Advanced Granulomatous Lesions in *Mycobacterium bovis*-infected Cattle are Associated with Increased Expression of Type I Procollagen, γδ (WC1⁺) T Cells and CD 68⁺ Cells

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Summary

The pathognomonic characteristic of tuberculosis (TB) is the formation of a tuberculous granuloma. The objective of this study was to classify lymph node granulomas from experimentally infected calves into different histopathological stages and characterize them further by studying cell types and markers of fibrosis associated with each of the stages. Four stages of granuloma were identified and mRNA and protein expression for cell markers, cytokines and pro-fibrotic markers were studied by immunohistochemistry (IHC) and in-situ hybridization (ISH). In advanced stage granulomas, there was an increase in the expression of TGF-β, and of type I procollagen as demonstrated by IHC and ISH. As the granulomas advanced, there were fewer CD3⁺ T cells and they tended to be more prominent towards the periphery of the lesions, with a steady increase in the number of CD68⁺ cells and γδ (WC1⁺) T cells. Granuloma classification and application of cell cytokine markers will assist in improving understanding of the pathogenesis of bovine TB and may help to identify the immunopathology of active disease versus contained or inactive disease. Such disease correlates may help to inform the development of improved diagnostic methods and support vaccine development programmes.

Keywords: bacterial infection; cattle; granuloma; *Mycobacterium bovis*; tuberculosis

Introduction

Bovine tuberculosis (TB) is currently of considerable importance in Great Britain. New herd incidents and culling of reactors to the tuberculin test is increasing annually by c. 18% and 20%, respectively. The cost to the Government for 2003/2004 was £88 million (DEFRA, 2004). An independent scientific review panel concluded that the development of a vaccine for cattle held the best long-term prospect for bovine TB control in GB (Krebs et al., 1997). To determine the efficacy of any such vaccine, however, identification of an accurate correlate of protection or pathogenesis is needed. Accordingly, there is a renewed interest in developing tools to study pathogenesis in relation to efficacy of vaccination.

The pathognomonic lesion of bovine tuberculosis is the granuloma. Granuloma formation in mammals is thought to be the result of chronic antigenic stimulation and to represent an attempt by the host to localize the disease process (Fuller et al., 2003). A granuloma can be defined as a focal accumulation of inflammatory cells in which macrophages, epithelioid macrophages, multinucleated giant cells and lymphocytes predominate (Cotran et al., 1999; Fuller et al., 2003). Both B and T lymphocytes participate in granuloma formation and evidence from mouse models of *Mycobacterium*...
tuberculosis infection suggests that CD4 and CD8 T cells play a role in lesion development (Gonzalez-Juarrero et al., 2001). Studies in cattle suggest a role for WC1+γδ T cells in the early host response to M. bovis infection (Pollock et al., 1996; Cassidy et al., 1998), and it has been speculated that in man these cells play a role in granuloma development (Modlin et al., 1989; Kaufmann, 1990). Characteristically, M. bovis granulomas are surrounded by connective tissue and in cases of human tuberculosis transforming growth factor (TGF)-β, which promotes collagen synthesis, has been detected near new collagen (Marshall et al., 1996). Newly synthesized collagen is detected by immunohistochemical labelling for procollagen (McDonald et al., 1984) or in-situ hybridization for mRNA of type I procollagen (Peltonen et al., 1991; Wangoo et al., 1995).

Granuloma stages were described in the lungs of mice and guinea-pigs infected experimentally with M. tuberculosis (Rhoades et al., 1997; Turner et al., 2003). These stages were delineated on the basis of criteria that included the extent and cellular composition of the granulomas. To the best of our knowledge this type of classification scheme has not been applied to bovine tuberculosis granulomas. In the present study, granulomatous lesions in lymph nodes of calves experimentally infected with M. bovis were classified and divided into four developmental stages and further characterized by inflammatory cell composition, expression of growth factors, and the formation of new collagen.

**Materials and Methods**

**Experimental Infection of Calves**

The study was approved by the local Ethical Review Committee and carried out under a Home Office project licence. Eighteen castrated male calves (Friesian or Friesian cross) aged 5 months were infected intratracheally, as described by Vordermeier et al. (2002), with 70 to 62 000 colony-forming units (cfu) of a UK field strain (AF2122/97) of M. bovis. The animals were humanely killed 29 weeks after infection.

At necropsy, all animals used in this study showed comparable gross lesions restricted to the lung and cervical and thoracic lymph nodes, regardless of the size of the infective dose. Samples were obtained from the following lymph nodes: mandibular, parotid, lateral and medial retropharyngeal, caudal and cranial mediastinal, bronchial and tracheobronchial.

**Tissue Preparation**

Tissues were fixed in neutral-buffered formalin (4% formaldehyde) for 7 days and embedded in paraffin wax. Sections (4 μm) were cut and placed on Vectabond-treated slides (Vector Laboratories, Peterborough, UK) for immunohistochemistry, and on positively charged, RNase-free slides (Shandon Scientific, Runcorn, UK) for in-situ hybridization. Lymph node sections were stained with haematoxylin and eosin (HE) for histopathological examination and by the Ziehl-Neelsen (ZN) method to detect acid-fast bacilli (AFB).

All the lymph nodes (234 in total) were screened for lesions by light microscopy and 55 found to contain granulomas. The granulomas from these 55 lymph nodes were then subjected to histopathological scrutiny with the aim of discerning broad developmental stages that could be used in classification. Four stages were identified and termed Stage I (Initial), Stage II (Solid), Stage III (Minimal Necrosis), Stage IV (Necrosis and Mineralization). Full descriptions of these stages are given in the Results section.

Lymph nodes often contained granulomas of different stages. For immunohistochemistry and in-situ hybridization studies, 22 of the 55 lymph nodes were selected in such a way as to ensure that at least five granulomas of each stage were examined.

**Immunohistochemistry (IHC)**

The avidin-biotin-complex (ABC Vector Elite; Vector Laboratories) method was used. The sections were dewaxed, rehydrated and treated with hydrogen peroxide 3% in methanol for 15 min to eliminate endogenous peroxidase activity. Sections were next pretreated for antigen retrieval by enzymatic digestion with trypsin 0.5%/chymotrypsin 0.5% (Sigma, Poole, UK) at 37 °C for 10 min or by microwaving in citric acid buffer, pH 6.0, for 3×6 min at 100 °C (780 W). Table 1 shows the epitope demasking method used for each antibody. The sections were then mounted in a Sequenza Immunostaining Centre (Shandon Scientific) and rinsed with 0.005 M Tris-buffered saline (TBS), pH 7.6 (Sigma). A blocking step, with 1.5% normal serum from the host species in which the link antibody was raised, was applied to the sections for 20 min. The primary antibodies, which were mainly anti-human antibodies that cross-reacted with bovine tissues, were applied to the sections either overnight at 4 °C or for 1 h at 20–22 °C. All primary antibodies had previously been screened to determine the optimum dilution and incubation temperature (Table 1). The sections were washed in TBS and incubated for 30 min with
the appropriate biotinylated secondary link antibody (Vector Laboratories) before being washed twice in TBS again. They were then incubated for 30 min at room temperature with ABC, the signal being detected with 3,3′-diaminobenzidine tetrahydrochloride (DAB), which produces a brown colour at the site of the reaction. Sections were lightly counterstained with Mayer’s haematoxylin.

### Table 1
Antisera and oligonucleotide probes used for immunohistochemistry and in-situ hybridization

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody or probe</th>
<th>Target cell type or cytokine</th>
<th>Source</th>
<th>Epitope demasking</th>
<th>Primary antibody or probe concentration (and incubation time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell marker</td>
<td>Anti-human CD68 (MC)</td>
<td>Macrophages</td>
<td>Dako*</td>
<td>Trypsin</td>
<td>4.0 µg/ml (O/N at 4 °C)</td>
</tr>
<tr>
<td>Anti-human CD3 (PC)</td>
<td>Trypsin</td>
<td>T cells</td>
<td>Dako*</td>
<td>Trypsin</td>
<td>1.0 µg/ml (O/N at 4 °C)</td>
</tr>
<tr>
<td>Anti-human CD73a (MC)</td>
<td>Trypsin</td>
<td>B cells</td>
<td>Dako†</td>
<td>Microwave</td>
<td>2.1 µg/ml (1 h at RT)</td>
</tr>
<tr>
<td>Anti-bovine CC15 (MC)</td>
<td>Trypsin</td>
<td>γδ T cells</td>
<td>Serotec†</td>
<td>Trypsin</td>
<td>0.9 µg/ml (O/N at 4 °C)</td>
</tr>
<tr>
<td>Anti-human Type 1 Pro-collagen (MC)</td>
<td>Trypsin</td>
<td>Pro-collagen 1 protein</td>
<td>Chemicon‡</td>
<td>Trypsin</td>
<td>2.5 µg/ml (1 h at RT)</td>
</tr>
<tr>
<td>Oligonucleotide Cocktail</td>
<td>Trypsin</td>
<td>Pro-collagen 1 mRNA</td>
<td>Genedetect¶</td>
<td>Proteinase K</td>
<td>200 ng/ml</td>
</tr>
<tr>
<td>Growth factors</td>
<td>Anti-human TGF-β (PC)</td>
<td>TGF β 1 protein</td>
<td>Santa Cruz§</td>
<td>Microwave</td>
<td>0.8 µg/ml (1 h at RT)</td>
</tr>
</tbody>
</table>

PC, polyclonal antibody; MC, monoclonal antibody; O/N, overnight; RT, room temperature.

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† Oxford, UK.
‡ Temecula, CA, USA.
¶ Auckland, NZ.
§ Santa Cruz, CA, USA.

The immunolabelled sections were examined by light microscopy and by digital image analysis (Lucia; Laboratory Imaging Ltd, Prague, CZ). In each slide, granulomas as described above were examined at ×400 to determine the number of immunolabelled cells, expressed as a percentage of total cells in the area of interest. Areas with no obvious lesions, immediately outside the granulomas, were similarly examined, serving as controls. The results were expressed as mean ± SE of five samples per granuloma stage.

### Image Analysis

The immunolabelled sections were examined by light microscopy and by digital image analysis (Lucia; Laboratory Imaging Ltd, Prague, CZ). In each slide, granulomas as described above were examined at ×400 to determine the number of immunolabelled cells, expressed as a percentage of total cells in the area of interest. Areas with no obvious lesions, immediately outside the granulomas, were similarly examined, serving as controls. The results were expressed as mean ± SE of five samples per granuloma stage.

### In-situ Hybridization (ISH)

Oligonucleotide probes designed by GeneDetect (Auckland, New Zealand) were used to detect type I procollagen mRNA. These probes, which contained a cocktail of two antisense sequences (48 bp) labelled with digoxigenin, were taken from the published sequence for type I procollagen (Data Bank Accession No. 14041788). The sequences were: (1) CAGACACGGATCTGGCGAG GCACGG GTTTCCA CACGTCCTCGGTCTATGG, and (2) GGGGTCAGGGGTCCGGGAACTGGTTGTCGCCAGGA

TGGCATCCTC. The hybridization procedure was as recommended by GeneDetect. Briefly, the sections were dewaxed in xylene and rehydrated through graded ethanol solutions and diethyl pyrocarbonate (DEPC)-treated water (Sigma). They were post-fixed in 4% paraformaldehyde and treated with Proteinase K (Roche Diagnostics, Lewes, UK) 20 µg/ml for 30 min at 37 °C, before being incubated for 2 h at 37 °C in prehybridization buffer and 18 h at 39 °C in hybridization mixture in a humidified chamber. After washes, the sections were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche), the signal being detected with BCIP/NBT (Roche), which produces a blue/purple colour at the site of the reaction. The slides were counterstained with Vector Nuclear Fast red (Vector Laboratories) for 5 min and mounted with Supermount aqueous mounting medium (BioGenex, San Ramon, CA, USA).

### Results

#### Histopathological Analysis of the Four Different Stages of Granuloma Formation in Bovine TB

From histological examination of HE- and ZN-stained sections from the 55 granuloma-positive lymph nodes, it was possible to recognize four broad developmental stages in the granulomas. Descriptions of these follow and examples of each stage are shown in Fig. 1.

**Stage 1 (Initial).** Irregular unencapsulated clusters of epithelioid macrophages were seen, with interspersed lymphocytes and small numbers of neutrophils. Langhan’s multinucleated giant cells were sometimes present, but there was no necrosis (Fig. 1a).
Stage II (Solid). Granulomas at this stage were composed primarily of epithelioid macrophages and were enclosed partly or completely by a thin capsule. Haemorrhage was often noted, with infiltration of lymphocytes, neutrophils and often Langhan’s multinucleated giant cells. Minimal necrotic areas were sometimes present, generally composed of necrotic inflammatory cells (Fig. 1b).

Stage III (Minimal necrosis). The granulomas were fully encapsulated, with central necrotic areas, which were caseous and mineralized. Epithelioid macrophages admixed with Langhan’s multinucleated giant cells surrounded the necrosis. A peripheral zone of macrophages mixed with clusters of lymphocytes and scattered neutrophils extended to the fibrous capsule. (Fig. 1c).

Stage IV (Necrosis and mineralization). Thickly encapsulated, large, irregular, multicentric granulomas with prominent caseous necrosis were seen, extensive islands of mineralization occupying the greater part of the lesion. Epithelioid macrophages and multinucleated giant cells surrounded the necrosis, with particularly dense clusters of lymphocytes near the peripheral fibrotic capsule (Fig. 1d).

Further details of the characterization of each stage, including granuloma size, numbers of mycobacteria present, cellular composition and extent of fibrosis, necrosis and mineralization, are given in Table 2. It was possible to assign all granulomas to one or other of the four developmental stages. Distribution of granulomas of each
The stage among the 55 lymph nodes examined is detailed in Table 3.

**Distribution of CD3 and CD79 Cells as Demonstrated by IHC**

In interpreting the results of immunolabelling (IHC) and image analysis of early stage I granulomas, resident CD3⁺T lymphocytes could not be distinguished from T cells responding to *M. bovis* infection. CD3⁺T cells were abundantly labelled in stage I granulomas and were widely distributed throughout and surrounding clusters of epithelioid macrophages and giant cells (Fig. 2a). As the granuloma increased in size and advanced in stage, the number of CD3⁺T cells composing the viable cell population within the fibrous capsular borders of the granuloma decreased. As the area of caseous necrosis increased, T cells became widely dispersed throughout the granuloma, but were increasingly present in a marginal zone inside the fibrotic capsule. Fig. 3a demonstrates a stage II lesion with a high proportion of CD3⁺T cells widely distributed throughout the granuloma and a large T-cell population outside the peripheral capsule. By stage III (Fig. 3b), T cells were concentrated in a subcapsular zone within the granuloma margins.

**Table 2**

Four stage characterization of lymph node granulomas in *M. bovis*-infected calves

<table>
<thead>
<tr>
<th>Lesion stage</th>
<th>Mean lesion size (mm) ± SE, and number of bacilli</th>
<th>Cellular composition</th>
<th>Growth factor, collagen and fibrosis</th>
<th>Necrosis and mineralization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Initial)</td>
<td>0.49 ± 0.02 0–1 bacilli</td>
<td>Epithelioid macrophage clusters, including giant cells with interspersed and encircling lymphocytes, mostly CD3⁺T cells; a few γδ T cells and B cells</td>
<td>Minimal procollagen, no peripheral fibrosis; few, scattered TGF-β-expressing cells (average 10)</td>
<td>No necrosis</td>
</tr>
<tr>
<td>2 (Solid)</td>
<td>0.68 ± 0.07 1–5 bacilli</td>
<td>Epithelioid macrophages and giant cells dominate, with widely interspersed CD3⁺T cells, fewer (often peripheral) B cells, scattered γδ T cells and occasional neutrophils</td>
<td>Procollagen expression; incomplete/thin encapsulation; more TGF-β cells, often peripheral (average 40)</td>
<td>Minimal central necrosis or haemorrhage</td>
</tr>
<tr>
<td>3 (Minimal necrosis)</td>
<td>1.14 ± 0.16 1–5 bacilli</td>
<td>Epithelioid macrophages and giant cells surround necrosis; CD3⁺T cells present but mainly peripheral; γδ T cells widely dispersed within periphery; B cells within periphery, but intensely clustered about fibrous capsule; few neutrophils</td>
<td>Increased procollagen expression; complete encapsulation; TGF-β expression mainly peripheral in granuloma</td>
<td>Central caseous necrosis, often with mineralization (minimal)</td>
</tr>
<tr>
<td>4 (Necrosis &amp; mineralization)</td>
<td>3.49 ± 0.76 &gt; 5 bacilli</td>
<td>Epithelioid macrophages and giant cells surround necrosis; γδ T cells prominent among CD3⁺T cells at periphery; B cells cluster multifocally outside the capsule and at granuloma margins</td>
<td>Procollagen present within and at periphery; thick fibrous septation and encapsulation; increased TGF-β expression in capsular region</td>
<td>Extensive multicentric caseous necrosis with mineralization</td>
</tr>
</tbody>
</table>

**Table 3**

Distribution of granulomas of each developmental stage among the 55 lymph node samples examined

<table>
<thead>
<tr>
<th>Lymph node</th>
<th>Number of nodes showing lesions of stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Mandibular</td>
<td>5</td>
</tr>
<tr>
<td>Parotid</td>
<td>4⁠*</td>
</tr>
<tr>
<td>Lateral retropharyngeal</td>
<td>3</td>
</tr>
<tr>
<td>Medial retropharyngeal</td>
<td>4⁠*</td>
</tr>
<tr>
<td>Caudal mediastinal</td>
<td>6⁠*</td>
</tr>
<tr>
<td>Cranial mediastinal</td>
<td>15⁠*</td>
</tr>
<tr>
<td>Bronchial</td>
<td>12⁠*</td>
</tr>
<tr>
<td>Tracheobronchial</td>
<td>6⁠*</td>
</tr>
<tr>
<td>All</td>
<td>55</td>
</tr>
</tbody>
</table>

*Some lymph nodes showed granulomas of several different stages.*
As the granuloma advanced, however, more γδ T cells migrated to the site and by stage IV there was a much higher proportion of such cells within the granuloma. Fig. 3e demonstrates γδ T (WC1+) cells primarily within the peripheral zone of a stage III granuloma, as well as interspersed through and outside the fibrotic capsule.

**Distribution of CD68+ Cells as Demonstrated by IHC**

The macrophage marker CD68 identified increasing numbers of macrophages with lesion advancement. Fig. 2c demonstrates the increasing infiltration of epithelioid macrophages and giant cells, in contrast to the number present in small, stage I granulomas. The number of macrophages increased in stages II and III, and in stage IV macrophages formed the majority of cells within the granuloma (P<0.05). Fig. 3f shows a stage II granuloma, with numerous CD68+ cells dispersed throughout, and scattered Langhan’s multinucleated giant cells.

**Distribution of TGF-β and Type I Procollagen in Granulomas**

The growth factor TGF-β, which is a product of many cell types including macrophages, has a potent stimulatory effect on collagen production. IHC showed minimal expression of this growth factor in the early stages of granuloma formation, but in advanced granulomas there was a much higher proportion of TGF-β-positive cells (Fig. 4a), especially near the fibrotic capsule. Some Langhan’s giant cells strongly expressed TGF-β. Expression of this growth factor was also observed in the medullary region of the lymph nodes, to approximately the same degree in each of the four stages.

Type I procollagen is a precursor of new collagen synthesis. In the initial stages of the lesion, it was barely detectable by IHC. By stage II, however, the formation of irregular fibres around cells had become apparent. By stage III, some collagen fibres were visible near central areas of necrosis and a definite fibrous band had formed around the granuloma (Fig. 4b). Similarly mRNA expression for type I procollagen, as demonstrated by ISH, was minimal in the early stages of the lesion, but by stage IV, a thick band of collagen deposition completely surrounded the granuloma (Fig. 4c, 4d).

**Other Properties of Developing Granulomas**

The size increased with the advancement of the lesion, almost doubling from stage I to II and from stage II to III, and more than doubling on average from stage III to IV (Table 2). The area of necrosis and mineralization increased correspondingly, with a dramatic increase at stage IV.

The number of acid-fast bacilli within granulomas generally increased with the stage of advancement (Table 2). The trend within stage IV
Fig. 3 a–f. Immunolabelling for CD3$^+$ (a and b), CD79$^+$ (c and d), γδ T cells (e) and CD68$^+$ (f) in granulomas of lymph nodes infected with *M. bovis*. Arrows indicate examples of brown immunolabelled cells. ×100.
granulomas was towards greater numbers of mycobacteria; one granuloma in particular contained numerous bacilli, but several others had low numbers. In the early stages, bacilli were found within macrophages or giant cells, but in advanced necrotic granulomas they were generally seen within necrotic areas, or sometimes even in mineralized debris.

**Discussion**

Lymph node granulomas that formed in response to experimental intratracheal infection with *M. bovis* could be classified into four stages, from stage I (small, irregular and unencapsulated granulomas) to stage IV (large, multicentric, thickly encapsulated necrotic granulomas). The cellular composition and growth factor microenvironment of granulomas varied according to the stage of the lesion. In early lesions close apposition between predominantly CD3^+^ T lymphocytes and epithelioid macrophages facilitates “upregulation” of various cytokines, such as interleukin (IL)-12 and interferon (IFN)-γ, and chemokines in experimental models (Saunders and Cooper, 2000; Gonzalez-Juarrero et al., 2001). The dynamics of these interactions, together with the elaboration of growth factors such as TGF-β, promote the formation of collagen and of the early structural framework of granulomas. Mycobacterial antigen stimulates the recruitment, retention and proliferation of cells, leading to enlarged granulomas with

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**Fig. 4 a–d.** Demonstration by IHC of (a) TGF-beta in a stage IV granuloma (×100), and (b) Type I procollagen in a stage III granuloma with both peripheral and central procollagen signal. Arrows depict specific brown signal. (×100). Demonstration by ISH of mRNA expression for Type I procollagen. (c) Magenta signal (arrow) depicts procollagen encircling a stage III granuloma (×40). (d) Higher magnification of the procollagen (arrow) within the fibrotic capsule. (×400).
additional fibrotic structure. With lesion expansion, necrosis increases, reducing cell-to-cell apposition and influencing or diminishing cellular interactions. This study represents the first attempt to demonstrate the distribution of cytokine and inflammatory cells with granuloma advancement in *M. bovis*-infected lymph nodes.

Early granuloma development in lymph nodes is based on close contact between activated macrophages and T lymphocytes, which facilitates the production of cytokines, especially IFN-γ, IL-12 and tumour necrosis factor (TNF-α), and cellular activation (Saunders and Cooper, 2000). In this study formalin-fixed tissues resisted the demonstration by IHC of the T-lymphocyte subsets CD4 and CD8; as a result their distribution in granulomas at different stages of development could not be illustrated. Previous reports of *M. tuberculosis* lung granulomas in mice and human beings showed that CD4⁺ T cells predominated and were widely distributed as aggregates within the granuloma, whereas CD8⁺ T cells were less numerous and localized to granuloma margins (Gonzalez-Juarrero et al., 2001). In the present study, CD3⁺ T cells represented a major component of the granuloma cell population (Fig. 2a). In advanced granulomas the CD3⁺ T-cell population was pushed to the periphery by an expanding area of necrosis and a dense zone of epithelioid macrophages and giant cells.

B cells represented a significant lymphocyte population within each stage of granuloma development. Although B cells were never as well dispersed throughout the granuloma as T cells, they were evident in early lesions, and with granuloma advancement became localized to the outer fibrotic capsular wall. Cassidy et al. (2001) noted, in their study of pulmonary granulomas, prominent B-cell clusters at the periphery of large lesions at 42 days post-infection; they suggested that B lymphocytes played an important role in granuloma pathogenesis following prior WC1⁺γδ and CD2⁺ T-lymphocyte activity. B cells proliferate and differentiate through CD4⁺ T-cell stimulation and produce antibody, cytokines such as IFN-γ, and chemokines, IL-8, macrophage inflammatory protein (MIP)-1α and monocyte chemotactic protein (MCP)-1, suggesting their protective and formative role in granuloma development (Bosio et al., 2000; Gonzalez-Juarrero et al., 2001).

An active role in innate immunity is played by the γδ T cells, which represent an important subset of CD3⁺ T cells. In primate and rodent species, only a small percentage of circulating T cells consists of γδ T cells. In ruminants, however, 20–60% of peripheral T cells belong to the γδ T cell subset and possess a unique surface antigen, WC1 (Hein et al., 1990; Hein and Mackay, 1991; MacHugh et al., 1997). In-vitro studies revealed that γδ T cells secreted IFN-γ, TNF-α, IL-2 and granulocyte-macrophage colony-stimulating factor (GMCSF) (Orme and Collins, 1994) and were cytotoxic to BCG-infected macrophages (Dieli et al., 2004). Within the context of bovine tuberculosis, experimental infections have indicated a role for γδ T cells in the formation of lung granulomas (Cassidy et al., 2001). In the present study, the number of γδ T cells increased with granuloma development (Fig. 2b). The granulomas with the greatest number of γδ T cells were advanced granulomas with large numbers of acid-fast bacilli.

Stage I granulomas contained roughly equal numbers of lymphocytes and macrophages (Fig. 2a, 2c), facilitating cellular communication. Stimulated macrophages, or epithelioid cells and multinucleated Langhan’s giant cells, formed the core of the granuloma in the earlier stages, but with lesion advancement central necrosis developed and expanded, encircled predominantly by macrophages and giant cells. T cells produce IFN-γ, which acts synergistically with TNF-α in the activation of macrophages (Kaufmann, 2002). Fig. 3f depicts a stage II granuloma with initial central necrosis in proximity to multinucleate giant cells, the cytoplasmic cell membrane being damaged and incomplete. Often, clusters of multinucleate giant cells showed incomplete cell membranes and the extracellular caseous necrosis was indistinguishable from giant cell cytoplasm; this suggested that giant cell degeneration is an initial event in, or the nidus of, central necrosis.

Scattered neutrophils occurred in granulomas of all four stages; granulocytes, however, were not a major component of the lymph node granulomas. Neutrophils contribute to lung granuloma formation in badgers (Canfield et al., 2002) and are prominent in early stages of granulomatous lymphadenitis in *M. bovis*-challenged, BCG-vaccinated calves (unpublished observation).

All four stages of granulomatous lesions were examined for the appearance and distribution of new collagen synthesis. Type I collagen is a triple helix composed of two alpha1 (1) and one alpha2 (1) chains (Stryer, 1988). Since type I procollagen carboxyl and amino terminal domains are removed proteolytically during collagen secretion (Prockop et al., 1979a,b), the presence of type I procollagen is an indicator of new collagen formation. No expression of this matrix protein was observed in normal areas of lymph nodes or in stage I lesions,
and expression was minimal in stage II lesions. Type I procollagen increased in stage III granulomas and frequently formed the outer rim in stage IV. Several studies have demonstrated collagen formation in mature granulomas in bovine tuberculosis (Palmer et al., 1999) but not the stage at which collagen deposition occurs. The present observations indicate that type I procollagen expression may be associated with the stage of lesion development and therefore may be used as a marker of lesion advancement.

Increased expression of procollagen was also associated with an increase in the pluripotent growth factor, TGF-β, in later granuloma stages. TGF-β, which is an important product of many cells, causes increased collagen type-1 gene expression (Quaglino et al., 1990), collagen protein production by fibroblasts in vitro (Ignotz and Massague, 1986) and enhanced wound healing in vivo (Pierce et al., 1989). TGF-β1 is present in increased amounts in lungs with pulmonary fibrosis (Khalil et al., 1991) as well as in fibrotic diseases of other organs (Yoshioka et al., 1993). The present study supports the association between the increase in TGF-β expression and the increase in type I procollagen mRNA and protein concentrations in advanced granulomas.

In addition to enhancement of fibrosis, TGF-β is believed to play an even broader role in TB pathogenesis. TGF-β is reported to suppress T-cell responses and reduce macrophage bactericidal function by diminishing the production of IFN-γ, IL-12, TNF-α, IL-6, IL-1 and reactive oxygen and nitrogen intermediates (Barnes et al., 1994; Barnes and Wizel, 2000). Inhibition of host TGF-β reduced intracellular growth of M. tuberculosis in human monocytes and restored T-cell function (Hirsch et al., 1997). Hernandez et al. (2002) suggested that the immunosuppressive effects of TGF-β were beneficial to the host by reducing an overexuberant immune response to schistosomes.

Mycobacterial granulomas are dynamic, the cell populations changing over the course of infection. The classification used in this study is descriptive and provides a spatial context of development and inferences as to the chronicity of the lesion. The scheme should assist in standardizing descriptions of bovine TB lesions in lymph nodes and in identifying the stages of granulomas by light microscopy. This should facilitate recognition of deviations from the normal granuloma progression, such as those resulting from immunity, immunosuppression or mycobacterial strain variation.

Acknowledgments

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