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Cell-Mediated Immune Response in Cattle to *Mycoplasma mycoides* var. *mycoides*

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The cell-mediated immune response of cattle to *Mycoplasma mycoides* var. *mycoides* was studied. Sensitized lymphocytes in blood leukocyte preparations showed a significant degree of antigen-induced transformation, judged by the uptake of tritiated thymidine. The increase in tritiated thymidine uptake in sensitized lymphocytes in the presence of *M. mycoides* membrane antigen varied from 2- to 13-fold compared with the controls, and this increase in activity was observed from 3 days after artificial infection. Inhibition of leukocyte migration by *M. mycoides* membrane antigen commenced between 17 and 30 days after infection, and preliminary observations indicate that this test correlated with the intradermal allergic test. *M. mycoides*-induced unresponsiveness was demonstrated 23 and 30 days after infection. Unresponsiveness, in that the lymphocytes did not respond to phytohemagglutinin, was very marked in two of three animals and partial in the third animal, whereas the humoral antibody response did not appear to be affected. Antigen-induced transformation was demonstrated in only two out of six cattle vaccinated two months previously with *M. mycoides* T_1 broth culture vaccine, and one animal only gave a doubtful intradermal allergic reaction. A further six cattle vaccinated 15 months previously were negative to both the leukocyte migration inhibition test and the intradermal allergic test.

The cell-mediated immune response to *Mycoplasma hyopneumoniae* has been studied in pigs (D. H. Roberts, M. Tech. thesis, Brunell Univ., England, 1972). Lymphocytes in blood leukocyte preparations demonstrated a small but statistically significant degree of antigen-induced transformation of sensitized lymphocytes, as judged by the uptake of tritiated thymidine. It was further demonstrated that a positive intradermal reaction of the delayed type followed injection of *M. hyopneumoniae* antigen into infected pigs. A factor which inhibited the migration of guinea-pig macrophages was obtained by culturing lymphocytes from *M. hyopneumoniae*-infected pigs in the presence of specific antigens.

Allergic skin tests have been used in the diagnosis of contagious bovine pleuropneumonia (CBPP) in cattle. It was shown that reactions to antigens prepared from *Mycoplasma mycoides* var. *mycoides* often did not occur in infected cattle dying with large acute lesions and in certain old cases with chronic sequestra (8, 10). Windsor et al. (in press) developed a single comparative intradermal allergic test for the diagnosis of CBPP. Two antigens were used—an *M. mycoides* membrane antigen and galactan, a polygalactose polymer extracted from *M. mycoides*. The galactan was associated with an edematous immediate type hypersensitive reaction and the *M. mycoides* membrane antigen with a more delayed type hypersensitive reaction, which was a circumscribed pea-like nodule or lump. The immediate type reaction was apparent at 1 h, was decreasing by 6 h, and in most cases had disappeared within 24 h. The delayed type reaction reached its maximum by 24 h and thereafter slowly decreased.

This report is concerned with the cell-mediated immune response in cattle to *M. mycoides*. Three phenomena of cell-mediated immunity were studied: transformation of sensitized lymphocytes by specific antigen, inhibition of leukocyte migration by specific antigen, and the intradermal allergic reaction.

MATERIALS AND METHODS

Cattle. The cattle used in the investigation varied from 1.5 to 2.5 years of age and were obtained from an area free of *M. mycoides*; the sera of all gave negative

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complement-fixation reactions (see below). Preliminary studies were carried out on six control susceptible cattle, six cattle vaccinated 2 months previously in the tail tip with 0.5 ml of the *M. mycoides* T_1 broth vaccine (5), and six cattle artificially infected 3 months previously with the Gladysdale strain of *M. mycoides* by the endobronchial route (4). Six other cattle vaccinated 15 months previously with the T_1 broth culture vaccine and six control cattle were also studied. Following these preliminary studies, four cattle were artificially infected with the Gladysdale strain of *M. mycoides* by the endobronchial route (4). Blood samples were taken from the cattle before inoculation and at weekly intervals thereafter.

Mycoplasma membrane antigen. The method was that of Windsor and Boarer (in press). *M. mycoides* strain T_1 was grown in 5-liter batches in Newing tryptose broth (9) until the estimated number of organisms per milliliter was 10^9 as measured by the method of Windsor and Boarer (14). The broth culture was centrifuged at 18,000 × *g* for 10 min, and the deposit was resuspended and washed twice in sterile 0.01 M $MgCl_2$/0.25 M NaCl solution. The washed deposit was resuspended in sterile 0.15 M NaCl and homogenized for 8 min in a "45" homogenizer (Virtis Research Equipment, N. Y.), working at 75% of its maximum speed. The homogenate was then centrifuged at 18,000 × *g* for 15 min, and the supernatant fluid was carefully removed and preserved. The deposit was resuspended in 40 ml of 0.15 M NaCl and subjected to ultrasonic waves (Ultrasonic Disintegrator, MSE Ltd., London) for 3 min at 4 C; the reading on the scale was never allowed to rise above 1.5. The sonically treated material was then centrifuged at 18,000 × *g* for 15 min, and the supernatant fluid was removed and mixed with that from the previous centrifugation. The pooled supernatant fluids were then centrifuged at 40,000 × *g* for 30 min, and the resultant supernatant fluid was discarded. The deposit was resuspended in $MgCl_2$/NaCl solution and washed six times. The protein content was estimated by the method of Lowry et al. (12), the volume was adjusted to give 1 mg of protein/ml, and the membrane antigen was freeze-dried.

Blood leukocyte preparation. Blood was collected aseptically in preservative-free heparin (25 IU/ml of blood). Blood leukocytes were prepared by using a modified method of Aalund et al. (1). A 10-ml amount of heparinized blood was centrifuged at 500 × *g* for 10 min. After the plasma had been removed, the buffy coat and 3 ml of the adjacent red cell layer were harvested. The leukocyte/erythrocyte suspension was thoroughly mixed with 5 ml of sterile distilled water for 1 min to produce lysis of red cells. Isotonicity was then restored with an equal volume of 0.25 M NaCl containing 0.04 M NaH_2PO_4 at pH 7.0. The cells were then centrifuged at 250 × *g* and washed twice in Eagle minimum essential medium containing the following: Hanks balanced salt solution (Gibco); 0.035% $NaHCO_3$; 0.005% asparagine; 100 IU of penicillin per ml; 100 μg of streptomycin per ml; 10 μg of kanamycin per ml, and 10% bovine serum. The cells were resuspended in this medium and sampled for total and differential counting and for viability using the trypan blue dye exclusion test.

Lymphocyte culture. Cultures containing 5×10^6 leukocytes (60–85% lymphocytes, 95–100% viability) were grown in 4 ml of Eagle MEM-Hanks-bovine serum medium in bijou bottles. The cultures were inoculated in duplicate with either 0.1 ml of the *M. mycoides* membrane antigen or 0.1 ml of phytohemagglutinin (Wellcome Research Laboratories, England). Cultures without stimulant served as controls. The bottles were tightly capped and incubated at 37 C for 4 days. Transformation and proliferation of lymphocytes stimulated by specific antigen or by phytohemagglutinin (PHA) was measured by labeling DNA with tritiated thymidine (Radiochemical Centre, Amersham, England). The method was a modification of that described by Barile and Leventhal (2). Forty-eight hours before collection, 5 μCi of tritiated thymidine (methyl-T specific activity 22 Ci/mmol) was added to each culture. The cells were sedimented at 1,000 × *g* for 5 min, washed with physiological saline, and centrifuged, and 5% trichloroacetic acid was added to the cell pellet. The precipitate was washed with 95% ethanol and then dissolved in 1.0 ml of 2 N solution of methanolic KOH at 60 C for 30 min. A 0.4-ml amount of this sample was added to 10 ml of scintillation mixture (Butyl-PBD [Ciba], 6 g; toluene, 600 ml; methylcellosolve, 400 ml; naphthalene, 80 g) and counted in a scintillation counter. The uptake of thymidine was expressed as the mean values of duplicate determinations as a counts per minute per culture. The results were expressed as the lymphocyte transformation (LT) index obtained by dividing the mean counts per minute per culture in the presence of antigen by that obtained in duplicate cultures without added antigen. This technique gave duplicate values usually within 5 and 10%.

Leukocyte migration inhibition test. Blood leukocytes were prepared as described above and resuspended in the Eagle MEM-Hanks-bovine serum medium at 7×10^7 leukocytes per ml. The leukocyte migration test was carried out by using the micromethod described by Federlin et al. (7) and using disposable polystyrene leukocyte migration plates (Sterilin, England). The chambers were filled with Eagle MEM-Hanks-bovine serum medium (control) or with Eagles MEM-Hanks-bovine serum medium containing 0.1 ml of mycoplasma membrane antigen per ml. The results were expressed as the leukocyte migration index (7), which is the ratio of the mean migration area in the presence of antigen to the mean migration area in the presence of culture medium only.

Comparative intradermal allergic test. The comparative intradermal allergic test method was that described by Windsor et al. (in press). Two sites were selected in the middle third of one side of the neck. The sites were shaved, cleansed with methanol, and measured with tuberculin-testing calipers. A 0.1-ml amount of the mycoplasma membrane antigen was injected intradermally in the upper site, and 0.1 ml of the galactan solution was injected intradermally in the lower site. A comparison was made between the reactions to the membrane and galactan at 24 h. An increase in skin thickness of 3 mm at the membrane site was interpreted as a doubtful reaction whereas an increase of 4 mm or more was interpreted as a positive

reaction, provided there was no increase in skin thickness at the galactan site.

Complement fixation test. The complement fixation test method used was described by Campbell and Turner (6), using antigen prepared from the T_1 strain of *M. mycoides*.

RESULTS

Table 1 illustrates results of the intradermal allergic test and of lymphocyte transformation induced by PHA and *M. mycoides* membrane antigen in artificially infected, vaccinated, and control cattle. Stimulation of lymphocytes as indicated by tritiated thymidine uptake was regularly obtained with PHA in the three groups.

With the *M. mycoides* membrane antigen, stimulation nearly always occured in the artificially infected cattle; the increase in tritiated thymidine uptake varied from 2.23- to 6.30-fold (LT index) in five out of six animals. In the sixth animal there was no obvious increase in tritiated thymidine uptake, although this one, together with the other five cattle, gave positive intradermal reactions. In the control animals there was very little difference in the thymidine uptake in the mycoplasma antigen-stimulated cultures as compared to the controls. The LT index varied from 0.64 to 1.20. In all six animals the intradermal test was negative. In the vaccinated animals, cultures from only two cattle showed a significant increase in tritiated thymidine uptake in the presence of membrane antigen. In the other four, the LT index was less than 1.76. Five out of six of the vaccinated animals gave negative intradermal reactions, and in the sixth animal the reaction was doubtful.

A further six vaccinated cattle and six control cattle were studied, using the leukocyte migration inhibition test and the intradermal allergic test. The leukocyte migration index in the control cattle varied from 0.84 to 0.97 with a mean of 0.90. In the vaccinated cattle the leukocyte migration index varied from 0.87 to 0.94 with a mean of 0.90. The control and vaccinated cattle were all negative to the intradermal test.

In Table 2 are given the results following artificial infection of cattle. In all four animals lymphocyte transformation response to the membrane antigen was observed 3 days after artificial infection with *M. mycoides*. The increase in tritiated thymidine uptake varied from 2.01- to 11.57-fold (LT index) compared to the controls. At 10 days after infection, the LT index in two of the animals was greater than at 3 days, whereas in the other two animals the LT index had declined. In all animals the LT index had declined considerably by 17 days after infection and remained at less than 2 in all animals until 37 days after infection, when it again increased. One of the animals died at 21 days postinoculation, but at 18 days after infection this animal gave a negative reaction with the intradermal test. In Table 3 are given the results of the PHA and mycoplasma membrane-induced lymphocyte transformation between the 10th and 37th days after infection. The addition of PHA to lymphocyte cultures prepared on the 23rd and 30th day after inoculation did not stimulate the lymphocytes, as indicated by the uptake of tritiated thymidine. The inhibition of the mitotic effect of PHA was very marked in two of the cattle and partial in the third animal. Similarly, mycoplasma membrane antigen did not stimulate transformation of lymphocytes from these cattle during this period. The suppression of antibody response was not demonstrated (see Table 2). The leukocyte migration (LM) inhibition tests are expressed as the LM index in Table 2. The LM index in animal B at 17 days after infection was 0.82; at 18 days after infection this animal was negative to the intradermal test. In animal D at 43 days after infection, the LM index was 0.78 and this animal gave a positive reaction in the intradermal test at 30 days after infection. Following these observations, an LM index below 0.80 was regarded as positive and above 0.80 was regarded as negative. The control cattle, which all gave negative intradermal reactions, had LM indices greater than 0.84. Using these criteria, the artificially infected cattle gave positive leukocyte migration inhibition tests 2 to 4 weeks after infection. The results of the lymphocyte transformation tests did not, however, run parallel with the leukocyte migration inhibition tests.

Considering the cattle group that had been artificially infected 3 months previously (see Table 1), the lymphocytes of animal no. 6 were very active in that the thymidine uptake in the control tubes was substantial. Eighteen months previously the animal had recovered from a subclinical reaction, resulting from an experimental infection with *Theileria parva;* this is a protozoon parasite which infects lymphocytes and erythrocytes. The significance of the finding is being investigated.

DISCUSSION

The results confirm previous work with the intradermal allergic test, which indicated that cell-mediated immunity is involved in *M. mycoides* infection in cattle. Tests tracing the transformation of lymphocytes, the inhibition of leukocyte migration, and the production of an

TABLE 1. *PHA and Mycoplasma antigen-induced lymphocyte transformation and the results of the intradermal allergic test in artificially infected, vaccinated, and control cattle*

Stimulant	Thymidine uptake (counts/min)					
	1[a]	2	3	4	5	6
Control cattle						
Control	3,773	3,637	2,721	1,912	1,357	789
Control	3,354	3,481	3,003	1,710	1,599	971
Mean	3,564	3,559	2,862	1,811	1,478	890
PHA	30,463	23,021	17,985	27,142	35,376	8,659
PHA	30,355	20,765	21,427	30,784	30,132	10.671
Mean	30,409	21,895	19,706	28,963	32,254	9,660
Antigen	4,520	3,430	1,667	1,627	1,599	778
Antigen	3,232	3,017	1,955	1,869	1,957	942
Mean	3,876	3,224	1,821	1,748	1,778	860
LT[b] index	1.09	0.90	0.64	0.97	1.20	0.97
Intradermal Test	–	–	–	–	–	–
Artificially infected cattle						
Control	482	160	395	861	1,950	30,421
Control	430	245	459	777	2,430	37,263
Mean	456	203	427	819	2,190	33,842
PHA	18,930	26,315	12,406	26,531	35,217	104,422
PHA	17,399	25,301	14,608	28,773	41,641	128,664
Mean	18,165	30,808	13,507	27,652	38,429	116,543
Antigen	3,096	1,390	1,102	1,745	2,360	91,941
Antigen	2,642	1,090	908	1,909	1,918	79,521
Mean	2,869	1,240	1,005	1,827	2,190	85,731
LT index	6.30	6.11	2.35	2.23	0.98	2.47
Intradermal test	+	+	+	+	+	+
Vaccinated cattle						
Control	534	4,875	885	821	487	816
Control	628	8,762	467	883	339	643
Mean	581	6,817	678	852	413	730
PHA	7,052	9,246	3.915	2,173	5,532	5,895
PHA	5,852	11,484	6,405	1,531	6,536	6,739
Mean	6,452	10,360	5,160	1,852	6,034	6,317
Antigen	1,748	43,477	1,308	608	299	148
Antigen	1,073	30,002	1,070	758	175	376
Mean	1,411	36,740	1,189	683	237	262
LT index	2.43	5.39	1.76	0.80	0.57	0.36
Intradermal test	∓[c]	–	–	–	–	–

[a] Animal number.
[b] LT, Lymphocyte transformation.
[c] ∓, Doubtful reaction.

TABLE 2. *Mycoplasma membrane-induced lymphocyte transformation, leukocyte migration inhibition, and complement-fixation results in M. mycoides-infected cattle*[a]

Animal no.	Test	Pre-inoculation date	Days postinnoculation						
			3	10	17	23	30	37	43
A	LT index	0.78	2.58	2.10	0.99	0.85	0.78[b]	2.70	2.40
	LM index	1.01	C	0.86	0.80	0,80	0.58	NS	0.72
	CF titer	<10	<10	<10	<10	320	320	160	160
B	LT index	0.94	5.58	13.24	1.17	Animal died 21 days postinoculation. At 18 days, intradermal test negative.			
	LM index	0.92	C	0.91	0.82				
	CF titer	<10	<10	<10	1,280				
C	LT index	0.84	2.01	8.98	2.10	0.98	0.94[b]	1.91	3.44
	LM index	0.89	C	0.84	0.75	0.68	0.63	NS	0.74
	CF titer	<10	<10	>2,560	>2,560	640	320	160	160
D	LT index	0.97	11.57	3.86	1.53	1.27	0.67[b]	3.26	2.88
	LM index	0.85	C	1.08	0.84	0.80	0.70	0.64	0.78
	CF titer	<10	<10	320	320	640	320	80	40

[a] Abbreviations: LT, lymphocyte transformation; LM, leukocyte migration; NS, test not successful; CF, complement fixation; C, clumping of leukocytes observed associated with failure of cells to migrate.
[b] At thirty days, all positive to intradermal test.

TABLE 3. *PHA and mycoplasma membrane-induced lymphocyte transformation in M. mycoides-infected cattle*

Animal no.	Stimulant	Mean counts/min				
		10[a]	17	23	30	37
A	Control	1,770	2,119	771	956	1,187
	PHA	50,233	61,257	1,972	1,466	14,715
	Antigen	1,614	2,088	651	741	3,210
LT index		2.10	0.99	0.852	0.78	2.70
C	Control	403	1,148	340	1,233	1,335
	PHA	67,293	41,297	3,398	6,877	14,409
	Antigen	3,611	2,398	334	1,160	2,544
LT index		8.96	2.10	0.982	0.94	1.91
D	Control	623	1,078	656	808	441
	PHA	60,169	85,086	14,034	12,062	11,241
	Antigen	2,406	1,651	834	543	1,441
LT index		3.86	1.53	1.271	0.67	3.26

[a] Days postinoculation.

intradermal reaction all gave positive results in artificially infected cattle.

After infection with *M. mycoides*, lymphocyte transformation in the presence of membrane antigen occurred 3 days after infection in all the cattle. Only one animal, however, had a positive result in the leukocyte migration inhibition tests at 17 days after infection, but all animals gave positive results by 30 days. This is in general agreement with the intradermal allergic test in which 3 weeks elapse after infection before the majority of cattle give positive reactions (Windsor et al., in press). Positive and negative results in the leukocyte migration inhibition test were based on a cut-off point of 0.80 (LM index). The test used in these investigations was similar to the test described by Federlin et al. (7), and their results indicated that an LM index of 0.80 determined between positive and negative subjects. As our findings,

which were based on fewer results, were similar to those of Federlin et al. (7), an 0.80 (LM index) cut-off was adopted. In cattle vaccinated with *M. mycoides*, an intradermal reaction was not observed in the majority of cattle examined 2 months after vaccination. Lymphocytes from two of the six animals transformed in the presence of mycoplasma membrane antigen.

The difference in the results of the three tests might indicate merely a difference in sensitivity, lymphocyte transformation being the most sensitive and the leukocyte migration inhibition test the least sensitive test (3). The immune response to a specific antigen is characterized by the development of antibody-producing cells (B cells) and of sensitized lymphocytes active in cell-mediated immunity (thymus-derived cells). PHA-induced transformation of lymphocytes is generally considered specific for thymus-derived cells, but Phillips and Roitt (13) presented evidence to show that in man B cells might respond to PHA. It is possible that the early lymphocyte transformation response of the group artificially infected with *M. mycoides* might be associated with bovine B cells.

The partial or nearly complete inability of lymphocytes from *M. mycoides*-infected cattle to respond to PHA is an interesting phenomenon. Mycoplasma-induced immunosuppression in rats has been demonstrated with *M. arthritidis*, involving both the humoral antibody response and inhibition of lymphocyte blast formation (11). With *M. mycoides* infection, however, the humoral response did not appear to be affected. This unresponsiveness to PHA has been observed on another occasion in cattle artificially infected with *M. mycoides*. These findings were observed in cattle being used for other experiments. It should nevertheless be noted that, in the vaccinated cattle, the lymphocytes did not respond to PHA as well as lymphocytes from control cattle or from cattle that had recovered after artificial infection with *M. mycoides* (see Table 1). Why this should happen cannot be explained at the present time, but work is in progress to investigate the role of cell-mediated immunity in cattle vaccinated with *M. mycoides*.

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