Studying the pathogenesis of paratuberculosis: the enduring challenge

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INTRODUCTION

Pathogenesis is defined as the origination and development of disease, more specifically the cellular events and reactions and other pathologic mechanisms occurring in the development of disease. It is the product of the confrontation between a pathogen and its host. The pathogen strives to enter the host to replicate and then be released for dissemination whilst the host fights to reduce the damage and eliminate the pathogen. The host response and hence the outcome of infection is determined by a complex interplay of factors including the genetic background and immune status of the host. Animals have evolved highly efficient recognition systems and effective inflammatory and immune responses to restrain, contain and eliminate microbial pathogens. However, microbial pathogens continuously adapt and evolve sophisticated ways of circumventing the host immune defences and so the host-pathogen battle continues. Therefore, to study and understand pathogenesis we need to consider both the pathogen and the host.

Studying the pathogenesis of paratuberculosis is an enduring challenge. The causative organism, Mycobacterium avium subspecies paratuberculosis (MAP), replicates extremely slowly and many isolates are refractory to culture in vitro. The disease has a prolonged incubation period of two to five years during which time it is difficult to detect the pathogen and study host-pathogen interactions. Although MAP is a successful pathogen in nature it is difficult to reproduce the characteristic features of the disease by experimental infection. These factors have severely hampered studies on the pathogenesis of paratuberculosis. Consequently, we have little knowledge of the underlying mechanisms of pathogenesis from the perspective of both the pathogen and the host. A clearer understanding of pathogenesis is of paramount importance if we are to be successful in intervening in the process to either prevent or treat infected animals.

This paper will review the progress that has been made during the past five years in understanding the pathogenesis of paratuberculosis from the perspective of how we study pathogenesis, with particular emphasis on the use of animal models. During this time, novel approaches employing new technologies have been applied. In some cases, this has been possible as a result of the availability of the complete MAP genome sequence and advances with respect to the genetic manipulation of MAP. These technologies will be reviewed by John Bannantine (Bannantine, 2005) and will not be discussed in detail here. Pathogenesis is a huge topic and it is not possible to cover everything in one short paper. Therefore, I have selected those studies which I think have yielded important and interesting information or have used novel approaches. The immunological aspects of pathogenesis will be covered by Ad Koets in his paper entitled ‘Acquired immunity to bovine paratuberculosis: the host’s perspective’ (Koets, 2005) and similarly will not be covered in detail in this paper.

PATHOGENESIS

Identification of MAP virulence factors

An obvious approach to studying pathogenesis is to identify virulence factors produced by the pathogen that have a role in pathogenesis. A virulence factor is broadly defined as “a component that results in a pathogen’s being able to survive or exploit the special environment represented by the host, or which
results in damage to the host”. As such, virulence factors encompass diverse molecules and are involved in all stages of infection and pathogenesis including adherence to, ingestion by and survival in macrophages, modulation of the host immune response, persistence and dormancy. There have been a number of studies undertaken to identify MAP virulence factors and the molecular mechanisms underlying pathogenesis. Studies have tended to focus on the identification of antigenic proteins as these are likely to be key factors in pathogenesis, could be MAP-specific and potentially could be exploited for diagnostics or vaccine development. Immunogenic determinants were initially identified by screening MAP expression libraries with antiserum from experimentally infected animals (Stevenson et al., 1991; De Kesel et al., 1993; Inglis et al., 1994; El-Zaatari et al., 1994; Bannantine and Stabel, 2001;) or from naturally infected clinical cases of paratuberculosis (Cameron et al., 1994). The latter's advantage is that it is possible to detect immunogenic antigens expressed in vivo that may be absent from or poorly expressed in bacteria grown in vitro. This approach is now referred to as in vivo induced antigen technology or IVIAT. Some of the proteins identified using this approach have been further characterised and utilised in serological tests (Vannuffel et al., 1994; El-Zaatari et al., 1997; Shafran et al., 2002) but their precise role in pathogenesis has not been elucidated. The 70KDa heat shock protein has been extensively studied with respect to its immunogenicity and more recently, as presented in this Colloquium, with respect to protection and potential as a subunit vaccine against bovine paratuberculosis (Koets et al., 2005). The 34kDa putative serine protease identified by Cameron et al. (1994) has since been demonstrated to enhance the survival of MAP in macrophages (Heaslip, 2002). The 35KDa major membrane protein (Banasure et al., 2001; Bannantine and Stabel, 2001) has been shown to have a role in the invasion of bovine epithelial cells (Bannantine et al., 2003). More recently, Stratmann et al. (2004) identified MAP-specific ABC transporter proteins expressed on the mycobacterial cell surface in vitro and in vivo and are recognised by the immune response of infected animals. The ABC transporter operon was found to be located within a 38Kb pathogenicity island specific for MAP that also encompassed several iron uptake-related gene clusters potentially involved in pathogenesis.

The availability of the complete genome sequence of MAP has permitted the construction of a MAP partial protein array providing an alternative approach for identifying novel immunogenic antigens (Bannantine et al., 2005). Sixty four MAP proteins are represented on the array including unknown hypothetical proteins and previously characterised antigens. The array has been screened with antiserum from immunised rabbits and mice and from non- and MAP–infected cattle. Ten proteins were recognised by infected cattle of which seven represent novel antigens that have not been described previously.

Identification of antigens that elicit a Th1 type response has been more difficult. Nagata et al. (2005) recently employed expression library technology to identify and isolate interferon-gamma (IFN-γ) –inducing antigens by screening a MAP expression library using the IFN-γ assay and peripheral blood mononuclear cells (PBMCs) from infected cattle. Three recombinant proteins were identified, two of which were members of the PPE protein family.

Progress also has been made regarding the identification of other MAP virulence factors. Hughes et al. (2005) have used a proteomics approach employing 2-D electrophoresis and Matrix Assisted Laser Desorption Ionisation time of flight (MALDI-tof) to identify potential virulence factors. They compared the proteomes of MAP grown in vitro and MAP isolated from the ilea of clinical cases of ovine paratuberculosis and identified a set of ten proteins whose expression is upregulated during natural infection. These proteins appear to be involved in metabolism and adaptation to physiological change expressed in the host environment and further studies will determine their precise role in the pathogenesis of paratuberculosis. No doubt with the availability of the complete MAP genome sequence, further virulence factors will be identified by searching for homologues of genes and proteins that have been found to be involved in the pathogenesis of other related organisms.

In order to demonstrate that a particular gene is important for pathogenesis it is necessary to fulfil molecular Koch’s postulates (Falkow, 2004). This requires the construction of mutant strains that lack the gene of interest. Such studies had been hindered by the inability to genetically manipulate MAP. Two groups have succeeded recently in producing mutant libraries by transposon mutagenesis (Cavaignac et al., 2000; Livneh et al., 2005). These libraries have been screened to identify additional genes that may be involved in pathogenesis and also could be used to isolate mutants of putative virulence genes identified in the studies outlined previously.
Studying host-pathogen interactions

Studying host-pathogen interactions in paratuberculosis is particularly difficult due to the protracted period over which the disease develops. Subclinically infected animals are difficult to diagnose so investigation of the early events in pathogenesis and the mechanisms of progression from subclinical to clinical disease requires experimental models. The use of in vitro tissue culture systems has permitted elucidation of some important host-pathogen interactions but they can never reproduce the dynamic and complex interactions that occur within the whole animal. The ultimate model system therefore is infection of the intact animal host.

In vitro systems

In vitro systems have proved extremely useful for studying specific events in the early stages of pathogenesis, particularly entry and intracellular survival of the pathogen. The primary entry point for MAP infection is believed to be the M cells, specialised cells within the follicle associated epithelium (FAE) lining the Peyer’s patches in the intestine. After entering the M cells, MAP is probably transported to the subepithelial lymphoid tissue where it is phagocytosed by subepithelial macrophages. M cells are very difficult to maintain in vitro and therefore most in vitro studies investigating attachment and uptake have been conducted using a variety of cell lines including Caco-2 cells that can be induced to develop an M cell like phenotype in vitro, T-24, and HT-29 intestinal epithelial cells. The mechanisms of attachment and uptake of MAP are poorly understood but progress has been made in this field in recent years. Two mycobacterial invasion proteins have been identified recently (Secott et al., 2001; Bannantine et al., 2003; Secott et al., 2004). Secott et al. (2002) showed that MAP expresses a fibronectin attachment protein (FAP) that binds soluble fibronectin, which facilitates attachment and uptake in T-24 and Caco-2 cells. Their results suggested also that α5β1 integrin may serve as the host cell receptor for fibronectin bound FAP. As the distribution of receptors on cells in culture can often be different to that in intact tissue, it was necessary to conduct further investigations in an animal model. The researchers injected wildtype and antisense FAP mutant MAP strains independently or together with blocking peptides or antibodies into murine gut loops and monitored invasion of M cells by immunofluorescence microscopy (Secott et al., 2004). These studies showed that MAP targeting and invasion of M cells is via formation of a fibronectin bridge between FAP and integrins on M cells. Bannantine et al. (2003) conducted experiments in cultured Madin-Darby bovine kidney cells and showed that the MAP 35KDa major membrane protein is a surface exposed protein that may play a role in the invasion of bovine epithelial cells. Schleg et al. (2005) developed an in vitro model for investigating attachment of MAP to bovine intestinal organ cultures. They found significant differences in the ability of different MAP strains to attach but could demonstrate no differences in attachment among different regions of the intestinal tract. Coating of the organisms with fibronectin affected attachment but appeared to be strain related. Therefore it is likely that there will be other factors governing the preferential targeting of M cells and that these will be revealed in future studies. Likewise, as will be described later, M cells may not be the only cells through which uptake of MAP occurs.

There have been a considerable number of in vitro studies investigating the entry and survival of MAP within macrophages. These studies make use of bovine monocyte-derived macrophages or murine bone marrow-derived macrophages and cell lines including J774 derived from a mouse tumour that has characteristics typical of macrophages. The molecular mechanisms by which MAP enters macrophages are unknown. Cheville et al. (2001) investigated the binding of MAP to murine macrophages by incubating with monoclonal antibodies to complement receptor CR3 and adding the tripeptide sequence RGD which is the binding site on bacteria for integrins. The results suggested that CR3 appears to be one receptor for the attachment of MAP to macrophages facilitating phagocytosis.

More progress has been made in characterising host macrophage responses to MAP internalisation and how MAP resists killing. Studies using J774 murine macrophage cell cultures have shown that phagosomes containing live MAP fail to mature and acidify whereas killed MAP were identified in acidified phagosomes and phagolysosomes (Cheville et al., 2001; Hostetter et al., 2003; Kuehnel et al., 2001). Phagosomal maturation can be evaluated using stage specific markers detected by immunofluorescent labelling and confocal microscopy. Phagosomes containing live MAP exhibited increased levels of the early endosomal marker transferrin receptor and decreased levels of the late endosomal marker lysosome associated membrane protein 1 relative to those containing killed MAP (Hostetter et al., 2003). Kuehnel et al. (2001) reported that phagosomes containing live MAP were reduced in their ability to acquire internalised calcein,
BSA-gold or lysosome associated membrane protein 2. The effects of cytokine activation on the ability of monocytes and macrophages to phagocytose and kill MAP have also been studied. Cytokine activation of J774 cells prior to infection appears to restore the ability of the cells containing MAP to proceed to the late phagosomal stages (Hostetter et al., 2002).

Coussens and coworkers (2002, 2003, 2004b) have pioneered an alternative approach and applied microarray and quantitative real-time PCR (Q-RT-PCR) technology to investigating the host-pathogen interactions in naturally MAP infected cattle (Coussens et al., 2002; Coussens et al., 2003; Coussens et al., 2004b). Microarray technology can provide information on the molecular basis for MAP pathogenesis and has the advantage of providing a global picture of gene expression. EST and cDNA microarrays were developed for studying bovine immunobiology (Yao et al., 2001). Coussens et al. (2002) used a bovine specific total leukocyte (BOTL) cDNA microarray to evaluate the response PBMCs from subclinically and clinically MAP infected cattle. This study demonstrated predominant repression of immune cell gene expression in PMBCs from clinical MAP infected cattle and that this was not due to a general loss of immune cell function. A total of 83 genes showed decreased expression including those encoding microsphereul protein 1, fibroblast growth factor and Lyn B protein kinase. Only eight genes exhibited up regulation including those encoding bovine CD40L, IFN-γ, interleukin 10 and tissue inhibitor of matrix metalloproteinases. In contrast, MAP stimulation of PBMCs from subclinical cattle revealed a predominate trend to activate immune cell gene expression. A total of 71 genes showed increased expression including those encoding bovine CD40L, several matrix metalloproteinases and SPARC (secreted protein, acidic and rich in cystine) whereas only 16 genes were down regulated including those encoding the bovine orthologues of cytochrome oxidase subunit III, interleukin 1 (IL-1) receptor type I and fibrinogen-like 2 protein. Further experiments using an expanded BOTL cDNA microarray demonstrated that there were major differences in the gene expression patterns between PBMCs isolated from infected and uninfected cattle regardless of in vitro stimulation with MAP (Coussens et al., 2003). The observed differential gene expression could not be explained by gross differences in the relative immune cell populations in the two groups of animals. Aho et al. (2003) extended the microarray studies to compare the gene expression profiles of ileal tissues from MAP infected and uninfected control cattle (Aho et al., 2003). These studies demonstrated upregulation of IL-1α and tumour necrosis factor receptor-associated protein 1 (TRAF1) mRNA and protein expression in ileal tissues of MAP infected cattle. IL-1 is a proinflammatory cytokine associated with granuloma formation and maintenance and TRAF1 is a proapoptotic intracellular signalling molecule. Both proteins may have an important role in MAP pathogenesis and have been the subject of further investigation, the results of which have been presented at this Colloquium (Coussens et al., 2005). High levels of TRAF1 are located primarily within infiltrating macrophages and IL-1α appears to be secreted from cells within lesions in MAP infected animals. In addition, Q-RT-PCR has been used to study the expression of selected proinflammatory cytokines in PBMCs from MAP infected cows (Coussens et al., 2004a). The microarray approach has been used also by Weiss and coworkers to characterise the response of bovine monocyte-derived macrophages to MAP infection and to investigate sequential gene expression patterns by bovine monocyte-derived macrophages associated with ingestion of MAP (Weiss et al., 2004a; Weiss et al., 2004b). The results of these studies revealed discrepancies with those of Coussens et al. (2002) possibly due to the different microarray platforms used and the use of a mixture of lymphocytes and monocytes rather than monocyte-derived macrophages. This highlights the importance of defining the experimental conditions when using microarray technology and verifying the results with additional assays measuring function or protein quantification.

Animal models
Small animal models for paratuberculosis offer a number of advantages. Small animals are easy to house, maintain and handle and generally the pathogenic features can be measured after a much shorter post infection incubation period than in ruminants. The disadvantage is that they do not always mirror the features of ruminant paratuberculosis. The murine model is particularly useful for studying host-pathogen interactions involving the immune response. Mouse strains have been produced with mutations that render them deficient in specific components of the immune response and there is a host of immunological reagents and assays available for studies. A number of small animals have been investigated as models for paratuberculosis including chickens (Larsen and Moon, 1972; Valente et al., 1997), guinea pigs (Francis, 1943), hamsters (Hirch, 1956; Beran et al., 2005) and rabbits (Mokresh and Butler, 1990). There has been more interest in developing the rabbit model in recent years following reports of paratuberculosis in wild rabbits (Greig et al., 1997; Greig et al., 1999; Raizman et al., 2005) and the ability of rabbit isolates of MAP.
to produce paratuberculosis in experimentally infected calves (Beard et al., 2001). A recent study by Vaughan et al. (2005) orally challenged adult and juvenile rabbits with a wildtype bovine MAP strain and followed disease progression by bacterial and histopathological examination over 36 months. Unfortunately, as with previous studies, it was not possible to consistently reproduce the disease as bacteria were cultured from the tissues of only two of four adult and three of 16 juvenile rabbits and microscopic lesions were detected in four of the diseased rabbits. All studies so far have employed New Zealand White rabbits and the outcome of experimental infection may be influenced by the genetic background of the host or host preference of the MAP strains selected.

In contrast, experiments using mice have made a valuable contribution to our understanding of the pathogenesis of paratuberculosis. These have been reviewed elsewhere (Harris and Barletta, 2001) and only recent experiments will be highlighted here. The mouse model is very versatile and can be used to study different aspects of MAP pathogenesis by selecting the appropriate strain of mouse, age at time of, dose and route of inoculation. Knockout mice have been exploited during the past five years to investigate the interaction between MAP and the host immune system. Tanaka et al. (2000) used BALB/c T-cell receptor (TCR) γδ knockout mice to study the role of γδ T cells in MAP infection. The results of the study suggested that γδ T cells may be important for the development of epithelioid granulomata during MAP infection but are probably not involved with the elimination of the mycobacteria from the host. Stabel and Ackermann (2002) also utilised TCR knockout mice to investigate the role of αβ and γδ T cells in resistance to MAP infection. They demonstrated that irrespective of bacterial strain or infection period, TCR-α- knockout mice had higher levels of colonisation of MAP in their tissues compared to TCR-γ-knockout mice or control mice suggesting that αβ T cells are critical for bacterial containment. Another interesting study was conducted by Mutwiri et al. (2001) who investigated the intestinal pathophysiological changes induced by MAP infection in beige/scid mice. This research group used Ussing chambers to show that MAP infection produced significant abnormalities in intestinal transport parameters. The results suggested that intestinal tissue from infected mice was less permeable to ions but that there was increased ion secretion. There was also evidence that the inflamed intestinal tissue had neural and/or epithelial damage. The model shows that mucosal pathophysiological changes consistent with chronic inflammation following MAP infection can occur via T cell independent mechanisms.

Although mouse models offer unique opportunities to study interactions of MAP with the host immune system, mice are not a natural host for MAP infections and they cannot accurately mimic all aspects of MAP pathogenesis in ruminants. Ultimately, pathogenesis has to be studied in the natural host. The calf model has been used extensively for studying the interaction of MAP with the bovine immune system and has been reviewed recently (Coussens, 2004). As with other animal models, the parameters used for MAP infection can influence the pathogenic features observed and a cautionary approach should be taken when interpreting results. Waters et al. (2003) investigated the usefulness of the intrarotonillar inoculation route as an experimental model for MAP infection. These experiments challenged the dogma of a Th2 response occurring late in pathogenesis as mycobacteria-specific antibody was detected as early as 134 days post challenge and concurrently with a cell mediated response. Koo et al. (2004) demonstrated that high-dose exposure via the oral route lead to a uniform and reproducible infection consistent with that described in animals directly inoculated with MAP. A proliferative immune response to MAP was not readily detected until five months post inoculation suggesting that MAP interferes with pathways of activation associated with antigen-presenting cells. This study also highlighted the advantages of using multicolour flow cytometry for monitoring immune responses during disease progression.

The calf model has not been widely used to study uptake of MAP or the histopathological features of MAP pathogenesis. The most notable progress in the past five years in this field has come from studies in goats. Subclinical paratuberculosis in goats following experimental infection has been characterised using immunological assays and microbiological culture (Storset et al., 2001). Sigurdardottir and co-workers have described some very elegant experiments to study the uptake of MAP through the distal small intestinal mucosa (Sigurdardottir et al., 2001; Sigurdardottir et al., 2005). MAP was injected into isolated loops of ileum and after one hour the segments were excised and processed for light and electron microscopic analyses (Sigurdardottir et al., 2001). MAP was observed in the FAE both in the cytoplasm of M cells and in the cytoplasm of intraepithelial leukocytes in M-cell pockets and occasionally between M cells in the ileal dome. MAP was not found in association with the absorptive epithelium. Uptake of MAP was found to be rapid, cells being detectable after just 30 minutes. More recently, the research group has used the everted sleeve method initially developed for the in vitro measurement of intestinal absorption to look at the uptake...
of MAP in more detail (Sigurdardottir et al., 2005). This method is particularly useful in that it provides a quantitative as well as a qualitative method of studying bacterial uptake although it does have limitations relating to how long mucosal integrity can be preserved. Small everted sleeves derived from areas of the intestine with and without Peyer’s patches were incubated for one hour in a suspension of radiolabelled MAP. The study showed that MAP could enter the intestine through areas with and without Peyer’s patches and therefore that uptake is not restricted to M cells and can occur via enterocytes. Valheim et al. (2002) undertook studies to investigate the association between the morphology of the Peyer’s patches and the lesions of paratuberculosis in goats. The observed morphological similarities between the ilealcaecal-valve Peyer’s patch and the jejunal Peyer’s patches but a different morphology for the ileal Peyer’s patch which undergoes involution during the first 12-18 months of life. The persistent organised lymphoid tissue of the ilealcaecal valve and jejunal Peyer’s patches but not the involuted ileal Peyer’s patch could sustain the development of granulomatous lesions during subclinical paratuberculosis. The characterisation of macrophages and occurrence of T cells in the intestinal lesions of subclinically infected goats has also been reported by Valheim et al. (Valheim et al., 2004).

Sheep models also have been used to study ovine paratuberculosis, particularly the early immunopathological events in infection (Begara-McGorum et al., 1998) but it is difficult to reproduce early histological lesions between experiments. This could be due to a number of reasons including differences in the breed of sheep or the viability, dose, or MAP strain used. It has been reported that infections are more easily established using MAP prepared directly from mucosal tissue rather than from in vitro cultures (Stewart et al., 2004). Whether this is a dosage effect (it is very difficult to count MAP in intestinal tissue) or due to upregulation of virulence factors in in vivo grown cells is yet to be established. Previous experimental infections have employed cattle-type strains that are easier to propagate in the laboratory but it has recently been demonstrated that sheep can be experimentally infected with ovine-type strains (Reddacliff and Whittington, 2003; Stewart et al., 2004). Hein et al. (2004) have adopted a novel approach for studying regional immune responses in naturally MAP infected sheep with clinical signs of paratuberculosis (Hein et al., 2004). They cannulated the afferent and efferent lymphatic vessels draining the jejunum enabling continuous sampling of lymph up to a period of 107 days. This enabled phenotypic analysis and cytokine gene expression profiling of the intestinal lymph cells. There is no doubt that this approach will prove extremely useful for gaining real-time insights into host-pathogen interactions as they occur in vivo within the gut associated lymphoid tissue.

CONCLUSION

Our knowledge and understanding of the pathogenesis of paratuberculosis has improved over the past five years but there are still many unanswered questions and much more to learn. The availability of the MAP genome sequence and novel molecular technologies including those for the genetic manipulation of MAP will accelerate further studies but will need to be combined with appropriate in vitro and in vivo models to evaluate pathogenesis. Although current animal models offer a range of approaches for studying different aspects of the disease, further refinement and development of novel models is a future area for research. An approach that has not yet been adopted for paratuberculosis is the application of non-invasive techniques for studying pathogenic bacteria in the whole animal (Camilli, 1996). Bioluminescence monitoring has been used successfully for monitoring Salmonella and Citrobacter rodentium infection in mice (Contag et al., 1995; Wiles et al., 2004; Burns-Guydish et al., 2005) and for detecting Yersinia pseudotuberculosis in dissected Peyer’s patches and spleen (Forsberg and Rosqvist, 1993). The technique has limitations imposed by its requirement for oxygen but could nevertheless be extremely useful. Once reliable and reproducible systems for genetically manipulating MAP are in place, it should be possible to construct reporter strains of MAP for bioluminescence studies. There is a promising and exciting future for paratuberculosis research.
REFERENCES


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