Immunohistochemical detection of C-reactive protein, serum Amyloid-A, caspase and Tumor Necrosis Factor-α in mediastinal lymph nodes in cattle with tuberculosis

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SUMMARY

The expression of the C reactive Protein (CRP), the Serum Amyloid A protein (SAA), the caspase 3 and the Tumor Necrosis Factor α (TNFα) was investigated in mediastinal lymph nodes from tuberculous cows (n = 29) and from healthy controls (n = 10) by specific immunolabelling. Mediastinal lymph nodes systematically with typical gross tuberculous lesions and control tissues were histologically analysed after Haematoxylin-Eosin and Ziehl-Neelsen staining. After confirmation of the tuberculosis diagnosis by histology, immunohistochemistry using specific antibodies against each antigen was performed. Strong positive reactions for CRP, SAA, caspase 3 and TNFα were found in macrophages and inflammatory cells mainly located near the center of caseous necrosis and more scarcely in surrounding areas. The giant cells poorly expressed the TNFα and the caspase 3 and very rarely the 2 positive acute phase proteins. These results suggest that an inflammatory response coupled to the macrophage apoptosis is involved in the formation and maintenance of the tuberculous granulomas and that some acute phase proteins can be produced for a long time during a chronic active disease, like tuberculosis.

Keywords: Immunohistochemistry, bovine tuberculosis, inflammation, apoptosis, CRP, SAA, Caspase, TNFα.

INTRODUCTION

Infection and injury provoke a range of physiological changes collectively known as the acute phase response. The acute phase proteins (APPs) are proteins whose plasma concentration changes during inflammatory disorders [6]. In response to injury, local inflammatory cells (neutrophil granulocytes and macrophages) secrete various cytokines into the bloodstream, in particularly the interleukins IL-1, IL-6, IL-8 and the TNFα [16, 18]. Some of the positive APPs (whose the serum concentration increases during the acute phase) mainly synthesized by the liver are C-reactive protein (CRP), serum amyloid A (SAA) and fibrinogen [21]. These APPs have immunomodulatory and anticoagulant properties [15]. The best known of these positive acute-phase proteins is probably the CRP abundantly produced and secreted by hepatocytes but also by other cells including lymphocytes, Kupffer’s cells, monocytes and macrophages [3]. An elevation of circulating CRP concentration is not, however, a tell-tale sign pointing to just one disease [16]. The SAA positive acute phase protein is one of the other major components of the acute-phase response. This protein is the circulating precursor of amyloid A protein, involved as a fibrillar form in amyloid deposits [8, 26]. The main production site is the liver but extra-hepatic production has been demonstrated in many species, including humans [9]. The TNFα is a proinflammatory cytokine produced primarily by mononuclear phagocytes after stimulation by immune complexes or bacteria and bacterial products [18, 24, 25]. In cattle, this cytokine is
likely an important mediator of the acute phase response and the SAA is a more rapid positive acute phase protein than the others [18].

With growing attention to the topic of macrophage apoptosis in tuberculosis an increasingly complex picture of this host-pathogen interaction is emerging involving TNFα dependent and independent apoptosis responses, depending on the intracellular bacterial load [22]. During apoptosis, the cell is killed by a class of endogenous proteases called caspasases, and caspasases 3 and 8 are the main forms activated during apoptosis [10-12]. Apoptosis can be triggered by either an intrinsic pathway, involving mitochondrial release of cytochrome c, or by an extrinsic pathway, involving the stimulation of membrane death receptors and direct activation of caspasases. The expression of some pro-apoptotic proteins (bax and fas) has been evidenced in macrophages within tuberculous granuloma [12].

The most known mycobacterial infection is tuberculosis (TB) and it occurs in a wide range of mammalian species [7, 19]. Bovine TB is a chronic disease characterized by progressive development of specific granulomatous lesions or tubercles in lung tissue, lymph nodes or other organs [2, 13, 22]. Although cytokine and acute phase protein concentrations were frequently investigated in the serum of subjects with tuberculosis [17, 24], there are limited studies available for immunohistochemical detection of TNFα [13, 25] and caspase [10, 23] in the tuberculosis lesions and, according to the author knowledge there is no available report about the detection of the CRP and SAA in mycobacterial lesion.

The aim of this study was to investigate the expression of the CRP and SAA proteins, of caspase 3 and of the cytokine TNFα by immunohistochemistry in mediastinal lymph nodes with typical lesions of bovine TB.

Materials and Methods

In this study, the lymph nodes from slaughtered cattle, 3-7 years old and stemming from closed stables were removed and sliced into thin sections of 2 mm thick and inspected for the lesions. The mediastinal lymph nodes of 29 cattle with characteristic granulomatous lesions for TB (white to yellowish hard nodules with caseous necrosis) were examined. Ten healthy cattle mediastinal lymph nodes were used as control. Infection with Mycobacterium organisms was confirmed by histopathological examination: for this purpose tissue samples from granulomatous lesions were fixed in 10% buffered formalin and processed routinely for light microscopy. Five µm sections were taken from paraffin embedded tissues and stained with Haematoxylin-Eosin (HE) and with the Ziehl-Neelsen (ZN) method specific for Mycobacteria [24].

Selected tissue sections were stained immunohistochemically in order to demonstrate the expression of the CRP [Santa Cruz Biotechnology, Inc.- California (CRP (N-14):sc-18304), 1/100 dilution], SAA [Santa Cruz Biotechnology, Inc.- California (SAA (C-16):sc-20275), 1/100 dilution], TNFα [Santa Cruz Biotechnology, Inc.- California (TNFα (N-19):sc-1350), 1/100 dilution] and caspase 3 [Neomarker –California (Caspase 3 (CPP32) Ab-4, 1/100 dilution] using a routine streptavidin-biotin peroxidase technique according to manufacturer instructions. Tissues were counterstained with Mayer’s Haematoxylin, washed in water, and cover slips were applied with mounting media. To evaluate the percentage of immunopositive cells, 10 different microscopic high powered fields of each tubercle were examined under the 40x objective of a Nikon E-600 trinocular microscope coupled to a UIII microphotography apparatus.

Results

GROSS FINDINGS

Some granulomatous foci formations were seen in lymph nodes in 29 cattle and were characteristic of TB. These formations, named tubercles, consisted in white to yellow nodules measuring 0.1 to 2 cm in diameter. More than one tubercle was generally found in lymph nodes. More severely infected animals had multiple nodules or large cheesy masses in the same areas. Caseous necrosis and sometimes calcification were observed in granulomas. Tubercles were seen in both the cortex and medulla of the lymph nodes and a marked lymphadenopathy was the prominent finding. Typical tubercles were observed in lungs from all the 29 animals and the lymph nodes of the lungs were swollen and necrotic. Adhesions to the lungs and mediastinum were seen in four cases. Numerous granulomas with different sizes were found in mediastinal lymph nodes and characteristic caseous necrosis was observed in the center of the big granulomas. By contrast, no gross lesion related to TB in lungs and in lymph nodes was evidenced in control animals.

HISTOPATHOLOGICAL FINDINGS

Caseous necrosis and giant cell formations were common in the scattered nodules. The accumulation of the macrophages and of other inflammatory cells and giant cells were the prominent findings (figure 1). A thick capsule formed around the tubercle walling it off from other tissues was usually observed. Calcification was a common histological finding in the granulomas. In addition to these characteristic lesions, all tissue sections were positively stained with the Ziehl-Neelsen method specific for Mycobacteria which appeared as short red rods (figure 2). On the other hand, no characteristic histological lesion for TB was observed in the control lymph nodes and no bacteria were detected with the specific staining method.

IMMUNOHISTOCHEMICAL FINDINGS

The mediastinal lymph nodes from cattle with TB were positively immunostained for TNFα (figure 3), SAA (figure 4), CRP (figure 5) and caspase 3 (figure 6) and the labelling of each antigen was restricted to cytoplasm. The number of positive cells and the intensity of staining within a cell were particularly intense and prominent closer to the necrotic center but some positive cells were also evidenced in the fibrous

tissue areas. Positively stained cells were essentially macrophages and inflammatory cells. By contrast, giant cells in the granuloma remained negatively stained for CRP and SAA except for some rare cells and a weak expression of TNFα and of caspase 3 compared to macrophages was also noticed in this cellular type. In control lymph nodes, only one or two TNFα and caspase positive macrophages or lymphocytes were observed but no CRP and SAA positive cell was detected.

**Discussion**

Tuberculosis is an ancient, communicable, world-wide, chronic disease of human beings and domestic animals. The TB has typical gross and histopathological characteristics [19]. The granuloma typically consists in a necrotic center surrounded by inflammatory cells (epithelioid macrophages, multinucleated giant cells, T and B lymphocytes) and scattered

![Figure 1](image1.png)  **Figure 1**: Histological appearance of a tubercle with giant cells (arrows), cow, Haematoxylin-Eosin, Bar = 50 µm.

![Figure 2](image2.png)  **Figure 2**: Zielh-Neelsen staining of Mycobacteria, numerous red rod shaped bacteria in macrophages (arrowheads) and in giant cells (thick arrows), and free bacteria in the tubercle (thin arrow). Bar = 100 µm.

![Figure 3](image3.png)  **Figure 3**: Immunohistochemical detection of TNFα in macrophages (arrows) from lymph nodes of a cow with TB. Streptavidin-biotin peroxidase technique, Bar = 50 µm.

![Figure 4](image4.png)  **Figure 4**: SAA expression in macrophages (arrows) in bovine TB. Streptavidin-biotin peroxidase technique, Bar = 50 µm.

![Figure 5](image5.png)  **Figure 5**: Intense CRP immunolabelling within macrophages (arrows) in a mediastinal lymph node from a cow with TB. Streptavidin-biotin peroxidase technique, Bar = 50 µm.

![Figure 6](image6.png)  **Figure 6**: Positive caspase staining in macrophages (arrows) indicating apoptosis in bovine TB. Streptavidin-biotin peroxidase technique, Bar = 50 µm.
foci of fibroblasts. The typical granulomas in lymph nodes from affected cattle were grossly and histopathologically seen in the present study. Caseous necrosis, giant cells and fibrous tissue proliferation that indicating a chronic reaction, were prominent. Abundant newly formed foci were observed by histological examination. Moreover, the acid fast stained bacilli, Mycobacteria, were demonstrated in all sections using the Ziehl-Neelsen stain. These histopathological findings undoubtedly confirmed the tuberculosis diagnostic.

The specific immune system is generally successful in containing, although not necessarily in eliminating, the pathogen tubercle bacilli mainly throughout macrophage and T cell responses. The granuloma formation is important in the host-defence against intracellular bacteria and other types of foreign antigens. Indeed, the survival of the host against M. tuberculosis may depend on the ability to form effective granulomas that limit microbial proliferation [1]. Furthermore, it is known that M. tuberculosis can persist for a long time in macrophages within granulomas. The formation of the tuberculous granuloma is complex, dependent on the migration and activation of an array of immune cells, initiated by cytokine and chemokine expression in response to mycobacterial antigens. Among these inflammatory mediators, the TNFα is considered as very important for the formation of tuberculous granulomas [3]. It is significantly involved in the macrophage activation leading to the intracellular killing of Mycobacteria after phagocytosis [5]. Both beneficial and deleterious effects of TNFα towards the host have been observed in the tuberculosis pathophysiology [4]. The activation of macrophages needed to killing Mycobacteria is mainly driven by the T helper type-1 (Th1) cytokines, such as the interferon γ and the TNFα [14]. In addition to T-cell-derived cytokines, a variety of other cytokines and chemokines are produced during the response to mycobacterial infection [20]. The host becomes hypersensitive to the mycobacterium, which enhances the cell-mediated immune defences in early or mild infections, but can result in host-tissue destruction in the form of caseous necrosis and granulomatous reaction [19, 22]. In this study, macrophages exhibited a strong TNFα expression whereas the giant cells poorly synthesized this cytokine. This result was similar with previous studies [13, 25]. Activation of macrophages and synthesis of TNFα were marked in all tubercles and were seen in both small and big granulomas. The TNFα expression can be attributed to the presence of mycobacterial particles in the granuloma and indirectly indicate the maintenance of the immune response leading to granuloma formation and development.

Macrophages are the primary target and a critical reservoir of the infection of Mycobacteria in the granulomas. After phagocytosis of the TB bacilli, macrophages express several antimicrobial mechanisms for limiting the intensity of the intracellular infection. Among these innate defence mechanisms, apoptosis also named programmed cell death has been linked to the killing of intracellular Mycobacteria [26]. Interestingly, several clinical studies have shown that apoptotic macrophages can be recovered from individuals with TB. Moreover, the increase of the caspase activity in mycobacterial infection due to the ESAT6 protein has been recently reported [11] and some studies have shown that exposure of macrophages to M. tuberculosis can increase the rate of apoptosis. However, despite an abundance of results linking apoptosis with mycobacterial infections, many questions including the identity of the mycobacterial factors responsible for evoking the apoptotic response, the TB-induced apoptotic mechanisms, and the impact of apoptosis on the overall infection remain unresolved [10, 23]. In the present study, the increase of apoptosis rate in macrophages from tuberculous granuloma was evidenced by the enhanced expression of the caspase 3, an enzyme specifically involved in the apoptosis pathway. The TB induced apoptosis was more severe in macrophages than in the other inflammatory cells. The giant cells presented a weak apoptotic activity, suggesting that this cellular type would be more resistant to apoptosis than macrophages and would constitute a continuous source for production of pro-inflammatory cytokines.

The CRP and SAA are the most sensitive indicators of inflammation among the acute phase proteins and plasma concentrations of these proteins are highly correlated in various inflammatory conditions [9]. This study demonstrated the presence of these 2 positive acute phase proteins into inflammatory lesions and their direct coupled production by inflammatory cells, mainly by macrophages. By contrast, the great majority of giant cells remained negative for these 2 proteins, probably because they become unable to synthesize them. As no CRP and SSA expression can be evidenced in macrophages or in lymphocytes from control lymph nodes, it is probable that their production by activated macrophages is necessary for the local inflammatory response in tuberculous areas and for granuloma formation.

As a conclusion, a combined intense expression of CRP, SAA, TNFα and caspase 3 mainly in macrophages was evidenced in tuberculous granulomas in all affected cows investigated. The production of these markers of inflammation and of apoptosis was maximal near the necrotic center but was also observed in inflammatory and fibrous surrounding areas. This descriptive study provides evidence that CRP, SAA, TNFα and caspase-3 are involved in the formation or maintenance of the characteristic lesions observed in bovine tuberculosis and that CRP and SSA can also be produced for a long time in chronic diseases such as tuberculosis.

References


