

Immunological characteristics of cattle with *Mycobacterium avium* subsp. *paratuberculosis* infection

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ABSTRACT: Several years lasting clinical and microbiological monitoring of a cattle herd infected with paratuberculosis allowed to identify three groups of animals showing different courses of the infection. Group N (non-shedders) included animals negative by faecal culture throughout the monitoring period. Animals of Group L (low shedders) shed sporadically small quantities of mycobacteria (1 to 9 CFU), but remained clinically healthy throughout the monitoring period. Group H (high shedders) included animals shedding repeatedly large quantities of *Mycobacterium avium* subsp. *paratuberculosis* (≥ 10 CFU) with a progressive deterioration of the state of health in most of them. Animals with specific antibodies detected by agar gel immunodiffusion and complement fixation test were found in all groups, but the percentage of serologically positive animals was higher in animals of Group L and significantly higher ($P < 0.01$) in animals of Group H than in animals of Group N. Specific cell-mediated immunity was demonstrated especially in the group of low shedders (Group L), the differences in the percentages of interferon gamma assay positive animals in this group and other groups was highly significant ($P < 0.01$). Only insignificant differences in the counts and activity of peripheral leukocytes were found among groups of clinically healthy animals differing in intensity of *M. paratuberculosis* shedding. However, the progressive development of clinical signs of paratuberculosis in Group H was associated with a significant ($P < 0.01$) decrease of lymphocytes and monocytes, a non-significant decrease of neutrophils, a significant ($P < 0.05$) increase in the percentage of CD4⁺ cells and a insignificant decrease in the percentage of CD8⁺ cells; consequently the CD4/CD8 ratio increased.

Keywords: Johne's disease; cattle; resistance; cell-mediated immunity; interferon-gamma

Paratuberculosis is a serious bacterial disease of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*), developing as chronic granulomatous enteritis and manifested clinically by emaciation and diarrhea (Chiodini *et al.*, 1984). Cattle become infected at an early age and clinical signs develop after a long incubation period lasting years. Most animals in an affected herd are clinically healthy and the causative agent is demonstrable in faecal samples only occasionally (Whitlock and Buergelt, 1996; Yayo Ayele *et al.*, 2001). Although culture methods for *M. paratuberculosis* have been developed, the identification of infected animals is rather difficult (Collins, 1996; Whitlock and Buergelt, 1996).

Like in other mycobacterial infections, the immunity to paratuberculosis depends on the cell-mediated

immune response (Chiodini, 1996; Merkal *et al.*, 1970; Buergelt *et al.*, 1977; Coussens, 2001). The development of protective immunity to intracellular organisms involves a cooperative action of Th lymphocytes and macrophages. It is assumed that macrophages, activated by interferon as a product of Th1 lymphocytes play the key role in the control of infection (Sweeney *et al.*, 1998). While phagocytosis without an involvement of specific cell mechanisms is less effective in the elimination of mycobacteria, the interferon gamma (IFN- γ)-activated macrophage is the major effector cell of protective immunity. It has been demonstrated in animals affected by paratuberculosis that the production of IFN- γ by peripheral mononuclear cells is higher in symptomless (without emaciation and/or chronic enteritis), low *M. paratuberculosis*

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shedding cows than in those that have developed clinical signs of infection (Stabel, 1996; Sweeney *et al.*, 1998).

The occurrence of paratuberculosis has been monitored in the farm NT of herd DS, for 10 years (Pavlik *et al.*, 1999). Examinations by culture of faecal samples collected from all the animals were launched in 1993 and the introductory tests demonstrated the presence of *M. paratuberculosis* in 2.3% of them. By the end of 1997, 86 animals positive by faecal culture or serological tests were culled. A relatively large proportion of them (23.1%) were positive by faecal culture only once during the investigation period, and their intestines and mesenteric lymph nodes were negative by culture and no lesions were found upon *post mortem* examination. An increased incidence of paratuberculosis was recorded towards the end of 1997 and in 1998. The long-lasting clinical and microbiological monitoring of this herd allowed us to identify three groups of animals showing different courses of the infection at least partly dependent on the degree of resistance.

The aim of this study was to compare the counts of peripheral leukocytes and subpopulations of lymphocytes and the antibody and cell-mediated immune responses in three groups of animals showing different courses of *M. paratuberculosis* infections.

MATERIAL AND METHODS

Animals

A set of 53 Holstein cattle born and stabled on the farm NT were monitored serologically and microbiologically (three times a year) for a period of 4 to 7 years, and were grouped according to the following characteristics:

N (non-shedders) – animals negative by faecal culture throughout the investigation period, but with a highly probable past infection (occasionally serologically positive, dubious, or negative, all these animals had a close contact with infected individuals at the time of birth).

L (low shedders) – animals shedding very sporadically (ones to three times during the investigation period) low quantities of *M. paratuberculosis* (1 to 9 CFU), but clinically negative throughout the investigation period.

H (high shedders) – animals shedding repeatedly higher quantities of *M. paratuberculosis* (≥ 10

CFU), mostly with progressive deterioration of the state of health (emaciation, recurrent diarrhea).

During the one-year investigation period, all the animals were observed daily and 4 to 6 faecal culture tests were done. Moreover, two types of serological tests and two types of cell-mediated immunity tests were done twice in all the animals. In twenty seven animals (8 to 10 from each group) the leukocyte counts and CD markers were determined three times during the investigation period. Interval between sampling in group H depended on clinical signs of the disease and was two months at minimum.

The slaughtered animals were examined for macroscopic lesions (corrugated intestinal mucosa – gyrification) and culture from several intestinal segments and adjacent lymph nodes was made.

Faecal and tissue culture

Faecal samples were processed as described by Whipple *et al.* (1991) modified by Pavlik *et al.* (2000). Tissue samples (three samples of ileal mucosa and three samples of mesenteric lymph nodes) were examined from each animal. Approx. 1 g of tissue was homogenized (Lab Blender, Stomacher, Germany) and treated overnight with 0.75% Hexadecyl Pyridinium Chloride (N-cetylpyridinium chloride monohydrate, No. 102340 Merck). The intensity of infection was expressed in terms of colony forming units (CFU) grown on Herrold's egg yolk medium (HEYM) supplemented with Mycobactin J (own product according to Merkal and McCullough, 1982) and incubated at 37°C for 14–16 weeks (Pavlik *et al.*, 2000).

All mycobacterial isolates were identified by subculture on media containing a growth factor (Mycobactin J) and free of it (Mycobactin J)-dependence test, and by PCR using primers for the detection of IS900 specific for *M. paratuberculosis* (Kunze *et al.*, 1992).

Total and differential leukocyte counts

Total leukocyte counts were determined using the Digicell 500 cell counter (Contraves AG, Switzerland). Differential leukocyte counts were enumerated from blood smears stained with May-Grünwald and Giemsa-Romanowski.

Lymphocyte subsets identification

Lymphocyte subsets were counted by flow cytometry using the indirect single-colour whole blood lysis technique. Fifty μ l of blood was incubated with a monoclonal antibody at room temperature for 15 min. After hemolysis, centrifugation and a removal of the supernatant, a second antibody was added and the suspension was incubated at 4°C for 20 min. The cells were washed and centrifuged and the sediment was resuspended in the washing solution. The enumeration was done using the flow cytometer FACS-Calibur (Becton Dickinson, Mountain View, CA, USA). Monoclonal antibodies shown in Table 1 were used as the primary antibody and caprine F(ab)₂ anti-murine IgG₁, IgG₂, or IgM, labelled with FITC or PE (Southern Biotechnology Associates, Inc., USA) as the second antibody.

Identification of antibody production

Blood serum antibodies to *M. paratuberculosis* were demonstrated by two tests. In the first antibodies were detected by agar gel immunodiffusion (AGID) using sonicated protoplasmic antigen. Positive result was evaluated according to the specific precipitation line. In the second antibodies in the complement fixation tests (CFT) using sonicated protoplasmic antigen was used. Sera with titres higher than 10 were considered to be positive. Both kits were produced by Bioveta, Ivanovice na Hane (Czech Republic) and the manufacturer's protocols were followed (Pavlik *et al.*, 2000).

Lymphocyte transformation test (LTT)

Antigen-specific activity of peripheral blood lymphocytes separated from blood in a cell separating medium with a density of 1 084 g/ml (Verografin,

Leciva Praha, Czech Republic) was tested after stimulation with antigen. Two hundred μ l cell suspension (1×10^6 cells/ml RPMI 1640 supplemented with penicillin, streptomycin and 10% precolostral calf serum) was pipetted in triplicates into microtitre plate wells. Twenty μ l of specific antigen *M. paratuberculosis* (PTB-3) or control antigen *M. bovis* (PPD-B) at concentrations of 30, 10 and 3 μ g/ml was added to each well. The microplates were incubated for 5 and 1 days. The incorporation of ³H-thymidine was measured using a liquid scintillation counter (Packard Tricarb CA 600, Canberra, Packard). The results were expressed in terms of stimulation indices calculated as the ratio between the activities of the stimulated and non-stimulated cells and the ratio higher than 3 was considered to be positive.

Interferon gamma release test

The production of interferon gamma (IFN- γ) was detected by enzyme immunoassay using Bovigam (CSL Veterinary, Australia). Triplicate 1 ml volumes of whole blood were incubated with 10 μ g specific *M. paratuberculosis* antigen (PTB-3), control *M. bovis* antigen (PPD-B), or without antigen for 18 hrs. Plasma was collected and assayed for release of IFN- γ by ELISA according to the manufacturer's protocol. In accordance with the manufacturer's protocol, the sample in which well's extinction with a specific antigen was at least by 0.1 higher than extinction of well with control antigen was considered positive.

Statistics

Evaluation of the results showed normal distribution of the obtained values. The significance of differences in non-specific activity of the immune

Table 1. Monoclonal antibodies to bovine leukocyte antigens used for flow cytometry

mAb	Source	Specificity	Ig isotype	Dilution
MM1A	VMRD, Inc.	CD3	IgG ₁	1 : 50
CC30	Serotec	CD4	IgG ₁	1 : 100
CC63	Serotec	CD8	IgG _{2a}	1 : 100
VPM30	Serotec	B cells	IgM	1 : 20
CACT61A	VMRD, Inc.	TcR1-N12	IgM	1 : 100

systems among Groups N, L, and H was evaluated by *t*-test, significance in changes during the development of clinical signs in Group H by paired *t*-test, and significance of differences in specific humoral and cell-mediated immunity by χ^2 -test (Matouskova *et al.*, 1992).

RESULTS

Clinical and *post mortem* examinations

The animals of Group N were clinically healthy throughout the investigation period. Four animals of this group, which were culled for other than health reasons, were negative in pathological and microbiological examinations of the intestines and mesenteric lymph nodes.

All the animals of Group L were clinically healthy or showed only minor alterations that could not be interpreted in terms of clinical paratuberculosis. Six animals were culled during the investigation period and moderate intestinal lesions, corresponding to sub-clinical paratuberculosis, were found in two of them. Samples collected from intestines and mesenteric lymph nodes were slightly positive (1 to 9 CFU in 1 to 3 sites) for *M. paratuberculosis* in four of the animals.

Eight animals of Group H developed clinical paratuberculosis during the investigation period.

In these animals we observed in the whole course of the infection from the asymptomatic stage with shedding of *M. paratuberculosis* through the stage of subclinical infection manifested by non-specific clinical signs up to the fully developed paratuberculosis characterized by emaciation and intermittent diarrhoea. *Post mortem* examinations revealed marked gyration of the intestinal mucosa and a massive infection of the small intestine and the corresponding lymph nodes (≥ 10 CFU, often more than 100 CFU) by *M. paratuberculosis* in these animals.

Antibody and cell-mediated immune responses

The presence of specific antibodies was demonstrated in some animals of Group N (non-shedders), but the percentage of animals positive by CFT was higher in the animals of Group L (low shedders) and significantly higher ($P < 0.01$) in the animals of Group H (high shedders) (Table 2). The results of serological tests (CFT and AGID) overlapped only partially so that the number of animals positive in at least one test was higher than the number of animals positive only in CFT or in AGID. In the Group H with the highest seropositivity, 7 animals (30.4%) were positive in both the tests, 10 animals (43.5%) were positive in one test, and 6 animals (26.1%) were negative.

Table 2. Frequency of humoral and cell-mediated immunity reactions in cows with different courses of *M. paratuberculosis* infection

Group	Number of animals tested	Serology tests ¹		Cell-mediated immunity tests ¹	
		AGID	CFT	LTT	IFN- γ
N (Non-shedders)	15	4 (26.7)	2 (13.3)	2 (13.3)	0 (0)
L (Low shedders)	15	5 (33.3)	4 (26.7)	6 (40.0)	10 (66.7)
H (High shedders)	23	11 (47.8)	13 (56.5)	4 (17.4)	3 (13.0)
	N vs. L				<0.01
χ^2 -test	N vs. H		<0.01		
	L vs. H				<0.01

Explanations:

AGID = agar-gel immunodiffusion, CFT = complement fixation test, CMI = cell-mediated immunity, LTT = lymphocyte transformation test, IFN- γ = interferon-gamma production assay

¹results expressed as numbers (%) of positive animals

Table 3. Total and differential leukocyte counts of cows with different courses of *M. paratuberculosis* infection

		Non-shed- ders <i>n</i> = 10	Low shed- ders <i>n</i> = 9	High shedders clinically healthy <i>n</i> = 8 ¹	High shedders non-specific signs <i>n</i> = 8 ¹	High shedders clinical signs <i>n</i> = 8 ¹
Leukocytes	(×10 ⁹ /l)	8.7 ± 2.6	7.5 ± 1.3	8.5 ± 3.4	6.4 ± 3.5*	5.8 ± 3.9**
Lymphocytes	(×10 ⁹ /l)	4.0 ± 1.3	4.0 ± 0.7	3.9 ± 0.8	3.5 ± 1.6	3.0 ± 1.3**
Neutrophils	(×10 ⁹ /l)	3.2 ± 2.1	2.7 ± 0.5	3.1 ± 2.4	2.1 ± 2.4	2.2 ± 2.1
Monocytes	(×10 ⁹ /l)	0.6 ± 0.3	0.4 ± 0.2	1.0 ± 0.5	0.5 ± 0.3**	0.4 ± 0.5**
Eosinophils	(×10 ⁹ /l)	0.9 ± 0.7	0.5 ± 0.3	0.4 ± 0.2	0.3 ± 0.3	0.2 ± 0.3

Explanations:

Statistical significance vs. high shedders clinically healthy (evaluated by paired *t*-test): **P* < 0.05, ***P* < 0.01

¹data were obtained from the same animals during the development of clinical signs

Specific cell-mediated immunity was demonstrated only sporadically in Group N (2 animals positive by LTT) and in Group H (five animals – 21.7% positive in at least one CMI test) (Table 2). On the contrary, a large number of the low shedders (Group L) reacted positively in cell-mediated immunity tests (40% in LTT; 66% in IFN- γ test, 73.3% in total – at least in one CMI test). The differences in the percentages of animals positive by IFN- γ assay between Group L and the Groups N and H were highly significant (*P* < 0.01).

Leukocyte counts and lymphocyte subsets

Only non-significant differences in the leukocytes counts (Table 3) and in the percentages

of lymphocyte subpopulations (Table 4) were found among the groups of clinically healthy animals differing in the intensity of shedding *M. paratuberculosis*.

On the other hand, significant changes occurred in Group H during the development of clinical paratuberculosis. The progressive deterioration of the state of health was accompanied by a decrease in leukocyte counts which was significant (*P* < 0.01) in lymphocytes and insignificant in neutrophils (Table 3). The progressive development of clinical signs was associated with a significant increase (*P* < 0.05) in the percentage of CD4⁺ cells and an insignificant decrease in the percentage of CD8⁺ cells; consequently the CD4/CD8 ratio increased (Table 4). The percentage of B lymphocytes, and $\gamma\delta$ T cells decreased insignificantly.

Table 4. Distribution of lymphocyte subsets in the peripheral blood of cows with different courses of *M. paratuberculosis* infection

Type of lymphocytes		Non-shed- ders <i>n</i> = 10	Low shed- ders <i>n</i> = 9	High shedders clinically healthy <i>n</i> = 8 ¹	High shedders non-specific signs <i>n</i> = 8 ¹	High shedders clinical signs <i>n</i> = 8 ¹
T cells (CD3 ⁺)	(%)	74.9 ± 5.4	71.9 ± 6.7	68.1 ± 6.8	67.8 ± 4.9	71.4 ± 8.2
Th cells (CD4 ⁺)	(%)	42.5 ± 4.8	39.3 ± 4.2	37.7 ± 5.6	41.1 ± 4.4*	44.0 ± 5.6*
Tc cells (CD8 ⁺)	(%)	20.0 ± 4.9	20.4 ± 5.5	18.8 ± 3.6	17.5 ± 3.7	15.9 ± 5.8
B cells	(%)	11.0 ± 3.3	9.3 ± 4.5	16.9 ± 9.3	7.2 ± 3.5*	13.2 ± 7.4
TcR1 ⁺ ($\gamma\delta$ T cells)	(%)	8.5 ± 3.6	7.8 ± 4.5	8.8 ± 4.3	7.6 ± 4.9	5.3 ± 3.5
CD4/CD8	ratio	2.3 ± 0.7	2.1 ± 0.5	2.0 ± 1.6	2.3 ± 1.2	2.8 ± 1.0

Explanations:

Statistical significance of differences between the same 8 high shedders in period with “non-specific signs” and “clinical signs” vs “clinically healthy” was calculated from absolute counts and evaluated by paired *t*-test: **P* < 0.05

¹ data were obtained from the same animals during the development of clinical signs

DISCUSSION

Resistance or susceptibility to infection is not absolute phenomena. Congenital resistance is controlled by the polymorphism of genes encoding components of the immune system and is further modulated by a number of environmental factors to develop into actual resistance of individuals (Horin, 1998). The actual resistance of individuals results from the interaction of congenital resistance with environmental effects. Data for specific resistance to paratuberculosis are limited yet (Horin *et al.*, 1995; Veazey *et al.*, 1995; Koets *et al.*, 2000). Routine diagnostics of paratuberculosis do not yield enough information for assessing the dependence of the manifestations on the degree of resistance, or stage of infection (Whitlock and Buergelt, 1996). In our study we were not able to determine the level of congenital resistance in individual animals. However, we were able to differentiate groups with different course of infection (and assumed different resistance) from the viewpoint of different level of specific immunity.

One of the major factors having influence on clinical manifestation is the infection pressure in the herd. Differences in the development of the infection were observed among five herds of which one was the object of this study (Pavlik *et al.*, 1999). The infected cows on the farm NT affected new-born and young calves during the first strong infection wave in the years 1993–1994 and it became apparent at the end of 1997 and in 1998. Although most animals were infected in the first months of their lives, only some of them developed clinical signs of paratuberculosis (Group H) while others were resistant to clinical disease (Group L) or overcame the infection completely (Group N).

The long-lasting microbiological monitoring of the herd and individual animals under study enabled us to group the cows in terms of the course of infection. However, the three defined groups do not represent strictly separate populations. Some of the animals were on the dividing line and could not be unequivocally classified into any of the groups. Therefore, the number of the analysed animals is smaller than the total number of the actually monitored animals.

Group N (non-shedders) included resistant animals that overcame the infection, if there was any, in the first months of life and no signs of it were detectable at the intestinal level at the time of our monitoring. The signs of humoral or cell-mediated

immunity were only weak at the time of investigation.

Group L (low shedders) included resistant and/or immune animals that did not develop clinical paratuberculosis. Although infected, they were capable of defence against the infection and of reducing the count of mycobacteria. *M. paratuberculosis* appears in faeces when the host fails to control the infection of intestinal tissues and infectious agent is released from disintegrating macrophages (Bendixen *et al.*, 1981). Typical markers of this group were increased parameters of cell-mediated immunity. The test of IFN- γ production was more significant in this sense than LTT.

Positive results of IFN- γ assay in clinically healthy animals shedding sporadically *M. paratuberculosis* correspond to the results published by other authors (Stabel, 1996; Sweeney *et al.*, 1998; Storset *et al.*, 2000). This assay is seemed to be suitable for the identification of subclinically infected animals (Stabel, 1996; Jungersen *et al.*, 2002). On the other hand, the test cannot be considered suitable for the diagnostics of paratuberculosis in general as suggested by Billman-Jacobe *et al.* (1992), although it has proved to be effective as a test for bovine tuberculosis (Wood *et al.*, 1992). Our results show clearly that the number of animals with positive IFN- γ test is lower than the number of infected animals and it does not include susceptible animals shedding large amount of germs in faeces (group H) that have low level of cell-mediated immunity (Table 2).

Group H (high shedders) included susceptible animals shedding repeatedly large quantities of *Mycobacterium paratuberculosis* of which some developed clinical paratuberculosis during the investigation period. They had a low level of cell-mediated immunity and humoral immunity response was demonstrated in most of them by at least one of the tests used.

We succeeded in detecting the changes of peripheral leukocyte counts and activity in the course of natural infections in eight cows of the Group H which, within a year, proceeded from the stage of permanent shedding of mycobacteria to the stage of fully developed clinical disease. The changes included a gradual decrease of peripheral lymphocyte and neutrophil counts associated with the onset of diarrhoea, described also in other infections (Toman *et al.*, 1998), an increase in CD4⁺ and decrease in CD8 counts resulting in an increase of the CD4/CD8 ratio, variations in the percentage of B lymphocytes, and a decrease in the $\gamma\delta$ T cell counts.

In our study we did not succeed to differentiate animals with different course of the infection based on the count differences and activity of the immune system cells. No differences were either found in non-specific activity of lymphocytes or phagocytic cells (data not shown). This does not mean, however, that the counts and activity of the immune system cells remains unchanged during the development of the infection. At an early stage of paratuberculosis, Chiodini and Dawis (1992) demonstrated an inhibition of CD4⁺ lymphocytes by $\gamma\delta$ T cells compromising their potential to activate macrophages. The differences between normal and infected animals in responses to T lymphocytes were studied by Bassey and Collins (1997) who demonstrated that the major subpopulation producing IFN- γ are CD4⁺ lymphocytes, and that, in the animals affected by paratuberculosis, the effect of $\gamma\delta$ T lymphocytes is not mediated through the production of IFN- γ . Koets *et al.* (2002) demonstrated a significant decrease in CD4⁺ T-cell frequency and a significant increase in TcR-N12⁺ gamma delta T-cell frequency in ileum lamina propria lymphocytes of symptomatic animals compared to the asymptomatic shedders. These authors also demonstrated shift of the immune reaction from Th1 type towards the Th2 type at the time of the development of clinical signs (Koets *et al.*, 1999, 2001).

Our results confirmed that actual resistance to mycobacterial infection and its clinical signs depend on the level of cell-mediated immunity (Chiodini, 1996; Coussens, 2001). In our study immunity correlated with the production of IFN- γ , which is the Th1 type of immune response (Sweeney *et al.*, 1998). On the contrary, humoral immunity response (Th2 type) did not assure protection against the infection. The highest percentage of infection was recorded in animals with massive shedding of mycobacteria; in some of them i.e. in animals with low level of immunity, clinical signs of the disease developed. However, even in that group antibodies were not detected in all the animals, which confirms the limited ability of detection based exclusively on serological examination.

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