Immunohistochemistry of Lymphocytes Subsets in Subclinical Experimental Paratuberculosis in Goats

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ABSTRACT

The present experiment was carried out to find out the lymphocytes subsets reactions in experimentally induced subclinical paratuberculosis in goats. Twelve goats of 8-12 weeks age were infected with 4.23 x 10^9 Mycobacterium avium paratuberculosis on 8 occasions. Seven goats were kept as in-contact controls and 4 as uninfected controls. Immunohitochemistry for detection of cellular reaction of CD2+, CD4+, CD8+, CD25+, MHC I and MHCII in the lymphocytes present in the intestine and lymph node revealed more reactive cells in the infected goats as compared with the in-contact and infected control goats.

Keywords: Paratuberculosis, Immunohistochemistry, Lymphocyte subsets MHC I and MHCII

INTRODUCTION

Johne's disease is one of the economically important chronic diseases affecting all domestic and wild ruminants and is caused by *Mycobacterium avium* subsp. *paratuberculosis.* The disease is characterized by chronic granulomatous *e*nterocolitis, lymphangitis and lymphadenitis. Clinical manifestations of the disease include incurable diarrhoea, progressive weight loss and decline in productivity of the animals (Williams *et.al.,* 1979; Chiodini *et.al.,* 1984; Benedictus *et.al.,* 1987; Buergelt and Ginn, 2000; Godfroid *et.al.,* 2000; Kramsky *et.al.,* 2000). The bacterium of Johne's disease has also been implicated with an inflammatory bowel disease in humans known as Crohn's disease (Stabel, 1998; Selby, 2000).

In India, paratuberculosis in goat was first reported by Pande in 1942 from Assam. The disease has now been reported from many parts of the country with prevalence varying from 2.5 to 18.9 % (Srivastava and More, 1987; Kumar *et.al.,* 1988; Singh *et.al.,* 1998; Tripathi and Parihar, 1999, Tripathi *et.al.,* 2002). Various authors have described lesions in paratuberculosis, which occurs due to host's inflammatory immune response to the invading mycobacteria (Paliwal and Rajya, 1982; Rubin and Habekar, 1999; Hirsh and Zee, 1999).

Navarro *et al.* (1998) demonstrated different lymphocytes subsets by immunohistochemistry in the intestinal mucosa and mysenteric lymph nodes of three goats with natural paratuberculosis using monoclonal antibodies against $CD2^+$, $CD4^+$, $CD8^+$, γ/δ, and MHCII. In the intestinal mucosa of goats with tuberculoid lesions, no significant changes were observed but in the cortical area of the mesenteric lymph nodes, the number of $CD4^+$ T lymphocytes increased. In two goats with lepromatous lesions, there was a decrease in the $CD4^+$ subpopulation and increase in the $CD8^+$ T lymphocytes in the lamina propria of the intestine and in cortical area of mesenteric lymoph nodes.

Little *et al*. (1996) performed immunohistochemistry in ileum of 12 normal and 18 naturally *M. avium* subspecies. *paratuberculosis* infected sheep, using monoclonal antibodies against ovine $CD4^+$, $CD8^+$, and γ/δ T cell receptor markers. Higher densities of lymphocytes were present in villus than in crypt areas. $CD8^+$ cells were located principally around the epithelial basement membrane where as $CD4^+$ cells were located towards the central villus of lamina propria.

Redcliff *et al.* (2004) used immunoperoxidase labeling for $CD4^+$, $CD8^+$, TCR γ/δ, WC1, CD1b, IFN γ, CD45 R, CD56 and lysozyme to investigate changes in cell mediated immune effector cell population in the intestinal Peyer's patches and mesenteric lymph node of lambs after experimental infection with *M.a.paratuberculosis*. Infected sheep had significantly more $CD4^+$ cells in the mucosa, domes and interfollicular areas of the terminal ileum and in the interfollicular areas of the jejuna Peyer's patch and increase number of $WC1^+$ cells in the ileal Peyer's patch.

MATERIALS AND METHODS

Experimental Design

Twenty four (24) goats were randomly divided into three groups namely the infected (n=12), the in-contact control (n=8) and uninfected control (n=4). The animals in the infected group were orally administered with 5 ml of bacterial suspension (4.23×10^9/ml) on eight occasions at 3 days interval within a month. Similarly, the uninfected control animals received 5 ml sterile PBS. The animals of the in-contact group were administered similarly with PBS and kept along with the animals of the infected group. The experimental goats were euthanized 3, 6, 9 and 12 months post infection by intravenous injection of Thiopentone injection (Anesthal, Jagsonpal Pharmaceutical, Faridabad, India).

Fixation and Sectioning of Tissues:

Tissue sections for microscopic examination included one section of duodenum, four sections of jejunum, three sections of ileum, one section each of mesenteric lymph node, ileocaecal lymph node, caecum, colon and rectum.All tissues sections were collected in 10% neutral buffered formalin. Representative tissue samples of intestines and mesenteric lymph nodes were processed and embedded in paraffin wax after going through all the steps of dehydration by different grade of alcohol and cleaning in Xylene as per the routine protocol. rendered for tissue sections which were cut into pieces of 5 µm thickness (Culling, 1968) and mounted on poly-L Lysine coated glass slides and dried overnight at 37ºC.

Immunohistochemistry for Identification of Lymphocytes Subsets

Immunohistochemical staining were performed for delineation of different lymphocytes sub population such as T cells subset, B cells and MHC class II bearing cells by using monoclonals against various CD molecules in tissues (lymph nodes and small intestines). Using different Immunohistochemistry of goat paratuberculosis monoclonal antibodies immunohistochemistry was carried out on intestinal and lymph node tissues. (Table-1)

Table1: Monoclonal antibodies (MoAb) and their specifications

MoAb	Ig isotype	Specipicity	Target species	Source
MUC2A	IgG_2	CD_2, αβ T cells and a subset of WC1 negative γδ T cells	Bovine, Ovine, Caprine	VMRD Pullman Inc. USA
GC5OA	IgM	CD_4	Bovine, Ovine, Caprine	
CACT80C	IgG_1	CD_8	Bovine, Ovine, Caprine	
CACT116A	IgG_1	CD_{25}	Bovine, Ovine, Caprine	
H58A	IgG_{2B}	MHC I	Bovine, Ovine, Caprine	
TH14B	IgG_{2B}	MHC II	Bovine, Ovine, Caprine	

Staining Procedure:

Immunohistochermistry procedure was followed as per the protocol described by Vector Lab. Inc. Burlingame CA94010, USA for demonstration of different types of surface markers of immune cells ($CD2^+$, $CD4^+$, $CD8^+$, $CD25^+$, MHC I and MHCII, VMRD Inc. Pullman, USA). The mounted tissue sections on slides were deparaffinized by treating in xylene I for 15 minutes and Xylene II for 5 minutes. Rehydrations of tissues were carried out in descending grade of alcohol I, II and III, 90% alcohol, 80% alcohol and finally 70% alcohol for 1 minute each.

The slides were rinsed in tap water for 5 minutes. Unmasking of antigen was achieved by boilimg in citrate buffer (0.1mM, pH6) for 2-3 min. The sections were treated with Phosphate buffered saline (10 mM Sodium Phosphate, pH 7.5, 0.9% saline) for 10 minutes. The sections were incubated in 0.3% H_2O_2 in Methanol for 30 minutes followed by washing for 5 minutes in PBS. The sections were incubated for one hour with diluted normal blocking horse or goat serum, 1-4 drops (50-200 µl) in 10 ml buffer. Excess serum was draied off the section and incubated overnight at 4ºC with optimally diluted (1:10) monoclonal antibodies. The sections were washed in buffer for 5 minutes and incubated for one hour with diluted secondary antibody ie biotinylated antimouse IgG/IgM (Vector laboratories, Burlingame, USA).

The sections were washed in buffer for 5 minute and again incubated for 45 minutes with Vectastain Elite ABC reagent (Vector laboratories, Burlingame, USA) prepared as per instructions. The sections were washed in buffered saline for 6 minutes and incubated (2-10 min) Iperoxidase substrate solution until the desired stain intensity was developed by using Diaminobenzidine (Sigma, USA). The sections were then rinsed in tap water and counterstained with the Hematoxylene for 3-4 seconds. Following brief rinsing in tap water the sections were dehydrated in 3 changes of absolute alcohol for 1 min each. These were left for 1 min in alcohol:

Xylene mixture followed by xylene I and xylene II for 1 min each and finally sections were mounted in DPX. Sections were examined at high power objective 400X to assess the reactivity of the cells in the sections of small intestines and mesenteric lymph nodes, For assessment of the reactivity of the labeled cells were scored a + (1-10 labelled cells), ++ (11-50 labelled cells), +++ (moore than 50 labelled cells). Scoring was made according to the protocol followed by Reddacliff *et al.* (2004) with slight modification.

RESULTS

The intensity of reaction of different lymphocyte subsets identified by various monoclonal antibodies are presented in Table 2.

Table 2: Reactivity of monoclonal antibodies (CD2, CD4, CD8, CD25, MHCI and MHCII) in intestine and lymph nodes of experimental goats

	Goat No.	CD2		CD4		CD8		CD25
		MHC I		MHC II				
		Int.	MLN	Int.	MLN	Int.	MLN	Int.
	MLN	Int.	MLN	Int.	MLN			
Infected	23	++	++	++	++	++	++	++
	++	++	++	++	++			
	28	++	++	+++	++	+++	+++	++
	++	++	++	+++	++			
		4	+++	++	++	++	++	++
		++	++	+++	++	++	++	
	1	++	++	++	++	+++	+++	++
	+++	+++	++	+++	++			
	3	+++	++	++	++	++	++	++
	+++	+++	++	+++	++			
In-contact	35	+	+	+	+	+	+	+
	+	+	+	+	+			
	33	+	+	+	+	+	+	+
	+	+	+	+	+			
Control	39	+	+	+	+	+	+	+
	+	+	+	+	+			
	40	+	+	+	+	+	+	+
	+	+	+	+	+			

Note : + 1 10 labelled cells, ++11- 50 labelled cells, +++> 50 labelled cells

CD_2+ labeled cells were present in the lamina propria of the villi and crypt regions of intestine. In mesenteric lymph nodes, more reactivity was seen in the paracortex and corticomedullary junction. CD_4+ cells were mostly distributed in the villus region of lamina propria of the intestine. labeled cells were seen in crypt region. In the mesenteric lymph nodes CD4+ cells were more in the cortical region.

CD_8+ cells were found mostly in the base of epithelium of intestine and paracortical region of the mesenteric lymph nodes. Crypt region also showed more reactivity. Labelled cells were also visualized in the cryptal region. CD_{25}+ cells were distributed in the lamina propria of the intestine. In the mesenteric lymph nodes cells were concentrated in the paracortex and medullary region

MHC I cells were found in the lamina propria and under the basement membrane of the epithelium, cortex and paracortical region of the mesenteric lymph nodes. MHC II cells expressing MHC II antigens were found in the villi and crypts of small intestine and paracortex and medullary regions of mesenteric lymph node.

DISCUSSION

There were increased reactivity of $CD2^+$, $CD4^+$, $CD8^+$, $CD25^+$, MHC I and MHCII labeled cells in the villi and crypts of the small intestine and cortex and medulla of the mesenteric lymph nodes in comparison to the in contact and control goats. These results were in line with those reported in naturally and experimentally infected in goat (Navarro *et.al.,* 1998; Gonzlez *et.al.,* 2001). The increase in $CD2^+$ labeled cells reflected the increased labeling of $CD4^+$and $CD8^+$ cells (Mackay *et.al.,*1988) and the importance of $CD8^+$ lymphocytes in paratuberculosis infection has been highlighted in sheep and goats (Navarro *et.al.,* 1998; Reddacliff *et.al.,* 2004). A decrease in $CD4^+$ lymphocytes and an increase in $CD8^+$ T lymphocytes have been observed in goats showing borderline lepromatous type lesions suggesting that deficit in macrophage activation mediated by $CD8^+$ T lymphocytes plays a role in progression of paratuberculosis lesions (Navarro *et.al.,* 1998). Navarro *et.al.,* (1998) also described an increae in $CD8^+$ T lymphocytes and decrease in $CD4^+$ lymphocytes in mesenteric lymph nodes and suggested that the progression of paratuberculosis lesions in the goat could be due to an ineffective host immune response, which was attributed to $CD8^+$ T lymphocytes subset that down regulates the activity of $CD4^+$ lymphocytes required for macrophage activation. In the present study an increase in the number of $CD8^+$ lymphocytes suggested strong CMI response in the animals, which could be responsible for asymptomatic paratuberculosis with mild lesions in goats. Koets *et.al.,* (2002) in a study of progressive bovine paratuberculosis indicated thast when the asymptomatic stage was compared with the clinical stage of Johne's disease, there was a decrease in the number of $CD4^+$ T lymphocytes where as $CD8^+$ T lymphocytes remain at comparable frequencies. The pathogenesis may be mediated predominantly by the loss of protective $CD4^+$ T lymphocytes responses during the course of the disease. The loss of $CD4^+$ and γδ cells through apoptotic cell death has been described for *M. tuberculosis* infection (Koets *et.al.,* 2002)

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