	<2	2
	RH[c]	<2	2
D657	Whey LF[d]	8	8
	LH[d]	<2	<2
	RF	<2	2
	RH	<2	2
C60	Whey RF[d]	2	2
	RH	2	2

[a] Abbreviations as in Table 1.
[b] ND, Not done.
[c] Quarters previously infected with virulent *M. dispar*.
[d] Quarters previously inoculated with avirulent *M. dispar*.

TABLE 3. *Cell response and conductivity of milk after challenge with Mycoplasma dispar*

Cow	Quarter[a]	Previously inoculated quarters				Quarter	Previously uninoculated quarters			
		Day before challenge		Day after challenge			Day before challenge		Day after challenge	
		Conductivity+ (μmho)	Cells[b]	Conductivity	Cells		Conductivity	Cells	Conductivity	Cells
C197	LH	5.1	≤5.0000	6.8	≥8.0000	LF	4.4	≤5.0000	5.8	6.8751
	RH	4.9	≤5.0000	7.1	≥8.0000	RF	4.3	≤5.0000	5.7	6.7634
B89	LH	6.0	5.9542	10.0	7.4624	LF	6.2	5.3010	8.5	7.7782
C182	LF	4.1	≤5.0000	7.0	≥8.0000	LH	4.8	5.6021	5.5	7.5185
M106	RF	5.0	≤5.0000	6.7	7.4232	LH	5.0	≤5.0000	5.5	6.9542
	RH	5.3	≤5.0000	7.0	7.0828					
D657[c]	LF	4.1	≤5.0000	4.7	6.7160	RF	4.2	≤5.0000	5.3	7.0607
	LH	4.3	≤5.0000	5.3	7.1271	RH	4.4	≤5.0000	5.6	7.0000
C60[c]	RF	4.0	≤5.0000	5.0	6.8451	RH	4.0	≤5.0000	4.4	6.0414

[a] Abbreviations as in Table 1.
[b] Log total cells per milliliter.
[c] Previously inoculated with avirulent *M. dispar*.

the day after challenge are shown in Table 3. The number of cells on the day before challenge in both the previously inoculated and uninoculated quarters was in all but three quarters $\leq 10^{5.0}$ cells/ml. In the other three quarters the numbers were $10^{5.3}$, $10^{5.6}$, and $10^{5.9}$ cells/ml, respectively. On the day after challenge a cell response was observed in all quarters, both those previously inoculated with the virulent and avirulent strains of *M. dispar* and those previously uninoculated. However, it was evident that the response, in all but one cow, was considerably greater in the quarters that had previously been inoculated with virulent *M. dispar*, being $\geq 10^{8.0}$ cells/ml in three of these quarters.

On the day after challenge all the quarters that had previously been infected with the virulent *M. dispar* strains produced grossly abnormal milk, it being yellow in color and separated into a clear whey and a floccular deposit. Examination of the cells in the deposit revealed the great majority to be polymorphonuclear, mainly neutrophils. Polymorphonuclear cells also predominated in the less severe cellular responses in the other quarters after challenge, including those that had not been previously inoculated.

The conductivity figures of the milk on the day before challenge in previously inoculated quarters averaged 4.75 μmho and in previously uninoculated quarters 4.66. The relevant average conductivity figures for the quarters previously inoculated with virulent *M. dispar* were: day before challenge, average figure 5.06 μmho; day after challenge, average 7.43. The figures for the previously uninoculated quarters were 4.94 and 6.20, respectively. The differences in conductivity in the quarters were analyzed as a randomized block experiment. A significant increase in conductivity was observed in both uninoculated and previously inoculated quarters compared to preinoculation milk samples ($P < 0.01$). Also the increase in conductivity on the day after challenge was significantly higher in previously infected quarters than in previously uninoculated quarters ($P < 0.01$). On the day after challenge milk from quarters previously inoculated with the avirulent Vic12 strain of *M. dispar* did not have conductivity values any higher than uninoculated quarters.

DISCUSSION

All quarters of the mammary gland previously infected with *M. dispar* were subsequently immune to reinfection with either the homologous or heterologous strains of *M. dispar*. It should, however, be noted that *M. dispar* appears to be a serologically homogeneous species, and all four strains used in this work cross-react serologically (7). On the other hand, previously uninoculated quarters in the same cow were not immune to challenge. Therefore, the immunity in this experimental system would appear to be a property of the individual quarter rather than of the whole mammary gland. This situation is in contrast to that observed in earlier studies with ureaplasmas, in the same experimental system, in which immunity was generalized throughout the mammary

gland and was not confined to the previously infected quarters (6).

None of the quarters of the two cows (D657 and C60) previously inoculated with an avirulent strain of *M. dispar* appeared to possess any immunity to challenge. It may be that an active infection is needed to produce immunity in the mammary gland to *M. dispar*. It should be noted that neither of these cows possessed in their whey any antibody detectable by the indirect hemagglutination test to *M. dispar*, thereby indicating that the antibodies detected in the whey of the other cows were not produced against medium constituents.

Preinoculation and prechallenge sera from two of the cows were examined. In both cows primary infection resulted in a considerable rise in antibodies in the sera detected by indirect hemagglutination (Table 2). One of these cows (C197) possessed a relatively high titer before infection, but this did not appear to affect the infectious process. Despite the possession of high serum antibody titers in cows C197 and B89, only the quarters that had had prior infection were immune to challenge; thus the presence of high serum antibody titers did not appear to correlate with resistance to infection in the mammary gland. Although the antibody titer in previously infected quarters was generally higher than that of uninfected quarters, the titers were still less than in the serum of the same cow. It is therefore possible that the higher titers in the previously infected quarters resulted from serum leakage into the quarter due to inflammation produced by the primary infection. No evidence was obtained that the antibody activity, detected by indirect hemagglutination, in whey and serum was due to different antibody classes. Thus no evidence for local antibody synthesis was obtained by the techniques used.

Challenge of quarters that had been previously inoculated with virulent *M. dispar* resulted in a massive cellular response that was not readily quantifiable due to the clotted nature of the floccular deposit produced but which was recorded as $\geq 10^{8.0}$ cells/ml. These cells were mainly polymorphonuclear. A response of this magnitude was not observed in previously uninoculated quarters. Besides this, the conductivity of whey from immune quarters taken the day after challenge was significantly higher than the conductivity of whey taken from previously uninoculated nonimmune quarters on the same day, a further indication of a more severe inflammatory response in the immune quarters on the day after challenge. It is, therefore, possible that some mechanism localized in the previously infected quarter of the mammary gland results in an increased polymorphonuclear cell response on challenge, and the local immunity produced may be dependent on these cells together with serum factors present as a result of the inflammation. Perhaps an allergic reaction could be involved. An increased cell response in the respiratory tract of hamsters was observed when previously infected animals were challenged with *M. pneumoniae* (3).

These results together with those previously published (6) may indicate the existence of two adaptive mechanisms effective against mycoplasmas in the bovine mammary gland. One mechanism, following infection in one quarter, results in immunity throughout the mammary gland and is observed in ureaplasma infections of the mammary gland (6). The other mechanism involves only the previously infected quarter and is evident in immunity to *M. dispar*.

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