New Discoveries in Immunology, with Direct Application in Veterinary Medicine and Deer Farming

Frank Griffin, Lynn Slobbe, Jan Hesketh, You-en Shi and Glenn Buchan

Deer Research Laboratory, Department of Microbiology, University of Otago, Dunedin, New Zealand

Introduction

The past decade has seen a phenomenal increase in our understanding of the basic mechanisms which define different pathways of immunity. While most of these studies have been based on genetically homozygous inbred mouse strains, a large body of confirmatory data has been produced in studies of human immunology using outbred populations. More recently, these findings are being extended to include domestic animals, where the basic observations from mice are directly applicable to other species.

The increased rate of progress is based on new methodology which allows for definitive studies of cell subpopulations (T-cell, B-cell, Monocyte-Macrophage) through our new found understanding of surface markers (phenotypes) which allow for separate functions to be attributed to different cell types.

The new level of precision in defining cell markers has relied on techniques using highly purified monoclonal antibodies (mAbs) which react specifically with single antigenic markers (epitopes) on proteins or biologically active molecules. These reagents are used with new analytical equipment such as Fluorescent Activation Cell Sorters (FACS) to identify cells labelled with Fluorescent - mAbs. Large numbers of cells, exposed to a laser beam, can be examined or isolated from a sample obtained from blood or lymphatic tissue. Increasingly, the use of mAbs and FACS technology has prescribed new subpopulations of cells for functional analysis.

Monoclonal Antibody (mAb)

The production of mAb (Kohler & Milstein, 1975) relies on the immunisation of a mouse and the isolation of specific (B-cell) splenic antibody-producing cells. Following immunisation, single antibody

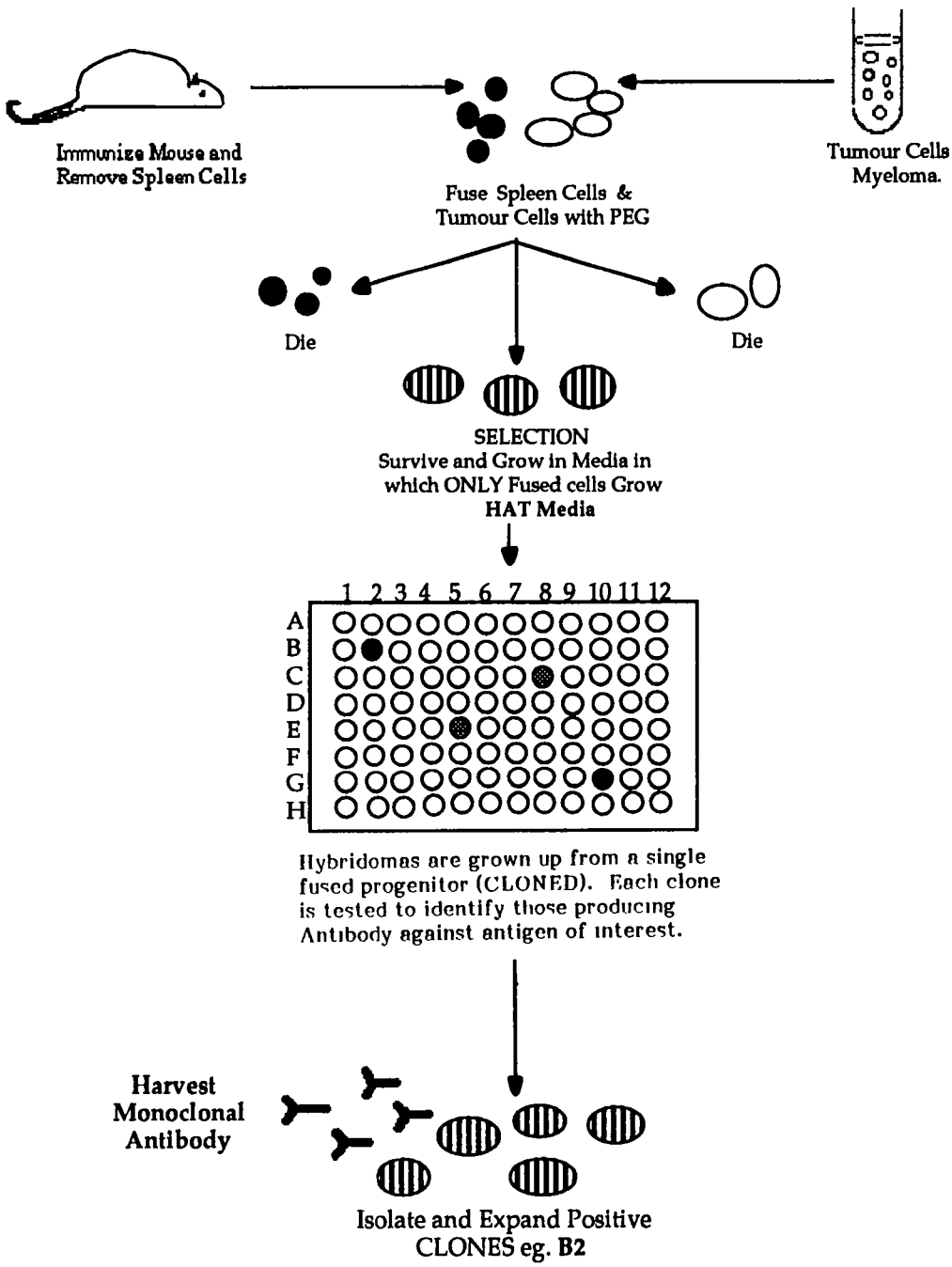
producing cells from the mouse spleen are isolated and fused with a malignant myeloma cell from similar inbred mouse strains (Figure 1). Hybrid cells, are produced by cell membrane fusion using chemicals (polyethyleneglycol-PEG) or virus (Simian virus). Clonal expansion of fused cells occurs in a restricted chemical medium (HAT), which allows growth only of hybrid cells. This is because the original spleen cells cannot grow in vitro in synthetic media while the immortalised malignant cell are selected againstbecause they contain a mutation which does not allow them to use HAT to synthesise DNA de novo. Only hybrid cells, which contain the intact enzymes to process HAT can grow. Hybrid antibody-producing clones excrete pure antibody of unique specificity. Culture of these clones produces large amounts (mg) of mono-specific **monoclonal** antibody. This technique allows for the production of large amounts of highly specific mouse monoclonal antibody against important markers such as deer surface markers (cluster determinant - CD), immunoglobulins; (IgM, IgG, IgG_2) or cytokines (IL-2, IL-4, IL-10 or IFNγ). Interleukins (ILs) are part of a family of specialised hormones produced by lymphocytes following antigenic activation. They have a diverse range of biological activity directed against different leukocytes and serve to amplify or suppress cell function.

Our laboratory has developed specific mAbs to study deer immunoglobulins (IgM/IgG_1/IgG_2). We also use mAbs specific for M.bovis antigens and are currently developing mAbs to deer IL-2, IL-4, IL-10 and IFN-γ.

Attributing specialised function to lymphocyte subpopulations has relied on a new level of understanding of the regulatory molecules (hormones) which orchestrate the complex interactions necessary to activate and control immune reactivity. The complex array of immune hormones (cytokines) from lymphocytes (lymphokines) and monocytes (monokines) have been characterised to levels which allows for integrated studies to be carried out on the immune physiology of cells and their products. While mAbs produced against cytokines have allowed initial studies to be carried out it has not been possible to critically study the structure and function of individual molecules until techniques became available for the isolation, expansion (cloning) and expression of the individual genes which produce the individual molecules.

Figure 1

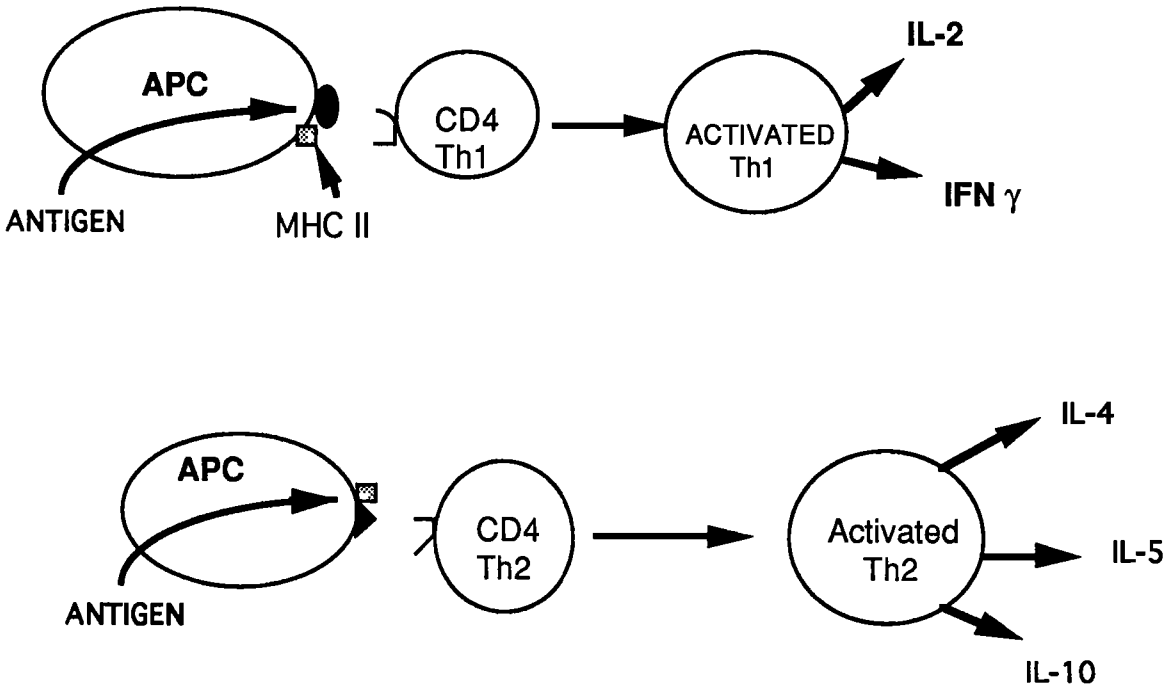
Monoclonal Antibody Production.



Surface Markers on Immunological Cells

A group of cell surface glycoproteins called cluster determinants (CD) are used to characterise immunological cells, especially T-cells, which are the most diverse populations of lymphocytes. CD4 markers characterise T-helper (Th) cells and cells involved in cell-mediated immune (CMI) reactions. CD8 markers are found on T-suppressor (Ts) and T-cytotoxic cells (Tc) involved in killer cell activity against intracellular parasites (such as Tb). $CD4^+$ and $CD8^+$ cells can be further subdivided functionally by the cytokines they produce in response to antigenic stimulation. $CD4^+$ cells can be divided into Th_1 and Th_2 cells (Figure 2) which have distinct regulator/effector functions in immunity (Mossman & Coffman, 1987). Th_1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ) which control CMI response to chronic intracellular infection. In contrast Th_2 cells produce IL-4, IL-5 and IL-10 which cause activation of B-cells and antibody production following exposure to acute infection (extracellular). The type of cell which is activated is influenced by the nature of antigen processing and presentation, which is mediated largely by monocytes. The function of Th_1 and Th_2 cells is mutually exclusive in that Th_2 cytokines down-regulate Th_1 function (Howard & O'Garra, 1992).

Figure 2



This technique has been used in our laboratory to isolate and culture $CD4^+$, $CD8^+$, B-cells and monocytes from deer in our Tb studies. We are currently looking at cytokines (INFγ vs IL-4) to characterise $CD4^+$ cell found in 'immune' and diseased animals exposed to M.bovis.

Polymerase Chain Reaction (PCR)

This technique (Erlich et.al., 1991) allows for the expansion of a single gene to produce at least one million copies, which can then be inserted into viral or bacterial genomes to allow the production and isolation of pure biologically active molecules encoded for by that single gene. Recently discovered techniques allow for the isolation of single genes using an indirect technique which relies on the transcription (DNA-mRNA) of genes in metabolising cells. For example it is possible to stimulate Th_2 cells in the laboratory to produce IL-4 mRNA. The mRNA, can then be transcribed in a reverse reaction (Reverse transcriptase) using viral enzymes to produce a single strand DNA copy of the gene (cDNA), which is identical to one strand of the DNA of the original gene under study (e.g. IL-4).

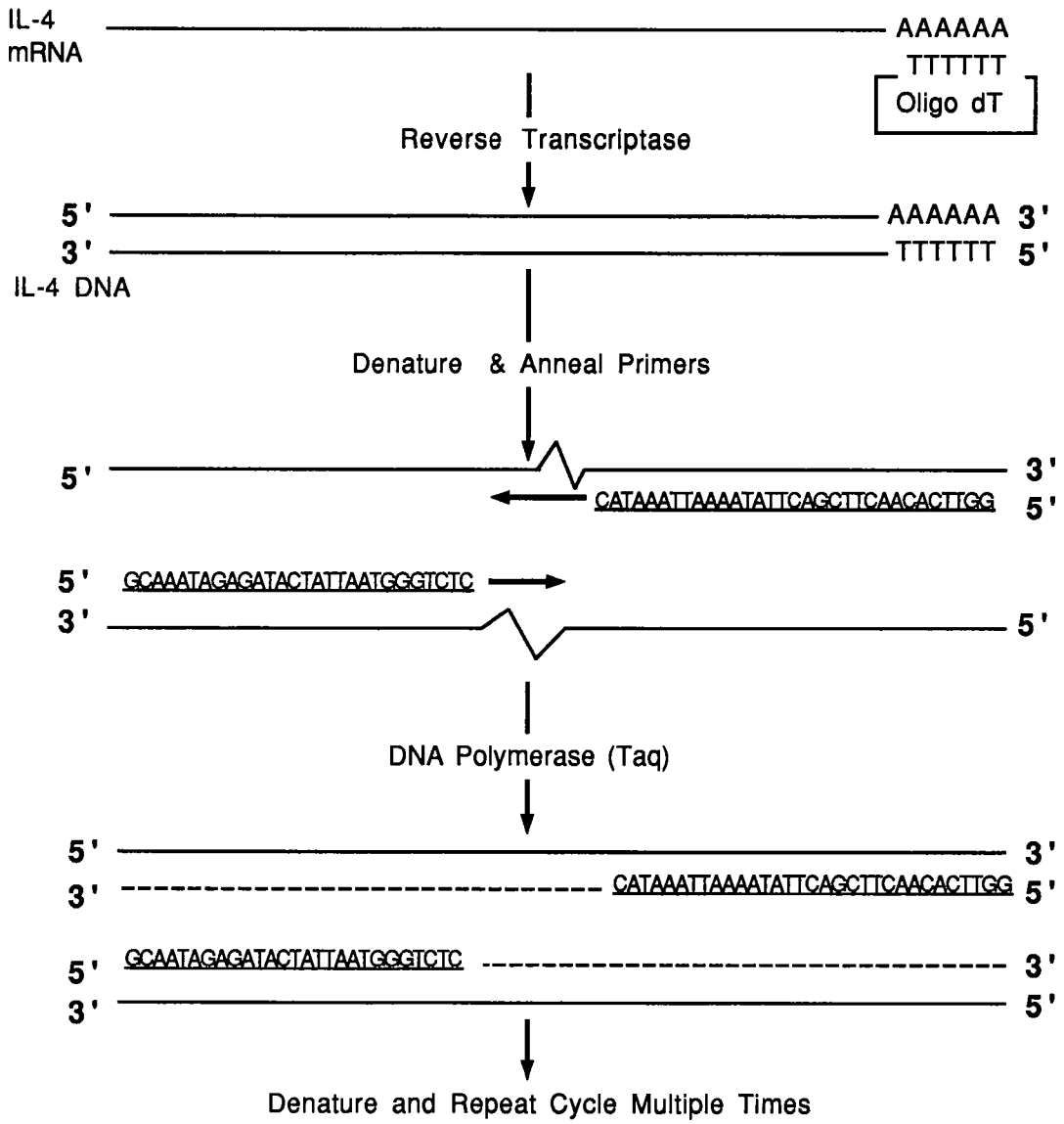
By using DNA primers for the specific gene, the original gene sequence can be duplicated by successive synthetic and denaturing (high temperature) cycles. A heat stable bacterial DNA polymerase (Taq) enzyme is used to faithfully reproduce the gene (Figure 3) from single strands of DNA. One million copies of the original gene can be produced synthetically within hours. The only limitation in this technique is access to the flanking sequence (**Primer**) for the gene under study.

Cloned genes produced by PCR can be inserted in viral recombinants which infect target cells, where the gene is expressed to produce large amounts of the original molecule encoded by the gene under study.

Nature provides a unique opportunity to exploit such technology because large parts of any given gene are conserved across species, especially between animals with close evolutionary development. This means that genes which have been mapped for well researched species; mice and man, can be used to design primer molecules of relevance for genes of domestic animals such as cattle and sheep. By extension this allows specific genes from more exotic animals such as

Figure 3

PCR AMPLIFICATION OF DEER IL-4 GENE



deer to be isolated and cloned. Known bovine primers can be used to clone deer genes.

In this way it is possible to use bovine IL-4 primers to reproduce deer IL-4 genes. This technology can be extended to allow for expression of any gene which is of interest in a new species of animal being studied.
Recently, work in our laboratory has resulted in the transcription and production of PCR copies of putative deer genes for IL-2, IL-4 and IFN-γ. Technical support by A. Crawford, MAF(Tech) Animal Genetics Laboratory, University of Otago, is acknowledged.

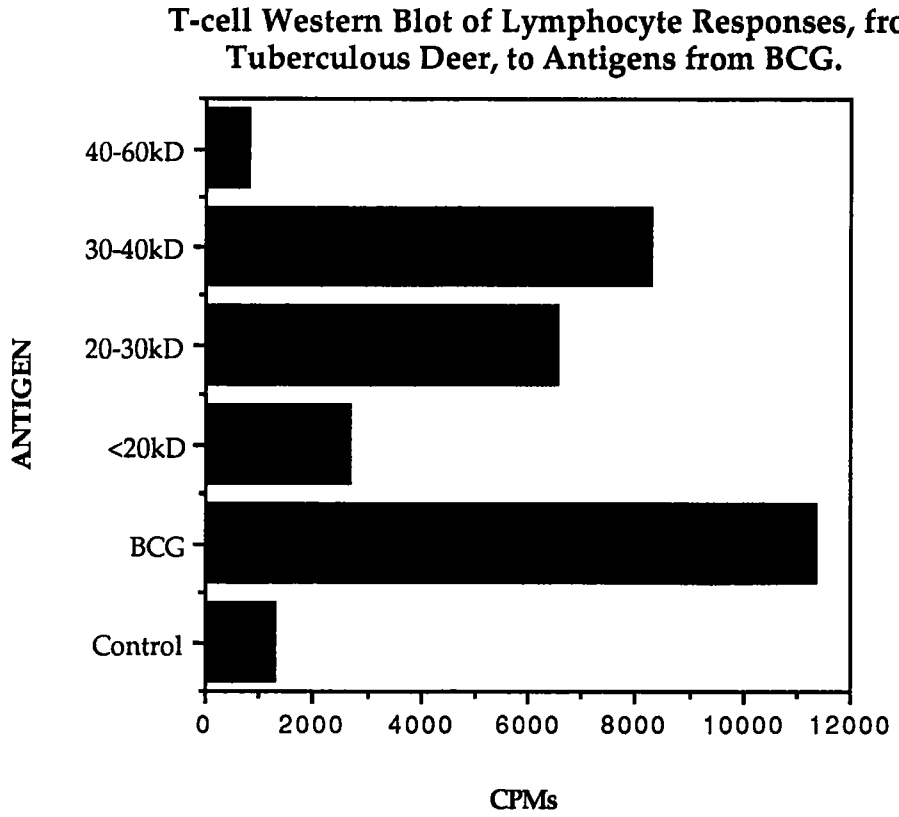
Western Blotting for Protein (Antigen) Separation

While it is vital to study the structure and function of lymphocytes and their products (cytokines/immunoglobulin) it is equally important to be able to isolate individual antigens from the disease causing organism. In any study of infectious disease there is the fundamental need to isolate pure antigens necessary for specific diagnostic tests or as target molecules to be used in vaccines.

Western Blotting gel separation techniques have been developed (Towbin et.al., 1979) which allow individual protein molecules to be separated in polyacrylamide gel exposed to an electrical current (**Electrophoresis**). Molecules are separated into individual bands based on their electrical charge and size. Following separation, bands of protein can be transferred directly to fine nitrocellulose membranes for further immunological characterisation. Proteins which are recognised by the immune system and against which antibodies have been formed will bind antibody from the sera of diseased or immunised animals. These antibodies (**Primary**) will bind to specific bands of antigens on nitrocellulose, where they can be detected using enzyme labelled anti-deer antibodies (**Secondary**). This is essentially a form of solid-phase ELISA, where enzyme reactivity produces a colour after the addition of a substrate, which can be seen visually. This reaction is called a Western Blot.

Individual groups of antigens with similar molecular weights can be separated on acrylamide gel and transferred to nitrocellulose which is then cut up to provide solid phase purified antigens to stimulate lymphocytes in cell culture assays.

Figure 4



An example of this procedure is shown in Figure 4, which antigens from mycobacteria which have different molecular weights are used to stimulate lymphocytes from deer, naturally infected by M.bovis or vaccinated with BCG. The results show that large (>45,000Kd) and small (<20,000Kd) molecules from M.bovis culture supernatants are not important stimulants for lymphocytes. By contrast molecules between 20-45,000 MW contain the major lymphocyte stimulatory antigens relevant in deer Tb.

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