Immunological and Molecular Markers which may be used to Distinguish Protective Immunity from Disease in *M.bovis* Infected Deer

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Immunological Markers of protective immunity and disease.

During the past decade there have been significant advances in our understanding of the fundamental immunological mechanisms which result from exposure to infection. Whereas the protective immune response to acute extracellular bacterial infection is usually mediated by antibody production, the response to intracellular infection by virus, bacteria and parasites, is considerably more complex. A solid body of evidence suggests that in many intracellular infections, the production of antibody occurs concomitantly with the emergence of disease and it has little role in protective immunity. In this respect antibody, when present following infection with intracellular pathogens, can be used to develop immunodiagnostic tests for disease. Data from our laboratory (Griffin and Buchan 1989, Griffin and Buchan 1993a) show that antibody response as measured by the ELISA, is an excellent marker for serious disease caused by *M.bovis* in deer. When this test is used serially with ST the combined assays have a sensitivity of 95.0% for Tb diagnosis. The ELISA also has a high positive predictive value (>75%) in identifying *M.bovis*(+) deer. While the ELISA is good at diagnosing serious disease it cannot detect early infection where the response is predominantly cell-mediated.

Laboratory assays of cellular immunity using the lymphocyte transformation (LT) assay, can be used in parallel with the ELISA to diagnose deer infected with *M.bovis*, (Buchan and Griffin, 1989, Griffin and Buchan1993a). Patterns of immune reactivity compatible with disease [LT(+)/ELISA(+)] are different from the 'putative' protective response [LT(+)/ELISA(-)], seen in deer which are disease-free following exposure to *M.bovis* . The LT is limited in its ability to provide more precise information whereby LT(+) reactivity typical of disease can be distinguished from protective immunity. Data from our laboratory using BCG vaccination of deer (Griffin, *et.al.* 1992a, Griffin and Buchan, 1993b) show that the LT response in vaccinated deer is much less specific for *M.bovis* than the reactions found typically in naturally infected deer. In an attempt to chart the pathways of reactivity which lead to protective immunity or disease it is necessary to look at each facet of the host response found in chronic intracellular infection to elucidate the fundamental mechanisms which influence the outcome.

I. Innate Resistance

Non-specific scavenger cell reactivity mediated by mononuclear phagocytes (macrophages) play a vital role in clearance of intracelllar pathogenic bacteria in the period immediate following infection (Crowle and Elkins, 1990). In tuberculosis it has been demonstrated that a single BCG gene (Forget, *et.al.* 1998), which is not linked to the major histocompability complex (MHC), has a vital role in non-specific innate resistance to *M.bovis*. This gene, first discovered in inbred mice, has been cloned and sequenced (Vidal, *et.al.* 1993) and is known to code for a protein which acts as a transport molecular for nitrous oxide radicals, to produce bactericidal activity within the phagosomes of the monocytic cell. A homologue of the BCG gene in mice has also been identified in humans (Schurr, *et.al.* 1990). This system of innate resistance is non-specific and appears to produce ubiquitous patterns of resistance to intracellular pathogens ranging from tuberculosis (Forget, *et.al.* 1981) to salmonellosis (Plant and Glynn 1976) and leishimaniasis (Bradley, 1977). The expression of the BCG gene may have a critical impact on the downstream immunological effects which result following exposure of an individual animal to pathogens such as *M.bovis*.

II. Pseudo-immune reactivity

In recent years a population of lymphoid cells has been identified which produce specific reactivity following exposure to infection or immunisation (Raulet, 1989). While these cells have surface glycoproteins which characterise them as T-lymphocytes they differ in function from classical immune cells in two ways:

1. They have unique specific antigen receptors, encoded for by $\gamma\delta$ genes, which are different from the typical T cell receptor for antigen (TCR) cells which is composed of $\alpha\beta$ chains (Brenner, *et.al.* 1988).

2. $\gamma\delta$ cells, though specifically activated following contact with antigens, do not show a classical enhanced memory response on re-exposure to homologous antigens.

Increased numbers of $\gamma\delta$ cells are found in the skin and mucosal epithelial tissues of the body. Increased numbers of these cells are found in mice following infection with *M.bovis* (BCG) (Inoue, *et.al.* 1991) and in the lungs of animals exposed to aerosol containing extracts of *M.tuberculosis* (Augustin, *et.al.* 1989). While $\gamma\delta$-cells may play a central role in protection (Boom, *et.al.* 1992) they may also contribute to the pathology associated with disease (Modlin, *et.al.* 1989). The $\gamma\delta$ cells produce a variety of cellular hormones (cytokines) including IL-2, IL-4 and γ-IFN. They may act as cytotoxic cells and could have an important role as a first line defence against invasion with various

pathogens (Janeway, *et.al.* 1988). Interestingly, whereas the number of γδ cells in peripheral blood of mice is low (1-3%), their relative incidence in ruminants may be up to ten times higher (Hein and MacKay, 1991, Buchan, *et.al.* 1990). This could markedly influence the patterns of responses in domesticated ruminants following infection with *M.bovis* and means that γδ cells should be examined closely when studying the tuberculosis response in *M.bovis* infected deer or cattle.

III. Classical Cellular Immune Reactivity

Lymphocytes generated within the embryonic and neonatal thymus develop into a population of mature T-cells which leave the thymus and colonise the peripheral lymphoid tissues. T-cells have diverse functions, which include a fundamental role in regulating all lymphocyte activity (antibody and cellular) and special effector roles in cell-mediated immunity. The key functions seen in cell-mediated responses include the amplification of mononuclear cell killing of parasites and cytotoxic activity against intracellularly parasitised cells. In addition adverse hypersensitivity responses may occur in association with activation of T-cells resulting in delayed type hypersensitive (DTH) reactions. DTH alone is not sufficient to protect the host against parasitic infection and if uncontrolled it may exacerbate the severity of disease. Recent characterisation of the cytokines produced by T cells suggest that they can be subdivided into two populations *(type-1 and type-2)* with disparate functions (Mosmann, *et.al.* 1986). The distinction between *type-1* and *type-2* cells first described by Mosmann's study has remainded central in distinguishing pathways of immune reactivity which characterise protective immunity and disease in a diverse range of intracellular infections, in many species of animals. The nett effect of activation of *type-1* or *type-2* cells is seen at a molecular level by the dominance of production of cellular hormones by either cell type to produce a response compatible with immune protection or disease, following infection with intracellular pathogens. *Type-1* cells produce IL-2 and γ-IFN while *type-2* cells produce IL-4 and IL-10. γ-IFN has a suppressive effect on *type-2* cells while IL-10 suppresses *type-1* cells, so the response of either cell type is mutually exclusive (Salgame, *et.al.* 1991). The recruitment and sustenance of a *type-1* or *type-2* response may be influenced markedly by the mononuclear phagocytic cells which act to process and present antigens to specific antigen receptors on lymphocytes. Theoretical models for activation of either type of response are given in Figure 1 and 2. Figure 1 provides a pathway of mononuclear cell and lymphocyte activation likely to stimulate *type-1* cell activation.

The pathways outlined in Figures 1 and 2 suggest model systems whereby protective immunity or disease may result from infection of macrophages by *M.bovis*. Figure 1 shows the selective activation of TH^1 cells facilitated by production of IL-1 and IL-12 by infected macrophages. Activated T_H1 cells

produce IL-2 and γ-IFN. IL-2 causes activation of cytotoxic cells (Tc) which kill infected macrophages and prevent the growth and spread of infectious organisms. Production of γ-IFN by T_H1 and Tc cells causes increased function of the macrophages. γ-IFN also causes suppression of T_H2 cell activity and prevents B-cell activation and antibody production.

Figure 1

THE PROTECTIVE IMMUNE RESPONSE

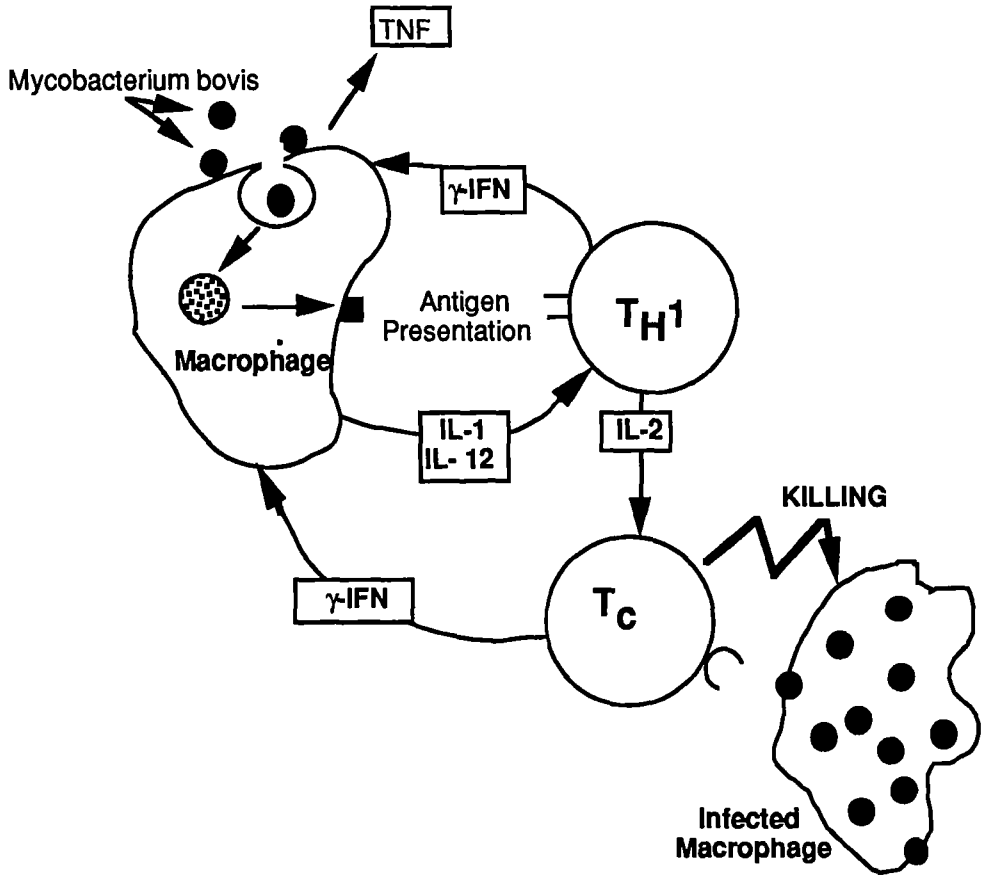
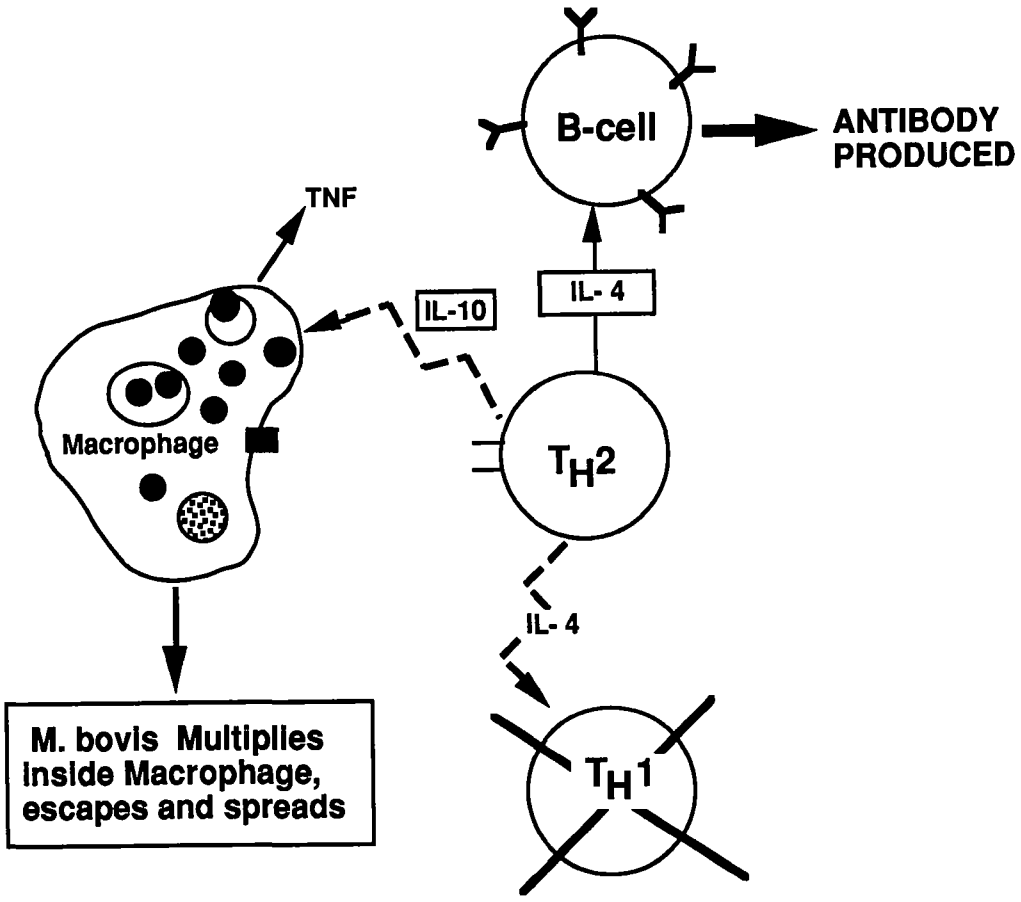


Figure 2 shows the outcome following uncontrolled replication of mycobacteria within the infected macrophage. This could be due to the lack of BCG^r gene, or the expression of altered phenotype (stress) affecting macrophage competence, resulting in impaired intracellular killing and antigen presentation by the macrophage. Release of tumour necrosis factor (TNF) by the infected macrophage may cause damage to cells within the local microenvironment and facilitate disease spread. Antigens presented by the diseased macrophage recruit T_H2 cells which produce IL-4 and IL-10 molecules. IL-10 downregulates

macrophage function so these cells do not produce IL-1 or IL-12. IL-4 produced by activated T_H2 cells serves to upregulate B-cell activity, resulting in antibody production, which is non-protective. Simultaneously, IL-4 acts as a suppressor molecule to exclude T_H1 cell involvement in the response. The nett effect of this cycle is that macrophages fail to control bacterial replication and no protective immune response results even though antibody production occurs.

Figure 2

The Disease Associated Response

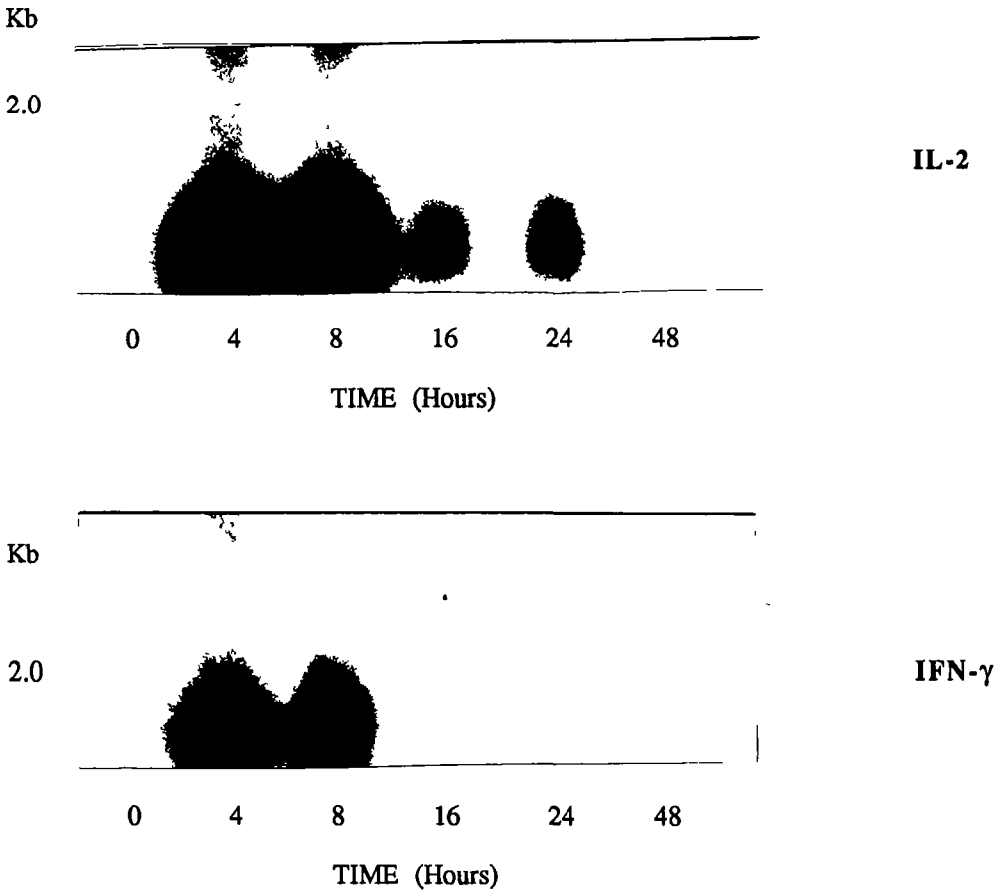


Molecular Marker of Immune Reactivity

Monitoring the production of cytokines by immunologically activated cells is the most definitive method for characterising the relative contribution of *type-1* or *type-2* cells following infection with virulent *M.bovis* or vaccination with BCG in deer. This can be done either by measuring the biological activity of the cytokines or detection of messenger RNA (mRNA) specific for the cytokine. The unique species specific patterns of biological reactivity of cytokines mean that it is simpler to use a molecular genetics approach to look for expression of cytokine mRNA, in the activated lymphocytes from individual animals. This technique involves the activation of blood lymphocyte in the laboratory using specific antigens and mitogens to produce increased quantities of mRNA which is isolated from the cultured cells. The mRNA is separated on agarose denaturing gel by electrophoresis and detected (Northern blot) using labelled cDNA probes which are identified by autoradiography (Griffin, *et.al.* 1992b).

Figure 3 shows an IL-2 and IFN-γ autoradiograph produced from lymphocytes in deer cells stimulated by mitogens (ConA).

Figure 3. Northern Blot analysis for IL-2 and γ-IFN in deer cells cultured from 0-48 hours with mitogen.



The striking observation is that the levels of γ-IFN mRNA in diseased animals is equivalent to the amount produced by cells from deer free of disease. This suggests that whereas γ-IFN is a good marker for protection against tuberculosis in mice it may not hold for ruminants where γ-IFN is found in both diseased and non-diseased deer. Independent studies in our laboratory using γ-IFN bioassays (virus neutralisation) also confirm that γ-IFN is produced at equivalent levels in diseased and disease-free deer lymphocytes (unpublished data). As IL-2 is not considered to be an ideal cytokine to define *type-1* cell activity we are left to ponder what approach will best elucidate the function of *type-1* cells in deer. It may be that we will have to look for IL-12 production by macrophages to target *type-1* cell activation (Figure 1). IL-4 and IL-10 are currently being studied as markers for *type-2* cell activity in *M.bovis* infected deer.

This data is presented to highlight the unique challenge in designing molecular techniques to look at the function of critical subpopulations of cells in the Tb response of deer. We have already highlighted (Buchan, *et.al.* 1992) the unique role of γδ cells in deer so it may be necessary to invoke new models for Tb reactivity in deer which differ qualitatively from that seen previously in mice and man. While the challenge is obvious our ability to design methods appropriate for the target species is vital if we are to define the critical pathways of immune reactivity which distinguish protective immunity from disease related hypersensitivity in *M.bovis* infected deer.

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