Studies on Ovine Interferon-gamma

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DECLARATION

The experiments and composition of this thesis are, unless otherwise stated, my own work. No part of this work has been, or is being, submitted for any other degree, diploma or other qualification.

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Abstract.

Interferons have been recognized as important mediators of cellular communication for many years. There are two types of interferon: Type I interferons have antiviral functions, but Type II interferon (IFN-g) is more important as an immunomodulating molecule. Type II interferon has effects on cellular MHC class II expression, immunoglobulin class-switching, macrophage activation, cellular proliferation and a number of other functions. The role of IFN-g during in vivo immune responses has not been studied in great detail, but the sheep is an ideal species in which to study these phenomena by using the efferent lymphatic vessel cannulation model. This allows access to cells and tissue fluid for cytokine analysis using antibody and genetic probes for the detection of IFN-g.

Bovine IFN-g peptides (amino-terminus, carboxy-terminus and central) were used to generate antibodies in rabbits. None of the anti-peptide sera reacted with denatured ovine or bovine IFN-g, nor neutralized their antiviral effect. Rabbit antibodies to bovine recombinant IFN-g neutralized ovine IFN-g and detected IFN-g in a sandwich ELISA when used in combination with a monoclonal antibody against a human IFN-g carboxy-terminal peptide. The sensitivity of detection was only 125ng/ml, insufficient for use with efferent lymph fluid samples.

The expression of MHC class II molecules on cell surfaces is increased by IFN-g on many cell types. This has been used previously to measure biologically active IFN-g concentrations in fluids. Measurement of ovine class II by slot blot was assessed as a method of adapting this to ovine IFN-g measurement, but the technique proved to be too problematic for regular use. The expression of class II on T lymphocytes is influenced by IFN-g in

the surrounding fluid. Analysis by FACS of resting ovine T lymphocytes shows them to express class II, a situation different to that in the human. Incubation of efferent lymph cells with IFN-g enhances the expression of ovine DR-like class II molecules especially on $CD8^+$ cells, but also on $CD4^+$ cells. The expression of ovine DQ-like class II molecules was much less influenced by IFN-g. This differential expression has been seen previously in human cells. It is likely that such differences are due to variation in transcriptional control between the two types of molecule.

The IFN-g genes of many species have been cloned, including bovine IFN-g. Nucleotide primers for the cloning of ovine IFN-g by polymerase chain reaction were chosen from the bovine sequence. Cloning of the central 300bp of the gene revealed 98% identity between ovine and bovine IFN-g at the amino acid level. Subsequent cloning of ovine IFN-g by other groups showed that allelic variation of the gene occurs with no alteration in the amino acid structure. This feature is not found in human IFN-g, but is described in other ruminant cytokines. Attempts to isolate a lambda clone expressing an IFN-g fusion protein using the rabbit anti-bovine rIFN-g sera were unsuccessful.

The immune response may be analysed by studying cells and fluid delivered by the cannulation of an efferent lymphatic vessel. A secondary response to ovalbumin was induced in a sheep by inoculation of a dependent area. The lymphocytes collected were isolated into $CD4^+$ and $CD8^+$ cells by a magnetic separation technique (MACS). Their mRNA was isolated, and cDNA generated from it was subjected to IFN-g-specific PCR. This revealed that both $CD4^+$ and $CD8^+$ cells contribute to the synthesis of IFN-g during the secondary immune response to a protein antigen in the sheep.

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Abbreviations.

IFN-g	interferon-gamma
IFN-α	interferon-alpha
IFN-β	interferon-beta
IFN-w	interferon-omega/alpha2 interferon
LV-IFN	lentiferon
IL-	interleukin-
TNF-α	tumour necrosis factor-alpha
LT	lymphotoxin
GM-CSF	granulocyte-macrophage colony stimulating factor
NK cells	natural killer cells
T_{H1}	T helper 1 cells
T_{H2}	T helper 2 cells
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
BSA	bovine serum albumin
PBS	phosphate buffered saline
HBSS	Hank's buffered saline solution
FCS	foetal calf serum
TBS	tris buffered saline
BBS	borate buffered saline
Con A	concanavalin A
CFA	complete Freund's adjuvant
Ig	immunoglobulin
MoAb	monoclonal antibody
MHC	major histocompatability complex
VSV	vesicular stomatitis virus
CAEV	caprine arthitis-encephalitis virus
TEA	triethanolamine
DOC	sodium deoxycholate
GTC	guanidinium thiocyanate
DEPC	diethylpyrocarbonate
OD	optical density
PEG	polyethylene glycol
MACS	magnetic activated cell sorter

1 INTRODUCTION.

1.1 DISCOVERY AND CLASSIFICATION OF INTERFERONS.

Interferon was discovered by Isaacs & Lindenmann (1957) when they were looking for a virus-induced factor which could interfere with viral replication. They described how heat-inactivated influenza virus induced chicken chorioallantoic membrane cells to secrete a factor which could prevent subsequent infection of fresh membrane by live influenza virus. They called this factor, which interfered with viral growth, "interferon".

Interferon has subsequently been grouped into two types as more individual interferons have been discovered. Type I interferons are produced in response to viral infection of cells and are typically acid and heat resistant. Type II is distinguished from Type I antigenically and by its heat (56°C for 30 minutes) and acid (pH2 for 30 minutes) lability.

Type I interferons are sub-divided into leucocyte or alpha interferon (IFN-α), fibroblast or beta interferon (IFN-β), and trophoblast, omega or alpha 2 interferon (IFN-w / IFN-α_{II}) (Pestka et al, 1987). The prime function of alpha and beta interferons seems to be antiviral. Trophoblast, omega, or class II alpha interferon has antiviral activity, but its prime role appears to be in signalling between the pre-implantation conceptus and the dam to prevent rejection (Imakawa et al, 1989).

[NOTE ON NOMENCLATURE: Type I and type II interferon refer to the early classification of interferons by antigenic and physical properties. Type I interferons are synonymous with alpha and beta

interferons, while type II interferon is gamma interferon. Class I and class II alpha interferons are subdivisions of the alpha group defined by nucleotide sequence comparisons. Class I alpha interferons are the interferons produced in response to viral infection of cells, while class II alpha interferons are produced by the placenta of pregnant animals.]

Type II interferon, also known as immune or gamma interferon (IFN-g), is synthesized by T lymphocytes and natural killer (NK) cells following exposure to mitogens or antigens (Sandvig et al, 1987). In addition to its antiviral activity, IFN-g has many immunoregulatory activities (section 1.4)

1.2 PRODUCTION OF INTERFERONS.

Interferons were first detected in vitro in supernatants from virus-infected cells. Subsequently Type I interferons have been detected from all nucleated cells examined, and alpha and beta interferons have been identified in many species. Interferon secretion can be induced in vitro by non-viral stimuli including protozoa, mycoplasma, Rickettsia, bacteria and double-stranded synthetic ribonucleic acids (Stewart, 1979).

Type II interferon is not induced by viral infection of cells, but is the product of antigenic or mitogenic stimulation of activated lymphocytes or NK cells (Croll et al, 1986). T cells may be activated polyclonally either by mitogen (Wheelock, 1965) or antibody (Falcoff et al, 1972), or in a clonally-restricted manner in response to antigen-specific recognition (Green et al, 1969). Lymphocytes exposed to antigen preparations only produce IFN-g if

the lymphocytes have been obtained from an individual previously exposed to the antigen in question (Green et al, 1969). A number of studies of lymphocyte and mononuclear cell suspensions have shown interferon-gamma to be produced by T-cells (Falcoff, 1972; Blanchard et al, 1986). Initially production of IFN-g was thought to be part of a positive feedback loop involving IL-1, IL-2 and IFN-g. IL-2 production is enhanced by the presence of interleukin 1 (IL-1), and IFN-g production is enhanced by IL-2. IFN-g has been shown to increase production of IL-1 by monocytes in response to lipopolysaccharide stimulation (Newton, 1985). However, the isolation of T helper subsets has allowed the autocrine requirements of T cells to be analysed more carefully. One subset, T_{H2} cells, use IL-4 as their autocrine growth factor, with IL-1 also being required for DNA synthesis (Lichtman et al, 1987). T_{H1} cells utilize IL-2 as their autocrine factor, and IL-1 is not required at all (Lichtman et al, 1988). IFN-g can act as a growth promoter for T cells, but the subset of T cells was not identified (Landolfo et al, 1988)

Natural killer cells are associated closely with the production of IFN-g in a mechanism requiring both IL-2 and a factor derived from macrophages (Kawase et al, 1983). Anti-IFN-g sera stain lymphocytes found within tumours which are thought to be NK cells (Saksela et al, 1980). Human peripheral blood lymphocytes (PBLs) enriched for NK cells were shown to be the major source of IFN-g after infection with influenza or herpes viruses (Djeu et al, 1982). Clones of NK cells when cultured in the presence of IL-2 secrete IFN-g (Handa et al, 1983).

During polyclonal T cell activation of ficoll-gradient isolated blood mononuclear cells (MNCs) by anti-CD3/Ti antibodies, double staining for IFN-g and cell markers demonstrated that up to 26% of IFN-g positive cells carried the CD16 molecule i.e. were putative NK cells (Sandvig et al, 1987). In these experiments, the $CD4^+$ and $CD8^+$ together only comprise approximately 50% of the IFN-g secreting cells. The remaining 25% of cells secreting IFN-g carried IL-2 receptors and the majority were erythrocyte-rosetting mononuclear cells but did not fulfill the criteria for either T-cell, B-cell or monocyte cell lineages.

1.3 PURIFICATION AND STRUCTURE OF INTERFERONS.

1.3.1 ALPHA INTERFERON.

Alpha interferon has been purified to homogeneity, and shown to form a family of alpha interferons, which in humans totals 16 or more unique protein species (Pestka et al, 1987). Two classes of alpha interferon have been identified genetically, IFN-α_I and IFN-α_{II} (Capon et al, 1985). IFN-α_{II} is also known as IFN-w and will be discussed in section 1.3.4.

The apparent molecular weights of the class I IFN-αs range from 16000 to 27000 and typically contain 165-166 amino acids. The class I IFN-αs are quite homologous in amino acid sequence with greater than 77% identity between the various individual interferons (Pestka et al, 1987). They were initially thought to be all unglycosylated, but there is now evidence for glycosylation in some species of IFN-α. This is postulated to be O-linked glycosylation as all but one human IFN-α sequence lacks an N-linked glycosylation site (Labdon et

al, 1984). The genes for human IFN-α have been cloned, and at least 15 separate loci have been identified. At least 14 of these encode functional proteins, but some of the loci represent pseudogenes. None of the genes identified so far contains an intron, in contrast to IFN-g. All animal species studied have a large IFN-α family of genes (reviewed by Weismann & Weber, 1986).

1.3.2 BETA INTERFERON.

Human beta interferon has been purified, and only one protein species was identified (Knight, 1976). It was of molecular weight 20000, and found to be a glycoprotein. The associated carbohydrate was thought to be N-linked. Target size determination has shown the functional unit to be a dimer (Pestka et al, 1983). Beta interferon is encoded by a single intron-less gene in the human and many mammalian species, but the bovine (Leung et al, 1984), equine and porcine (Wilson et al, 1983) IFN-β gene families are known to contain many genes.

1.3.3 GAMMA INTERFERON.

Gamma interferon has been purified to homogeneity from human peripheral blood lymphocytes using chromatographic techniques (Yip et al, 1982; Rinderknecht et al, 1984). Three forms have been identified by SDS-PAGE separation with apparent molecular weights of 15500-17000, 20000 and 25000 (Pestka et al, 1987). The molecule has two potential glycosylation sites. The 20000 molecule only has one site glycosylated, whereas the 25000 molecule has both sites

glycosylated (Rinderknecht et al, 1984). Isolation of IFN-g by gel filtration has identified it to have a molecular weight of 40000-60000, suggesting it exists as a dimer (Yip et al, 1982).

Murine IFN-g has been extensively studied to determine the basic structure of the molecule. Murine IFN-g is also glycosylated (Wietzerbin et al, 1979), and sensitive to pH2 (Wiranowska-Stewart et al, 1980). SDS-PAGE revealed a monomer structure of approximately 20500 molecular weight, but gel filtration gave a molecular weight of 41000, again suggesting the active structure is a dimer (Havell & Spitalny, 1983). Variable glycosylation occurs, as demonstrated by a change in molecular weight after production in the presence of glycosylation inhibitors such as tunicamycin, and the binding of the interferon to a lectin affinity column (Havell & Spitalny, 1984).

The gene for IFN-g has been cloned for human (Gray et al, 1982; Devos et al, 1982), mouse (Gray & Goeddel, 1983), rat (Dijkema et al, 1985), cow (Cerretti et al, 1986), and recently for sheep (McInnes et al, 1990; Radford et al, 1991) and pig (Dijkmans et al, 1990). The genetics of IFN-g are discussed in greater detail in section 4.1.

1.3.4 OMEGA INTERFERON.

The interferon-ws (also known as class II interferon-αs) are 172 amino acids long, and have 58% identity with the class I IFN-α genes. IFN-w (IFN-α_{II}) has molecular mass of 24500 and is presumed to be N-glycosylated (Adolf et al, 1990). From the sequence data it is believed to represent the human equivalent of the ovine

trophoblast protein-1, although no similar role for it has been defined in humans (Adolf, 1990). Six human loci have been identified, but five of these are pseudogenes (reviewed by Shephard et al, 1985).

1.4 IMMUNE FUNCTION OF INTERFERON-GAMMA.

Although IFN-g was first detected as an antiviral factor and is still defined as an interferon, it has many important functions which have no apparent connection with its antiviral activity. Prior to the synthesis of recombinant IFN-g the activities of IFN-g were difficult to define and separate from those of other lymphokines, as the source of IFN-g was cell supernatants. All three interferon species are able to increase expression of MHC class I molecules on many cell types (Heron et al, 1979; Fellous et al, 1979; Fellous et al, 1982; Basham et al, 1982), but IFN-g can also increase the expression of MHC class II molecules on macrophage lines (Steeg et al, 1982; King & Jones, 1983), B lymphoma cells (Wong et al, 1983), and many other cell types (see 3.1.3). Interferon-α/β interacts with IFN-g by antagonizing the increase in class II on many cells (Morris & Tomkins, 1989).
IFN-g acts as a potent macrophage-activating factor for tumour cell killing (Schulz & Kleinschmidt, 1983) and for enhancement of the killing of intracellular parasites (Nathan et al, 1983). Tumour cell lysis is increased in vitro by synergism with tumour necrosis factor α (TNF-α) and lymphotoxin (LT) (Stone-Wolff et al, 1984; Lee et al, 1984). IFN-g is pivotal in the development of macrophage resistance to infection by Lieshmania major, but requires the

presence of either granulocyte-macrophage colony stimulating factor (GM-CSF), IL-2, or interleukin 4 (IL-4) in the medium, as it is insufficient alone to induce resistance (Belosevic et al, 1988).

Zlotnik et al (1983a) analysed T-cell hybridomas, and subclones of them, for production of factors responsible for five different biological activities. They demonstrated that antiviral activity (IFN-g) from T cell clones was always associated with macrophage activating activity (MAF), MHC class II induction factor (IaIF), and B cell helper activity (IL-X), but is not generally associated with T-cell growth activity (IL-2). IFN-g, MAF, IaIF and IL-X were all sensitive to acid denaturation. The conclusion was that all these activities were caused by IFN-g. This was later confirmed for IaIF when recombinant IFN-g was found to induce Ia (Zlotnik et al, 1983b).

IFN-g has a suppressive effect on the MHC class II expression of small B cells. MHC class II molecules are increased on B cells by incubation with IL-4 (B cell stimulating factor-1) (Roehm et al, 1984; Noelle et al, 1984; Rousset et al, 1988), but this increase is antagonized by IFN-g (Mond et al, 1986). IFN-g is also competitive with IL-4 in the selection of immunoglobulin class-switching. IL-4 promotes immunoglobulin class-switching in mouse B cells from secretion of IgM, IgG3 and IgG2b to IgG1, IgG2a and IgE. IFN-g antagonizes this and promotes the class-switching to IgG2a (Coffman & Carty, 1986; Lebman & Coffman, 1988; Mosmann & Coffman, 1989).

IFN-g upregulates a number of cell surface molecules including intercellular adhesion molecule 1 (ICAM-1) (Dustin et al, 1988) and Fc receptor on monocytes (Perussia et al, 1983).

1.5 INTERFERONS IN CATTLE.

Interferons have been studied in cattle for much longer than in sheep because of the relative financial values of the two species. Bovine interferons should be good models from which to base information for use in sheep research.

Both type I and II interferons were reported in cattle many years ago. An acid-stable interferon was described with anti-viral activity on cells from several species (Pollikoff et al, 1962). An acid-labile interferon has been described which also was active on heterologous cells (Babiuk & Rouse, 1977).

Bovine IFN-α is similar to the human gene family, having two classes of gene. Multiple copies of both sub-families exist, and the two families have approximately 54% identity at the amino acid level (Capon et al, 1985). Bovine IFN-α_{II} (IFN-w) is also known as bovine trophoblast protein-1. It functions to signal to the dam that a conceptus is present in the uterus and that the corpus luteum should be maintained (Imakawa et al, 1989). It may also be involved in the suppression of the maternal immune response to the foetus.

Recombinant bovine IFN-α_I1 protein has been used in clinical work and found to have a beneficial antiviral and immunomodulatory effect in bovine respiratory disease due to parainfluenza-3 virus (Martinrod et al,1988), and shipping fever due to Pasteurella multocida (Lynn & Phillip, 1988).

Interferon beta has been purified by controlled pore glass chromatography, and when assayed by SDS-PAGE has a molecular weight of 21000 (Allen et al, 1988). There are at least five copies of the IFN-β gene in the bovine genome compared with a single IFN-β gene in the human genome (Leung et al, 1984).

Interferon gamma has been found to be a single copy gene encoding a precursor protein of 166 amino acids, and a predicted mature protein of 143 amino acids with a molecular weight of 16858. It has 63% amino acid identity with human IFN-g, and 47% with murine IFN-g.

1.6 INTERFERONS IN SHEEP AND GOATS.

Interferons were first reported to exist in sheep when sheep cells were infected in tissue culture by mycoplasma (Rinaldo et al, 1973), and Bluetongue, Chikungunya and Herpes simplex viruses (Rinaldo et al, 1975). These interferons were certainly Type I interferons. More recently ovine interferons have been produced from fibroblasts with poly inosinic: poly cytidylic acid, and from T lymphocytes by antigenic and mitogenic stimulation (Entrican et al, 1989). These two types of interferon had physical characteristics (heat and pH stability/lability) typical of IFN-β and IFN-g respectively.

Much work has been carried out on ovine trophoblast protein-1 which is secreted by trophoectoderm cells of the preimplantation sheep embryo (Roberts et al, 1989). This has antiviral activity and its cDNA sequence is similar to bovine IFN-α_{II} (Imakawa et al, 1987; Imakawa et al, 1989).

A seemingly unique interferon, lentiferon (LV-IFN), of sheep and goats has been described which is produced by lymphocytes in contact with lentivirus-infected macrophages (either maedi-visna virus- or caprine arthritis-encephalitis virus-infected) (Narayan et al, 1985; Lairmore et al, 1988). This interferon is similar to IFN-α in resistance to heat and acid conditions, whilst it is resistant to freeze-thawing and protects some cell lines from viruses not protected by known IFN-α. This LV-IFN also possesses the ability to induce expression of Ia antigens on macrophages (Kennedy et al, 1985). Although data has not been presented, it has been asserted that LV-IFN activity is associated with a 54- to 64-kilodalton nonglycosylated protein, its production requiring the interaction of monocytes and lymphocytes (Zink & Narayan, 1989). LV-IFN production is blocked by anti-MHC class II antibodies. LV-IFN inhibition of caprine arthitis-encephalitis virus (CAEV) replication is thought to be indirect by inhibiting monocyte maturation, and also direct by inhibiting viral gene expression in macrophages. LV-IFN consequently has properties of both IFN-α and IFN-g, and may even be a mixture of the two. Further biochemical and molecular data is required to define this factor(s).

The physical properties of goat interferons have been examined (Yilma et al, 1988). The stability/lability to both heat and pH were typical of Type I and II interferons. Also shown was that CAEV induced high levels of interferon in synovial fluid during acute exacerbations of the infection, and also in response to intraarticular injection of CAEV. The interferon had physical properties typical of IFN-g.

1.7 CLINICAL USES OF INTERFERON-GAMMA.

Murine IFN-g has been used as an adjuvant in the *Plasmodium yoelii* murine malaria model, when a Triton X-100 lysate of infected erythrocytes was used as a vaccine (Heath *et al*, 1989). Injection of IFN-g at the same time as the vaccine into mice with either cellular or humoral immune defects enhanced the number of mice protected compared with those receiving vaccine alone. Similar results have been demonstrated with the use of IFN-g as an adjuvant in cattle (Anderson *et al*, 1988). Cattle were more resistant to vesicular stomatitis virus (VSV) challenge when IFN-g had been used as an adjuvant with VSV "G" protein than when no adjuvant was used. They also demonstrated enhanced secondary antibody responses after boosters of the same antigen without IFN-g. The mode of action is unknown at present, but does not involve a second mediator stimulated by the interferon. Localization of lymphocytes to the vaccination site does occur, but the significance is unknown. The most likely mechanism is that of improved antigen presentation associated with elevated MHC class II expression.

Liposomes are membranous vesicles of naturally occurring phospholipids, separated by aqueous compartments. The incorporation of interferon-gamma into liposomes can activate blood monocytes to lyse virus-infected cells in both a human and murine system (Swenson *et al*, 1988).

The use of interferons as antiviral agents has been best studied for IFN-α, but bovine IFN-g has also been examined. IFN-g has more potential as an immunomodulatory substance, but its effect on prevention of bacterial infections has been controversial. Treatment of piglets 24 hours prior to *Actinobacillus*

pleuropneumoniae challenge abolishes disease, but similar treatment of calves with bovine IFN-g 24 hours prior to Pasteurella haemolytica challenge exacerbates the pneumonia, seeming to enhance the ongoing inflammation (Bielefeldt-Ohmann, 1990). Recombinant bovine IFN-g cannot prevent pneumonia caused by Haemophilus somnus challenge, but reduced morbidity and mortality in calves immunosuppressed by glucocorticoids (Chiang et al, 1990).

The immunomodulatory effect of IFN-g has been assessed on the mammary gland of cows. Infusion of bovine rIFN-g during the dry period increases the bacterial killing capabilities of phagocytes harvested 24 hours later (Fox et al, 1990). Experimental intramammary E. coli challenge was resisted much better by cows 24 hours after intramammary infusion of rIFN-g than by control cows, and clinical disease was less severe and of shorter duration. No treated cows died, whilst untreated cows suffered 42% mortality (Sordillo & Babiuk, 1991).

1.8 THE IMPORTANCE OF STUDYING INTERFERON-GAMMA IN THE SHEEP.

The size and docility of the sheep permits the cannulation of lymphatic vessels and the chronic collection of lymphatic fluid. This has allowed the analysis of lymphocyte migration through lymph nodes and the study of the development of immune responses. With the growing number of monoclonal antibodies to sheep lymphocyte surface antigens and the recent development of assays for lymphokines, the opportunity now exists for a much more detailed analysis of the cellular output from an antigen-stimulated lymph node and the cytokines produced by and which interact with such cells. These studies cannot be performed in small laboratory

animals because the chronic cannulation of lymphatic vessels leads to a progressive depletion of lymphocytes and so prejudices the physiological integrity of the animal (Miyasaka & Trnka, 1986).

The sheep popliteal lymph node efferent cannulation model allows access to the initial immune response of the sheep to an antigen injected into the distal limb. This may be a naïve or a memory response from the sheep. The cannulation delivers both lymphocytes exiting the node, which will reflect the response taking place, and also the supernatant of the cells, which will allow analysis of lymphokine and antibody levels exiting the node. The lymphocytes may be analysed in a number of ways, including FACS analysis of surface antigens (e.g. CD4, CD8, MHC class II), cytokine levels in lymphatic fluid and production on further culture, and mRNA levels of cytokines and surface molecules. A variety of techniques for studying the secretion of cytokines have been developed in the last few years, including ELISA assays and the intracellular staining for cytokines. These new types of assays have not yet been used in sheep immunology, but they represent a novel method of determining cellular involvement in the active immune response.

Unfortunately ELISA assays for ovine IFN-g were not available at the beginning of this thesis. The development of antibodies to ovine IFN-g was a prime requirement of the project so that characterization of the molecule and measurement of its presence in lymphatic supernatants could be performed. Initial attempts to obtain antibodies reactive to native IFN-g were by the use of peptides derived from the bovine IFN-g sequence for the immunization of rabbits. Subsequent work utilized recombinant bovine IFN-g and

polyclonal sera to it, and also a monoclonal antibody reactive with a human carboxy-terminal peptide. ELISA methodologies were developed. The techniques are described in chapter 2.

A second method for assaying IFN-g in supernatants is by the measurement of MHC class II upregulation on cell surfaces. The availability of both ovine cell lines and anti-sheep MHC class II monoclonal antibodies made this technique a second possibility for the quantitation of IFN-g. The study of different class II subsets and their differential upregulation by IFN-g was an area of interest as sheep T cells express class II at rest, unlike human T cells. The attempts to develop a MHC class II cell assay, and the regulation of class II on T cells are described in chapter 3.

The production of IFN-g can be assessed at the protein level or the mRNA level. The latter depends upon the availability of an appropriate DNA/RNA probe, or polymerase chain reaction protocol. Either technique requires the ovine IFN-g gene to be cloned in part. The cloning of part of the ovine IFN-g gene by PCR, and attempts to isolate a cDNA clone are described in chapter 4.

The production of IFN-g by human lymphocytes during an <u>in vivo</u> immune response has been assessed by intracytoplasmic fluorescent staining. The absence of appropriate monoclonal antibodies denied the application of this technique to sheep lymphocytes. The PCR in chapter 4 was optimized and the levels of IFN-g mRNA in Concanavalin A-stimulated lymphocytes were assessed using this technique. This protocol was then applied to IFN-g mRNA levels in T cell subsets in efferent lymph during an <u>in vivo</u> secondary immune response. A magnetic cell separation technique was used to isolate the T cell subsets. The results of this work are described in chapter 5.

2 PEPTIDE STUDIES ON OVINE INTERFERON-GAMMA.

2.1 INTRODUCTION.

Interferon-gamma is an important immunoregulatory lymphokine which displays pleiotropic effects (reviewed by Trinchieri and Perussia, 1985). Its major effects are thought to relate to its ability to activate macrophages, to induce Major Histocompatibility Complex molecules, and to influence antibody isotype switching. Its role in the development of primary and secondary immune responses at the lymph node level has not been ascertained to date. Access to this stage of the immune response is possible by chronically-cannulating the lymphatic vessels of sheep and analysing the draining cells and fluid (Hall & Morris, 1964). The ability to dissect the role of IFN-g in the immune response in this system is however dependent on the availability of reagents specific for ovine IFN-g. One of the major objects of this section of work was to develop antibody reagents which would react specifically and sensitively to ovine IFN-g.

Antibodies have previously been described which react specifically with human (Langford et al, 1981; Johnson et al, 1982), murine (Langford et al, 1983), and porcine IFN-g (Charley et al, 1988), but cross-reactivity with IFN-g of other species has been poor due to low sequence identities at the amino acid level (40% between human and murine IFN-g [Gray & Goeddel, 1983]; 59% between human and porcine IFN-g [Dijkmans et al, 1990]). Antibodies to IFN-g of these three species are consequently unlikely to react with ovine IFN-g. Bovine IFN-g has been cloned and expressed (Cerretti et al, 1986) and antibodies to it are more likely to bind ovine IFN-g due to the close phylogenetic origin of the two species. However neither

polyclonal antisera nor monoclonal antibodies to bovine IFN-g are readily available. Instead it was proposed that synthetic peptides derived from the bovine sequence be used for the generation of anti-peptide antibodies. The degree of identity between bovine and ovine IFN-g was unknown, so regions of close homology between the human, murine, rat and bovine sequences were considered. These homologous regions are also likely to be in regions of the molecule important for structure and function.

[NOTE ON NOMENCLATURE: the signal sequence of human IFN-g was originally thought to 20 amino acids long, but is now thought to be 23 amino acids in length. The amino acids of the mature cleaved protein have been numbered throughout this thesis on the basis of a signal sequence of 20 amino acids. The numbering of peptides cited in other papers has been altered if necessary to conform to this standard. A peptide is described by quoting its first and last amino acid e.g. peptide 1-10 is the peptide from amino acid 1 to amino acid 10]

2.1.1 IMPORTANT REGIONS OF INTERFERON-GAMMA DEFINED BY ANTI-PEPTIDE SERA AND AMINO ACID DELETIONS.

The first antibodies raised against IFN-g were derived from the immunization of rabbits with partially purified supernatant from Staphylococcal enterotoxin A-stimulated human lymphocytes (Langford et al, 1981). The sera were found to neutralize IFN-g but not IFN-α/β in an antiviral assay (50% Sindbis virus cytopathogenic effect reduction assay on human amnion WISH cells). IFN-g was therefore assumed to be antigenically dissimilar to IFN-α/β.

The cloning, sequencing and expression of the cDNA for human IFN-g (Gray et al, 1982) allowed study of the molecule by the use of synthetic peptides and antisera to them (Johnson et al, 1982). This approach eliminated the problem of partial purity of the immunizing protein which afflicted the previous polyclonal antisera. The amino terminal portion of the molecule was the first to be studied for its functional importance. The peptide synthesized represented the first twenty N-terminal amino acids of the mature molecule inferred from the cDNA sequence. Sera were tested by an anti-peptide ELISA and also by neutralization of the antiviral activity. Comparison with sera raised against native interferon-gamma showed that the anti-peptide sera reacted with peptide by ELISA much more strongly than did the anti-interferon sera, but that the anti-interferon sera was neutralizing in the antiviral assay to a much higher titre than the anti-peptide sera. This suggested that anti-interferon sera contained antibodies to additional determinants on the interferon molecule which are important in the antiviral action.

There is much evidence for the functional importance of the amino terminus of human IFN-g. Protease digestion of the amino terminus of IFN-g leading to the removal of 11 or 14 amino acids (numbered with the CYS-TYR-CYS as 1-2-3) renders the protein devoid of biological activity and causes a dramatic change in secondary structure (66% α-helix and 0% β-sheet before, and 6% α-helix and 36% β-sheet afterwards) (Hogrefe et al, 1989). Leist et al (1985) found that rabbit antisera to both peptides 1-59 and 24-59 bound to rIFN-g, but only anti-(1-59) neutralized the antiviral activity. Disruption of rIFN-g by acid treatment did not alter binding of anti-(24-59) indicating the structure in this region was little

disturbed by the acid treatment. Monoclonal antibodies to peptide 4-21 have been found to neutralize human IFN-g antiviral activity (Ichimori et al, 1987).

The cDNA cloning of murine IFN-g (Gray & Goeddel, 1983) allowed an N-terminal peptide to be synthesized and antisera produced. Additional experiments were carried out to compare the cross-reactivity of anti-murine peptide and anti-human peptide sera on both peptides and natural immune interferon in ELISA and neutralization assays. The degree of cross-reaction ranged from 4 to 30% for anti-murine peptide sera in a neutralization assay with human IFN-g, and 5 to 12% for anti-human peptide sera in the reciprocal assay. Cross-reactivity was similar in the anti-peptide ELISA. Anti-native murine or human IFN-g sera cross-reacted less than 1% in both neutralization assay and ELISA (Langford et al, 1983).

Studies with four monoclonal antibodies to recombinant murine IFN-g showed that binding of ^{125}I-rIFN-g by three of the four MoAbs could be inhibited by a synthetic peptide corresponding to amino acids 1-39 of IFN-g, but not with peptide 95-133. The monoclonal antibodies also bound labelled 1-39 peptide, and one of the three antibodies (5.102.12) inhibited both antiviral activity and macrophage activation of rIFN-g. Polyclonal antisera to the two peptides were neutralizing and were shown to inhibit binding of labelled IFN-g to the membrane receptor. These data suggest that the interaction between the cellular receptor and IFN-g involves both the amino and carboxyl termini of the IFN-g molecule (Russell et al, 1986). The monoclonal antibody (5.102.12) was further studied to define the reactive epitope at the amino terminus of IFN-g (Magazine et al, 1988). In a competitive RIA, peptides 1-39, 1-20, 3-20, and 5-20

all inhibited binding of 5.102.12 to recombinant IFN-g, whilst peptides 7-20, 1-10, 10-30 and 21-44 had no effect. It was predicted by the Chou and Fasman algorithm that IFN-g has a secondary structure of predominantly α-helical structure, with amino acids 12-20 in a loop formation on the surface of the molecule, and that the peptide inhibition data suggest that the epitope for monoclonal antibody (5.102.12) lies on this loop of IFN-g.

There is conflicting evidence for the importance of the carboxy terminus in receptor binding. Human IFN-g molecules proteolytically truncated by 13 amino acids at their C-terminus have been shown to have reduced biological activity (Arawaka et al, 1986; Leinikki et al, 1987). Similarly re-engineered human rIFN-g deleted of its C-terminal 14 amino acids was 100-1000 times less effective in anti-chlamydial, antiviral and antiproliferative activities than full length IFN-g, whilst rIFN-g modified to possess four serine residues at positions 129-132 was 10-50 times less potent than full length IFN-g in the same experiments (de la Maza et al, 1987). Others, however, have demonstrated that truncated molecules deleted of 15 amino acids (Honda et al, 1987; Rose et al, 1983) or 23 amino acids (Sakaguchi et al, 1988) retain full antiviral activity. The discrepancies in remaining antiviral activity may be due to differences in cells used in the assay and the IFN-g receptors on their surfaces.

A number of monoclonal antibodies against carboxy-terminal peptides have not possessed neutralizing activity (Ichimori et al, 1985; Altrock, 1986; Favre et al, 1989; Lord et al, 1989), whilst a monoclonal antibody to human rIFN-g which is reactive against peptide (132-137) is described as blocking antiviral and anti-proliferative activity (Seelig et al, 1988).

Russell et al (1986) produced a polyclonal serum against a carboxy-terminal peptide, amino acids 98 to 136 of the murine sequence, which was neutralizing in an antiviral assay and also in a macrophage activating assay. Similarly a monoclonal antibody made against the same peptide was found to neutralize both the antiviral and macrophage priming activities of IFN-g (Russell et al, 1988). Peptides encoding the terminal nine amino acids of murine IFN-g were shown to induce antisera which neutralized its antiviral and immunoregulatory activities (Langford et al, 1987). These results imply that the carboxyl terminus, as well as the amino terminus, is important in receptor binding.

Peptide studies have been of considerable importance in predicting the tertiary structure of human IFN-g. The preparation of peptides covering the whole of murine IFN-g and the generation of antisera to them all has led to the demonstration that only the N-terminal and the C-terminal peptides are important in receptor binding (Jarpe & Johnson, 1990). This data, circular dichroism spectra showing mainly alpha-helical structure, and the protein secondary structure predicted by the method of Chou and Fasman (1974b), predicted the tertiary structure to be six α-helices with five turns. This prediction was incorporated in a model, based on the four α-helix bundle model, as demonstrated by crystallography for interleukin 2 (Brandhuber et al, 1987). The model proposed that IFN-g be found as a monomer, contrary to much chromatographic evidence (Yip et al, 1982). The model allowed the N- and C-terminal helices to be closely associated, and to form the receptor binding site of the molecule. The accuracy of this model has been refuted by the recent description of the tertiary structure of human IFN-g derived from x-ray crystallography (Figure 1) (Ealick et al, 1991). Human IFN-g crystallized as a dimer of identical subunits, with each subunit

containing six alpha-helices and no beta-sheet regions. The first four helices of one subunit form a cleft that accommodates the carboxy-terminal helix of the other subunit. The structure of bovine IFN-g has been predicted using the human structure as a model.

The object of the work described in this chapter was to generate antisera that would specifically react with ovine IFN-g and may be used to quantitate ovine IFN-g concentrations in lymphatic fluid. The use of peptides as immunizing molecules would also allow an assessment of the important functional regions of the ovine IFN-g molecule.

2.2 MATERIALS AND METHODS.

2.2.1 GENERATION OF ANTI-BOVINE INTERFERON-GAMMA PEPTIDE POLYCLONAL RABBIT SERA.

The three peptides shown below were synthesized in the Department of Veterinary Pathology by Mr D. Thompson. They were made using a Cambridge Research Biochemicals Pepsynthesizer II by the fluoronylmethoxycarbonyl (FMOC) polyamide solid phase method (Dryland & Shephard, 1986). The amino acids were purchased as pentafluorophenyl esters (Milligen, Millipore, UK). The solid support used for peptide synthesis was polydimethylacrylamide resin (Pepsyn KA, Milligen, Millipore, UK). Cysteine was coupled to the resin by 4-hydroxymethylphenoxyacetic acid. This was deprotected with 20% piperidine in dimethylformamide before addition of a second amino acid. The cycle of esterification and deprotection was continued until the whole peptide was synthesized. It was cleaved from the resin with trifluoroacetic acid (TFA). Purification was by reverse phase chromatography on a Zorbax ODS column (Du Pont, Delaware, USA) using a Gilson HPLC system with an Apple II computer control unit. A gradient of acetonitril (Rathburn Chemicals Ltd., HPLC grade) was generated from 0% to 100% in 0.1% TFA pH2 over a period of 30 minutes. Absorbance was detected at 230nm and recorded on a Shimadzu C-R3A chromatopac. The peptide peak was collected and freeze-dried prior to use.

Each peptide was used to immunize two rabbits. Peptides were not coupled to a carrier protein. Initial immunizations were of 200µg of peptide in Complete Freunds Adjuvant (Sigma). A second immunization was of 200µg of peptide dissolved in PBS.

PEPTIDE	RABBITS	SEQUENCE
42-68	R144 + R145	CESDKKIIQSQIVSFYFKLFENLKDNQV
112-137	R146 + R147	CAINELIKVMNDLSPKSNLRKRKRSQN
1-23	R155 + R156	CSYGQGQFFREIENLKEYFNASSP

The rabbits were immunized on two occasions, two to three weeks apart, and then bled from the ear vein. Repeat immunizations of peptide in PBS were periodically performed to maintain antibody titres. Sera were tested for reaction to the peptide concerned and an irrelevant peptide of the same length (usually a tuberculin peptide) using the direct ELISA described below.

2.2.2 DIRECT ELISA TO TEST ANTI-PEPTIDE ANTISERA.

Peptide was diluted in 0.1M carbonate buffer pH9.5 to 20µg/ml. 75µl was applied to each well of a 96-well polystyrene ELISA plate which was left overnight at 4°C. The wells were washed at each step with PBS/0.05% Tween 20 five times. Wells were blocked with 2% BSA/PBS for 30 minutes at 20°C. A doubling-dilution series of rabbit antisera, diluted in 2% BSA/PBS, was applied to the wells for one hour at 20°C. The wells were washed before addition of 75µl of 1/1000 goat anti-rabbit alkaline phosphatase (Sigma). After incubation for 1 hour, the ELISA was developed with 0.1% p-nitrophenyl phosphate disodium (Sigma) in 0.1M glycine, 0.05N NaOH, 0.5mM $ZnCl_2$, and 0.5mM $MgCl_2$ for 1 hour at 37°C, and the colour reactions measured at OD 405nm on a Titertek Multiskan plate reader.

2.2.3 GENERATION OF CONDITIONED MEDIA CONTAINING OVINE INTERFERON-GAMMA.

Fresh spleen and mesenteric lymph nodes were obtained from young adult sheep slaughtered at Gorgie abattoir, Edinburgh. Lymphocytes were obtained from the tissues by gentle teasing apart with sterile forceps and scissors. Cells were washed three times in HBSS + 1% foetal calf serum, with centrifugation at 1500 x g for 5 minutes each time. Cells were counted, with viability being assessed by trypan blue exclusion. Cells were resuspended at 5 x 10^6 /ml in Iscoves medium (Sigma) supplemented with bovine serum albumin (500µg/ml), human transferrin (25µg/ml), palmitic, oleic and linoleic oils (all at 1µg/ml), fungizone (25µg/ml) and penicillin/streptomycin (10U/ml of each). Concanavalin A (Sigma) was added to all except one flask at 10µg/ml. The other flask had no con A added. Cells were incubated at 37°C in 5% carbon dioxide. After 24 hours the supernatant was collected by centrifugation and stored at -20°C until use. Each supernatant was tested in the antiviral assay and shown to be positive (G Entrican, personal communication). Titres of the supernatant ranged from 1/100 to 1/800. The flask that had been incubated unstimulated tested negative in the antiviral assay and con A was added to the supernatant at 10µg/ml after collection. This was the control medium.

2.2.4 DIRECT ELISA TO DETECT OVINE INTERFERON-GAMMA WITH ANTI-PEPTIDE ANTISERA.

Supernatant from both ovine and bovine concanavalin A-stimulated lymphocytes (75µl) was applied to a 96-well ELISA plate overnight at 4°C. The supernatant was removed and the plate washed five times with PBS/0.05% Tween 20. The rest of the ELISA followed the protocol of section 2.2.2.

2.2.5 INHIBITION ELISA FOR OVINE INTERFERON-GAMMA.

An inhibition ELISA was designed to measure ovine IFN-g concentrations. Sera from the rabbits (R144, R145, R146, and R147) previously described were titrated against peptide by direct ELISA, and they were subsequently used in the inhibition assay at four dilutions which fell on the linear part of the titration curve. Preimmune rabbit serum was used at the same dilutions. A number of different supernatants from conditioned media were tested to see if they could inhibit the binding of sera to peptides. In one experiment a positive supernatant was concentrated 23 times by ultrafiltration over a YM10 membrane (Amicon) before being used to inhibit the sera.

All wells of a 96-well ELISA plate were blocked with 2% BSA/PBS for 30 minutes at 20°C and then washed in PBS. 90µl of conditioned medium was added to the initial wells of four columns and double diluted down the columns in medium from unstimulated cells. Each column had 10µl of one of the four dilutions of sera added to each well. All the sera were now at their previously determined dilutions. Each medium and serum were incubated together for 60

minutes at 20°C. A similar set of ELISA plates had been coated with appropriate peptide as described for the anti-peptide ELISA. After these wells were washed, 75µl of the serum/medium from each well was added to the corresponding peptide-coated well, and the rest of the ELISA followed the protocol of section 2.2.2.

2.2.6 PURIFICATION OF RABBIT IMMUNOGLOBULIN.

Antibodies were precipitated from serum by the addition of 0.313g/ml solid ammonium sulphate at 20°C (50% ammonium sulphate final concentration). The serum was slowly stirred overnight at 4°C. The precipitate was collected by centrifugation at 3000xg for 30 minutes in a MSE high-speed centrifuge. The precipitate was dissolved in PBS and dialysed for 48 hours against three changes of 10mM sodium phosphate buffer, pH8.0. The dialysate was fractionated on a DEAE-cellulose (Whatman DE52) column previously equilibrated with 10mM sodium phosphate buffer, pH8.0. The protein-containing fractions were tested by the direct ELISA for anti-peptide activity and positive fractions pooled. Purity of the IgG was assessed by immunoelectrophoresis.

2.2.7 IMMUNOELECTROPHORESIS OF PURIFIED RABBIT IMMUNOGLOBULIN G.

Purified immunoglobulin G was electrophoresed through 1% agarose in 0.5X veronal buffer (see Appendix 2) at 40V for 1 hour. The parallel well was filled with a 1/4 dilution of goat anti-rabbit whole serum (Sigma) in PBS. The gel was incubated for 16 hours at 4°C in a moist atmosphere. The gel was then washed for 48 hours in two changes of PBS before staining with 0.025% Coomassie Brilliant

Blue R (Sigma) in a 50:45:5, methanol:water:acetic acid mix for 30 minutes. The gel was destained in a 87:8:5, water:acetic acid:methanol mix.

2.2.8 BIOTINYLATION OF PURIFIED RABBIT IMMUNOGLOBULIN G.

Purified IgG from the rabbits immunized with peptides was dialysed against 0.1M sodium hydrogen carbonate, pH 8.4, for 24 hours with three changes of buffer. The protein concentration of the dialysate was measured by spectrophotometry at 280nm. 4mg of each IgG sample was diluted with buffer to a final concentration of 1mg/ml. Biotin-amidocaproate N-hydroxysuccinimide ester (Sigma) was added to the IgG at 75µg biotin/mg IgG. The mix was rotated for 5 hours at 20°C and then dialysed against PBS/0.05% sodium azide overnight.

2.2.9 DEVELOPMENT OF SANDWICH ELISA FOR OVINE IFN-g.

The biotinylated polyclonal rabbit sera were used to attempt to develop a sandwich ELISA for interferon-gamma by capturing the interferon with one antibody and detecting with a second biotinylated antibody.

Each assay used three 96-well polystyrene ELISA plates. Each plate was coated with 75µl of the previously purified immunoglobulins (section 2.2.6) at 50µg/ml (280nM) in carbonate buffer, pH9.0, for 16 hours at 20°C. Columns 1-4 were coated with R144/5 immunoglobulin (peptide 42-68), columns 5-8 with R146/7 immunoglobulin (peptide 112-137), and columns 9-12 with R155/6 immunoglobulin (peptide 1-23). The wells were washed with PBS/azide + 0.05% Tween 20. Wells were blocked with 2% BSA/PBS/azide for 30 minutes at

20°C. 75µl of neat conditioned media was applied to columns 1-2,5-6, and 9-10, whilst control medium was applied to the remaining columns of wells. Thus each coating antibody was tested with interferon-containing medium and negative medium. Media were left in wells for 24 hours at 4°C and then washed out three times. 75µl of one biotinylated antibody was added to each well of one plate for 3 hours at 4°C. Row A of the plate was incubated with 5µg/ml of antibody, and each row down was a double dilution of this, i.e. Row B - 2.5µg/ml, Row C - 1.25µg/ml, etc. Each of the three plates was incubated with either R144/5 biotin, R146/7 biotin, or R155/6 biotin. The wells were washed as before and then incubated with 75µl of 1/4000 Extravidin Alkaline Phosphatase (Sigma) for 90minutes at 4°C. After a final round of washes the plates were developed with p-nitrophenyl phosphate disodium in 0.1M glycine, 0.05N NaOH, 0.5mM $ZnCl_2$, and 0.5mM $MgCl_2$ for 60 minutes at 37°C. The optical density of the wells was determined at 405nm with a Titertek Multiskan plate reader. The experiment was performed three times.

2.2.10 MONOCLONAL ANTIBODY HB 8291.

A monoclonal antibody, HB 8291, was obtained from the American Type Culture Collection. HB 8291 was originally generated in a fusion of the BALB/c myeloma cell line SP2/0-Ag14 [HRPT-] with spleen cells from a BALB/c mouse immunized with a 19-mer synthetic polypeptide identical to amino acid sequence 128-145 of human interferon-gamma, coupled to keyhole limpet haemocyanin (Altrock BW, 1986). The following is the sequence of the peptide.

NH_2-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Ser-Glu-Met-
-Leu-Phe-Arg-Gly-Arg-Arg-Ala-Ser-Glu-COOH

The monoclonal antibody HB 8291 is a mouse IgG1 which recognises the immunizing peptide, native IFN-g, recombinant IFN-g, and denatured IFN-g. It does not neutralize the antiviral activity. It was produced in both murine ascites and tissue culture cell supernatant. Immunoglobulin was purified from ascitic fluid on a protein G-sepharose chromatography column (Pharmacia). Binding buffer was borate buffered saline (BBS) (see Appendix 1) and elution buffer 2.5M glycine. Purified antibody was biotinylated in an identical fashion to rabbit immunoglobulin.

2.2.11 GENERATION OF RABBIT ANTI-BOVINE INTERFERON-GAMMA POLYCLONAL SERA.

Bovine recombinant IFN-g was donated by Ciba-Geigy. Two 10 week old, male New Zealand White rabbits were immunized subcutaneously once with 25µg of interferon in complete Freund's adjuvant (CFA), followed by 25µg of interferon in PBS intravenously two weeks later. One week later the rabbits were bled from the ear vein and the antibody titre assayed. Initial assay was by ELISA against two of the bovine interferon-gamma peptides (1-23 and 112-137) as previously described for testing the anti-peptide antisera. Antisera were purified by 50% ammonium sulphate precipitation and DEAE-cellulose chromatography as previously described for the rabbit anti-peptide sera. Immunoglobulin was biotinylated as previously described.

2.2.11.1 WESTERN BLOT ASSESSMENT OF RABBIT ANTISERA AND MOUSE MONOCLONAL ANTIBODY HB 8291 AGAINST RECOMBINANT PROTEIN.

The sera and monoclonal antibody were tested in a western blot against recombinant protein. Recombinant interferon-gamma preparation was boiled in loading buffer (Appendix 1) to denature the protein and 500ng loaded onto each track of a BIORAD minigel system. The preparation was fractionated on a 12% SDS-PAGE gel at 200V for 30 minutes in running buffer. The proteins were transfered electrophoretically to nitrocellulose membrane (Hybond C, Amersham, Aylesbury, Bucks) using a BIORAD Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell and transfer buffer at 120mA for 40 minutes (Bjerrum & Schafer-Nielsen, 1986). Following transfer, marker tracks were stained with 0.5% amido black (BDH, Poole, Dorset) and the remainder of the membrane cut into strips, blocked with 5% dried cow's milk (Marvel, Cadbury's), and incubated with anti-rabbit or anti-mouse Ig alkaline phosphatase. Blots were washed before development in alkaline phosphatase substrate (Appendix 1).

2.2.12 SANDWICH ELISA USING RABBIT ANTISERA AND MOUSE MONOCLONAL.

Sandwich ELISAs similar to those described in section 2.2.9 were performed using either R167/8 or HB 8291 as the coating antibody at 20µg/ml in PBS, and biotinylated R167/8 or HB 8291 as the detector antibody.

2.2.13 FUSION PROTOCOL FOR GENERATION OF ANTI-INTERFERON-GAMMA MONOCLONAL ANTIBODIES.

6 week old female BALB/c mice from the animal house at R(D)SVS, University of Edinburgh, were immunized with recombinant bovine interferon-gamma on several occasions.

DATE	PROCEDURE		
13/9/90	2.5µg bov rIFN-g	CFA	SUBCUT
1/10/90	5.0µg bov rIFN-g	IFA	SUBCUT
6/2/91	TEST BLEED: MOUSE 2 1/800; MOUSE 1 1/100		
15/2/91	2.0µg bov rIFN-g	PBS	I/P MOUSE 2
19/2/91	FUSION MOUSE 2		
4/3/91	2.0µg bov rIFN-g	PBS	I/P
14/3/91	TEST BLEED: MOUSE 1 1/3200		
30/4/91	2.5µg bov rIFN-g	PBS	I/V MOUSE 1
3/5/91	FUSION MOUSE 1		

Fusions were carried out essentially as described by Köhler and Milstein (1975). A mouse myeloma line was used, NS0/1 (Galfre & Milstein, 1981), which had been selected for non-secretion and non-expression of immunoglobulin heavy and light chains, and also contained a mutation of the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene. NS0/1 were grown in RPMI 1640 media with 15% foetal calf serum, 100mM sodium pyruvate, and 100mM 2-glutamine (Gibco, Scotland). Fusion of cells was achieved with 50% polyethylene glycol in PBS (PEG 1500, Boehringer Mannheim, Gmbh, W. Germany). Fused cells were plated out in 96-well plates at 2 x 10^5 cells per well in 100µl of selecting media. Each well also contained 100µl of mixed thymocyte medium (MTM) to provide growth

factors for the fused cells. Selecting medium was RPMI 1640 with the addition of 15% foetal calf serum, L-glutamine, sodium pyruvate and HAT (Sigma). The HAT supplement gave final concentrations of 1 x 10^{-4}M hypoxanthine, 4 x 10^{-7}M aminopterin and 1.6 x 10^{-5}M thymidine.

Tissue culture supernatants from confluent layers of hybridoma cells were tested in an interferon-gamma specific sandwich ELISA. This ELISA was designed using rabbit anti-bovine interferon-gamma IgG as the capture antibody. The IgG was purified by 50% ammonium sulphate precipitation and DEAE-cellulose chromatography as performed previously. 50µl of antibody was applied to the ELISA plate at 20µg/ml in 0.1M carbonate buffer, pH 9.5, for 3 hours at 4°C. Excess antibody was washed off and the wells were blocked with 2% BSA/ PBS for 3 hours at 4°C. 50µl bovine recombinant interferon-gamma was applied at 200ng/ml in 2% BSA/ PBS for 16 hours at 4°C. The excess was washed off with ice-cold PBS five times, one minute each time. Test supernatants were then applied and incubated for 2 hours at 4°C. The plates were again washed. A 1/100 dilution of anti-mouse Ig Horseradish Peroxidase (SAPU, Carluke, Scotland) was applied for 2 hours at 4°C. The anti-rabbit IgG reactive component had previously been removed by affinity chromatography on a rabbit IgG-sepharose column. The plate was washed prior to development using 20mg o-phenylenediamine (Sigma) in 0.05M Na_2HPO_4, 0.024M citric acid, and 0.005% H_2O_2. The reaction was stopped with 50µl of 2M sulphuric acid, and the results read on a Titertek Multiskan plate reader at an OD of 495nm.

2.3 RESULTS.

2.3.1 ANALYSIS OF BOVINE INTERFERON-GAMMA TO IDENTIFY PEPTIDES OF POTENTIAL USE.

The availability of the human, murine, rat and bovine IFN-g sequences allowed identification of conserved and variable regions of the IFN-g molecules. Figure 2 shows the four sequences aligned and the conserved regions are marked.

The amino acid sequence of bovine IFN-g (Cerretti et al, 1986) was examined for secondary structure (CF and GOR), hydropathicity (KD) and antigenicity (Jameson-Wolf) using the GCG7 computer program from the University of Wisconsin. The plotted results are shown in Figure 3.

A plot indicating a hydrophilicity and hydrophobicity of more than 1.3 according to the Kyte-Doolittle algorithm (Kyte & Doolittle, 1982) was generated by the GCG7 computer program and is shown (Figure 4).

Potential T cell epitopes may be predicted using the Rothbard algorithm (Rothbard & Taylor, 1988). The four amino acid motif (charged or glycine-/-hydrophobic-/- hydrophobic-/-polar or glycine or charged) and the five amino acid motif (charged or glycine-/-hydrophobic-/-hydrophobic-/-hydrophobic or proline-/-polar or glycine or charged) were used to predict the presence of T cell epitopes with bovine interferon-gamma (Figure 5).

The peptides synthesized are depicted within the bovine sequence (Figure 6).

2.3.2 TESTING OF ANTIPEPTIDE ANTISERA.

The peptides synthesized were 1-23 amino-terminal, 112-137 carboxyl-terminal, and a central region 42-68. The rabbits were immunized with uncoupled peptide. All the rabbits developed antibodies reactive to the peptides. The antisera could be diluted to a high degree before reactivity disappeared (Figure 7). A positive response in the ELISA was defined as being twice the average value generated by normal rabbit serum in the assay. The titre is thus the last dilution at which a positive response could be determined.

RABBIT	PEPTIDE	TITRE
R144	42-68	1/2560
R145	42-68	1/5120
R146	112-137	1/5120
R147	112-137	1/5120
R155	1-23	1/3200
R156	1-23	1/1600

2.3.3 DIRECT ELISAs AGAINST CONDITIONED MEDIA.

ELISA plates were coated with crude preparations of ovine interferon-gamma from Concanavalin A-stimulated lymphocytes (conditioned medium). The conditioned medium had been tested for the presence of interferon by the antiviral assay (Entrican et al, 1989). Some preparations were further concentrated by

ultrafiltration through a PM10 membrane (Amicon), which would retain all molecules with molecular weight more than 10 kDa (i.e. including IFN-g). In all the assays performed preimmune rabbit serum was as reactive to conditioned medium as the antipeptide sera, and all sera were unreactive to control medium containing no IFN-g (Figure 8). Sera at low dilutions bound non-specifically to the ELISA wells in which conditioned medium had been included.

2.3.4 DIRECT ELISA USING BOVINE CONDITIONED MEDIA.

Conditioned medium was generated using bovine lymphocytes as the IFN-g producing cells. This medium tested positive in the antiviral bioassay. The ELISA procedure was as before. There was no significant difference between the reactivity of the antipeptide sera and preimmune sera at any dilution (Figure 9). Interestingly none of the sera, even at low dilutions, gave the high results found when ovine conditioned medium was used.
The question of reactivity of the antisera with ovine or bovine IFN-g remained unresolved.

2.3.5 INHIBITION ELISA FOR OVINE INTERFERON-GAMMA.

The rabbit sera certainly possessed antipeptide activity and this may be able to detect native IFN-g, but a more sensitive and specific assay was thought to be required which did not rely on the binding of conditioned medium to a plate. The inhibition ELISA was developed as a theoretically more sensitive method. The sera against peptides 42-68 and 112-137 were used in this assay. The sera were used at final dilutions which lay on the linear part of the

titration curve against the specific peptide, and were hence present in limiting amounts (Figure 7). Sera from rabbit R144 (42-68) was used at 1/640, 1/1280, 1/2560 and 1/5120. Sera from the other three rabbits were all used at 1/2560, 1/5120, 1/10240 and 1/20480. A variety of conditioned media were used in the assay, including the Amicon-concentrated material previously described.

No consistent difference was observed between the signal generated after inhibition by neat medium and diluted medium, and that generated after negative control medium was included. This was true for all four rabbit sera at each of the dilutions. A representative graph for one of the sera is included (Figure 10).

2.3.6 PURIFICATION OF RABBIT ANTI-PEPTIDE IMMUNOGLOBULIN G.

Rabbit serum against peptide 42-68 (R144/5) was combined and then purified for IgG by chromatography. Sera against the other peptides were separately purified and subsequently assayed by the direct peptide ELISA. The pre- and post-purification reactivity to peptide were tested in a similar ELISA. The recovery rate of anti-peptide IgG is shown below.

SERA		TITRE 1/x	VOLUME ml	TITRE x VOL
R144/5	PRE	2560	24	61440
	POST	640	75	48000
R146/7	PRE	12800	30	384000
	POST	3200	51	163200
R155/6	PRE	5120	16	81920
	POST	640	63	40320

The above results show that R144/5 (42-68) had a recovery of 78%, R146/7 (112-137) had a recovery of 43% and R155/6 (1-23) had a recovery of 49%. The "lost" antibody may be immunoglobulin M which binds to the DEAE-cellulose column, or it may be immunoglobulin G which has bound to the column in a non-specific manner. Immunoelectrophoresis was performed to analyse the purity of the protein fraction recovered from the DEAE-column. All three preparations contained a single major band corresponding to IgG.

The anti-peptide IgG fractions were used in a direct ELISA against conditioned medium (section 2.2.3). For all three preparations, there was a higher reading against conditioned medium than against control medium at all or most of the IgG dilutions. The difference between the reactions was however not great enough to allow good discrimination between conditioned and control media. Unfortunately preimmune rabbit serum reacted identically in the ELISA. To avoid this problem a sandwich ELISA was developed using the IgG fractions as capture antibodies and the biotinylated IgG as detector antibodies.

2.3.7 DEVELOPMENT OF A SANDWICH ELISA FOR OVINE INTERFERON-GAMMA.

The sandwich ELISA was used with three different conditioned media preparations, including the bovine conditioned medium, as the reactivity of the antibodies to ovine interferon-gamma was still not resolved. In all the ELISAs, wells that included conditioned medium had higher readings than those that included control medium. The readings from the wells containing the conditioned medium were at most 10% higher than the readings from the control wells (Figure 12). This difference was insufficient to allow development of the sandwich ELISA in its current form.

The coating antibody had a profound effect on the result obtained. Wells which contained R144/5 (42-68) as coating antibody recorded results approximately one third that of wells coated with R155/6 (1-23). Wells coated with R146/7 (112-137) were approximately two thirds the value of R155/6 (1-23). This was consistent for all three detector antibodies and at all concentrations of coating antibody tested (0.15-10µg/ml).

2.3.8 ASSESSMENT OF THE PROTEIN CONTENT OF THE RECOMBINANT BOVINE INTERFERON-GAMMA PREPARATION.

On an SDS-PAGE gel stained with Coomassie Blue the preparation contained a major and a minor protein band with estimated molecular weights of 17,000 and 18,000 respectively (Figure 13). The mature recombinant protein is described as being of 16,858 molecular weight (Cerretti et al, 1986) which would correspond with the major band. The origin of the minor band was unknown. The preparation was examined using the antipeptide sera.

2.3.9 GENERATION OF RABBIT ANTI-BOVINE INTERFERON-GAMMA POLYCLONAL SERA.

Rabbit polyclonal antisera to the recombinant bovine IFN-g were generated as described (section 2.2.11). The sera from rabbits R167 and R168 were assessed for antibodies to the rIFN-g by a direct ELISA against peptides 112-137 and 1-23. Both rabbits were positive when tested against peptide 112-137. R167 was positive at 1/1600 dilution, and R168 was positive at 1/100 dilution. Both rabbits were negative when the sera were tested against peptide 1-23. In each ELISA the specific anti-peptide serum was highly positive against its peptide and negative against a tuberculin peptide. This initial ELISA not only measured an aspect of the antibody response to the bovine rIFN-g, i.e. recognition of peptide epitopes, but also provided good evidence that the recombinant protein supplied by Ciba-Geigy was bovine IFN-g. The IFN-g was tested for antiviral activity in the Semliki Forest virus/ ST6 ovine cell line assay and was highly potent in this test. The R167 antisera neutralized this effect to a titre of 1/1000 (G. Entrican, personal communication).

2.3.10 DEMONSTRATION THAT ANTIPEPTIDE SERA AND MONOCLONAL ANTIBODY HB 8291 REACT WITH BOVINE INTERFERON-GAMMA.

The hybridoma cell line HB 8291 (see section 2.2.10) was generated to a human carboxyl-terminal peptide and its reactivity to bovine or ovine interferon-gamma was unknown.

All the rabbit antisera (anti-peptide and anti-bovine rIFN-g) and the mouse monoclonal HB 8291 were tested by immunoblotting against recombinant bovine interferon-gamma which had been separated by SDS-

PAGE. The immunoblots are shown in Figures 14 and 15. Preimmune rabbit serum did not react with any proteins on the blot. Figure 14 shows that the rabbits immunized with the amino- and carboxyl-terminal peptides (1-23 and 112-137), and those immunized with the bovine rIFN-g, all reacted with a protein of molecular weight 17000. A faint band was visible on the original blot just above the major band at approximately 18000 MW. This was interpreted as being the 18000 MW minor band visible on the coomassie stained SDS-PAGE of the recombinant preparation. Figure 15 shows that the monoclonal antibody HB 8291 reacts with a broad protein band of approximately 18000 MW indicating that the larger band contains the carboxyl-terminal sequence and that the smaller band may represent a degradation product not containing the critical epitope for HB 8291. The reaction of the rabbit anti-carboxyl peptide antisera indicates that the 17000 MW protein still contains epitopes recognised by the poyclonal sera, and hence part or all of the peptide 112-137 is still present in the 17000 MW protein.

2.3.11 SANDWICH ELISA USING R167/8 AND HB 8291 IMMUNOGLOBULINS.

When R167/8 biotin was used as the detector antibody, rIFN-g was detected in wells coated with either R167/8 or HB 8291 (Figure 17), but not in wells coated with irrelevant antibodies and using the rIFN-g at 500ng/ml. Sensitivity with HB 8291 as the coating antibody was twice that with R167/8. IFN-g was detected down to a concentration of 125ng/ml. The recombinant preparation had a quoted activity of 2.5×10^6 units/mg (Ciba-Geigy), which gives a maximal sensitivity of 625 units/ml in this ELISA system.

When biotinylated HB 8291 was used as the detector antibody, IFN-g was not detected even at high IFN-g concentrations. The biotinylation was not at fault as blots of the antibody could be easily detected by an alkaline phosphatase method. The monoclonal antibody HB 8291 could be detected on a dot blot down to a level of 25pg.

2.3.12 RESULTS FROM HYBRIDOMA EXPERIMENTS.

Mice primed for a fusion with bovine rIFN-g were tested using the modified sandwich ELISA and one serum found positive was tested to assess the sensitivity of this system. A rIFN-g concentration of 62ng/ml was detectable, the best detection thus far in any of the sandwich ELISAs devised.

Three mouse hybridoma fusions were performed using this sandwich ELISA as the screening method. No monoclonal antibodies were detected. The number of total hybridomas grown was less than expected. This problem affected all hybridoma work performed within the Department of Veterinary Pathology at that time. It was subsequently determined to be the result of foetal calf serum which was capable of supporting established hybridoma lines but not the requirements of recently fused cells.

2.4 DISCUSSION.

Peptides used for the generation of antisera should represent regions of the molecule which are likely to be exposed on the surface of the molecule, and hence these regions are likely to be antibody-binding. Antibody production against peptides can also depend upon the presence of T cell epitopes within the peptide sequence, especially if uncoupled peptides are to be used. Historically, peptides have been chosen on the basis of the predicted secondary structure of the protein. Secondary structure predictions were initially based on the potential of amino acids to be present in α-helices, β-sheets or turns (Chou & Fasman, 1974b). Values for each amino acid were derived from X-ray crystallography (Chou & Fasman, 1974a). Helix or β-sheet initiation sites were identified where sufficient of each type of amino acid were clustered, and these conformations were deemed to continue until broken by a sequence of tetrapeptides which act as "breakers". A similar method was used by Garnier et al (1978) to derive regions of helix, sheet or turn. From Figure 3 it can be seen that the Chou-Fasman prediction of secondary structure (Chou & Fasman, 1974b) is for seven alpha helices and four beta sheet regions. The Garnier-Osguthorpe-Robson prediction of secondary structure (Garnier et al, 1978) is similar to that of Chou-Fasman but they predict that the second alpha helix goes from amino acid 30 to amino acid 48, whilst Chou-Fasman predict this helix ends by amino acid 39. Both CF and GOR predictions show an absence of alpha helix or beta sheet structure for the carboxyl twenty amino acids.

Later, exposed regions of proteins were located by plotting the hydropathic characteristics of the amino acids using a 6-10 amino acid window on the protein sequence of interest (Hopp & Woods, 1981;

Kyte & Doolittle, 1982). This approach was based on the concept that highly charged clusters of amino acids are almost always exposed to an aqueous environment and strongly hydrophobic clusters are usually buried in the interior of the protein. However, this method is poor at predicting whether regions of proteins with intermediate hydropathicity are exposed on the surface or hidden inside the protein. The predicted hydropathicity of bovine IFN-g was thus taken into consideration. The plot for bovine IFN-g is shown in Figure 3 as "KD hydrophilicity". Both the amino and carboxyl-terminal regions are predicted to be hydrophilic, but the central 44-68 region is hydrophobic. The surface probability shows two main regions predicted to be exposed to the exterior, at amino acid residues 37-49 and 125-135.

A similar type of prediction (Emini et al, 1985) can be made using values for the "solvent accessibility" of the individual amino acids (Janin et al, 1978). Amino acids with high "solvent accessibilities" are likely to be on the surface of the molecule. This method produces a "surface probability" plot with different values from the hydropathicity plots, but suffers from the same limitations. The plot for bovine IFN-g is shown in Figure 3 as "Surface Probability".

Highly mobile regions of proteins have been found to correlate with known antigenic determinants (Atassi, 1984; Tanier et al, 1984; Westhof et al, 1984) and consequently these flexible regions may be plotted, using values of atomic oscillation based on x-ray diffraction (Karpus & Schulz, 1985). Although these plots are informative they do not predict all antigenic peaks.
Antigenic predictions have usually utilized only one of the above methods, but a more recent method has been described which integrates all the previous methods to give an indication of

potentially exposed surface peaks of a protein and hence regions likely to be useful for the production of anti-peptide antibodies reactive to native protein (Jameson & Wolf, 1988). The plot for bovine IFN-g is shown in Figure 3 as "Jameson-Wolf (Antigenic Index)". It can be seen that this predicts high antigenicity for the amino- and carboxy-termini, but a region of low antigenicity for the central 42-68 peptide. This latter probably reflects the hydrophobic prediction for this region.

Although proteins with the same function in different species may have differences in amino acid sequence they will commonly retain the same functional regions. Consequently in these regions the amino acids are more likely to be conserved. Comparison of the IFN-g sequences of different species may allow prediction of the ovine IFN-g sequence. This feature is shown in Figure 2. The region with highest conservation of residues is the carboxyl region 109-135, where 20 of 27 residues are identical in three of the four sequences. Region 44-68 has a high degree of identity, with 18 of 25 residues the same in three of the four sequences. A region with less identity is 1-26 (12 of 26 residues).

A combination of the above methods determined which peptides were synthesized for the production of antisera reactive with native bovine IFN-g. The two regions with the highest degree of homology are in the central region, 42-67, and the carboxyl terminus, 112-135. Two-thirds of the amino acids of each region had sequence identity in three of the four sequences assessed. These sequences formed the basis for two of the peptides synthesized. The surface probability plot indicates the two regions likely to be found at the surface of the IFN-g molecule are 37-49 and 125-135. Two regions

predicted to be of likely high antigenicity by the Jameson-Wolf plot are the amino (1-27) and carboxyl (125-146) termini. Regions 35-47 and 84-97 are also thought to have a high antigenic index.

Peptide sequences which bind to mouse T cell receptors may be predicted by the method of Rothbard & Taylor (1988). This may indicate the likelihood of B lymphocytes reactive with uncoupled peptides receiving T lymphocyte help. Bovine IFN-g was examined in Figure 5 and the carboxyl-terminal, 109-135, and the central region, 44-68, contain two Rothbard 4 amino acid motifs, whilst the amino-terminal region, 1-23, contains one predicted epitope. These regions should thus be able to stimulate T cell responses if used without carrier proteins.

The anti-peptide sera generated against the amino- and carboxyl-termini of bovine IFN-g were reactive with denatured recombinant bovine IFN-g, but none of them seemed to react strongly with native or denatured ovine IFN-g when tested by ELISA, western blot or viral neutralization assay. The first two test methods suffer from the disadvantage that they rely on sufficient IFN-g being bound to an ELISA plate or membrane for subsequent detection by antibodies in the antisera. In the absence of large amounts of purified IFN-g protein this can be difficult to achieve convincingly. Consequently the absence of a reaction by these methods must be interpreted with caution. It may only reflect that too little IFN-g was bound to the ELISA plate or nitrocellulose membrane, which is difficult to confirm without a positive control antibody. A neutralization assay depends on the activity of the IFN-g preparation for its readout system (i.e. the presence of IFN is detected regardless of the presence of antibodies) but this assay only detects antibodies which disrupt the molecule-receptor interaction. It will not detect

antibodies which bind to native protein but fail to block this interaction, and these may be the predominant antibodies formed to peptides, depending on the location of the peptide regions in the native molecule.

The inhibition ELISA was an attempt to increase the sensitivity of the ELISA techniques being used. It relied upon native IFN-g blocking binding of antibodies to wells coated with peptide. The negative results can be explained by considering that the antipeptide sera is reactive to a linear sequence which may not exist as such in the native molecule. A high percentage of the antibodies would be reactive only to the peptide, with a remaining low percentage also being reactive to native interferon, possibly with low affinity. The inhibition ELISA relies on a significant percentage of the antipeptide antibodies being reactive to the native IFN. These antibodies are the only ones in which blocking by native IFN is possible, and hence a decreased ELISA reading will register. A low percentage change in ELISA reading would not be detectable in this ELISA system given the coefficient of variation inherent in the ELISA. It is unlikely that the antipeptide sera would contain enough antibodies reactive to native IFN-g to enable the assay to work.

The detection of bovine rIFN-g by HB 8291 on a western blot may be explained by the linear nature of the IFN-g molecule beyond amino acid 125 and the high degree of identity between human and bovine IFN-g in this region. The human peptide sequence used to generate monoclonal antibody HB 8291 is very similar to the carboxyl-terminal sequence of bovine IFN-g. If the three amino acids (128-130) at the amino end of the peptide are ignored, the two sequences share 14 of 16 residues, and one of the differences is at the extreme carboxyl

end. Antibodies to a linear peptide 128-146 of human IFN-g are likely to react with native IFN-g because the three dimensional structure of human IFN-g has been shown to be linear from amino acid 125, and bovine IFN-g is predicted to be similar (Ealick et al, 1991). This level of identity and linear nature should allow some cross-reactivity of the monoclonal antibody with bovine IFN-g. On a western blot HB 8291 only detected bovine rIFN-g which was present at more than 100ng per track. This suggests that it had low affinity for bovine rIFN-g and in the patent application HB 8291 is described as having higher affinity for the immunizing peptide than for native human IFN-g (Altrock, 1986). The sandwich ELISA using HB 8291 as coating antibody detected IFN-g down to 125ng/ml in 50µl, i.e. 6.25ng. This level of detection however remains several orders of magnitude below that required. Most commercial ELISAs for human or murine IFN-g are based on monoclonal antibodies used in a sandwich ELISA technique and can detect as low as 10pg.

The unreactivity of sera generated against peptide 42-68 suggest that the central peptide site is relatively inaccessible or that ovine IFN-g varies from bovine IFN-g in this region. Reference to the cloned sequences (described in chapter 4) shows the amino acid sequence is only different at one amino acid, where a leucine replaces a phenylalanine, a relatively conserved substitution. This may be sufficient difference to affect binding of a monoclonal antibody, but within a polyclonal antisera to a peptide there should be antibodies reactive to short amino acid sequences not containing this amino acid, or affected conformationally by it. The more likely reason is therefore that the site is inaccessible to antibodies. This is supported by the crystallographic structure of human IFN-g. The tertiary structure of bovine IFN-g was predicted from this structure and is shown in Figure 18. The amino- and

carboxyl-termini (helices A and F; 7-18 and 107-124 respectively) are on the molecule surface, while the central region (helix C; 44-64) is buried in the core of the dimer. Unfortunately this tertiary structure was not published until 1991, more than a year after the choice of the peptides used here for immunization.

The antisera generated against the amino- and carboxyl-terminal peptides are reactive with denatured bovine rIFN-g by western blot, but appear to be unreactive with native IFN-g by sandwich ELISA, and this adds to the evidence that the regions the antisera bind to are not linear in the native molecule, but have a degree of secondary structure. Reference to the three-dimensional structure of human IFN-g (Ealick et al, 1991) reveals that amino acids 7-18 and 107-124 are within alpha helical regions of the molecule. Antisera to a linear peptide 1-23 of bovine IFN-g are consequently unlikely to bind region 7-18 in native IFN-g but may bind this region when its secondary structure is removed by detergent denaturation. Similarly antisera to linear peptide 112-137 are unlikely to bind native IFN-g. Antibodies within this preparation may bind native IFN-g if they are reactive with the region 125-137 which is linear in the native molecule.

Antibodies to a peptide should have a high degree of specificity, but it may be difficult to obtain a good antipeptide serum which will react well with native IFN molecules. The difficulty in showing that the anti-peptide sera bind to native IFN-g has been experienced with rabbit sera to human peptides 1-20, 1-59, 24-59, 36-59 and 87-96 (Leist et al, 1985). Only two of 14 rabbits produced antibodies which recognised native IFN; one to unlinked peptide 1-59 and the other to unlinked peptide 24-59. Leist et al concluded that it was difficult to obtain antisera to peptides which

reacted well with native IFN molecules, and that hybridoma technology was a better method of obtaining specific high-affinity antibodies to IFN if a suitable immunogen is available.

Antibodies to peptides react primarily with linear epitopes which may not be present in the secondary and tertiary structure of the complete protein. Only five epitopes have been fully characterized by X-ray diffraction of antibody-antigen complexes (Amit et al, 1986; Sheriff et al, 1987; Padlan et al, 1989; Tulip et al, 1990). All five epitopes were of the discontinuous type comprising 15-22 amino acids split between several surface loops. Laver et al (1990) commented that anti-peptide antibodies that seem to bind native protein were most likely binding to denatured protein within the protein preparation, and that the reactivity of sera generated against native protein which binds to peptides is likely to be the result of contamination of the immunizing protein preparation with denatured protein. This theory may partly explain the reactivity of the anti-bovine rIFN-g sera (R167/8) with peptide 112-137 by ELISA as the presence of denatured IFN-g in the preparation cannot be excluded. Another explanation may lie in that amino acid sequence 125-137 is not predicted to be in helix F of the tertiary structure but probably extends away from the dimer and may be linear (Ealick et al, 1991). Antibodies against this linear region could therefore strongly bind a linear peptide.

There are many reports of the successful use of peptides as immunogens, but most of these use peptides coupled to large carrier proteins such as ovalbumin. The carrier protein is thought to serve two main purposes. The coupled peptides are more likely to assume conformations found in the native whole protein than are free peptides. Antibodies which bind these peptides are more likely to

bind native IFN-g. The carrier protein also provides the basis for the generation of immune help from T cells for the peptide-reactive B cells. T-cell recognition depends upon processing of the antigen and subsequent presentation of peptides with MHC class II molecules. T-cell recognition tends to be limited to a small number of immunodominant sites within the protein. Overlapping epitopes containing these immunodominant sites are seen by different T cells with different receptors. Large proteins such as ovalbumin are likely to contain several T-cell epitopes and will consequently stimulate helper T cells with a variety of T cell receptors. Algorithms have been devised to predict the sites of T cell epitopes within proteins. Berzofsky et al (1987) proposed that T-cell immunodominant sites tend to be amphipathic structures, usually α-helices but occasionally β conformations with turn and coil propensities. Rothbard & Taylor (1988) observed a 4 or 5 amino acid primary sequence pattern within many T-cell epitopes. This pattern is consistent with one turn of an amphipathic helix. Indeed Berzofsky et al (1987) summarized the requirements for a T cell epitope as the "potential to form stable amphipathic secondary structures". The structure of the class II histocompatibility molecules have been derived (Brown et al, 1988) from the crystallographic structure of the class I molecules (Bjorkman et al, 1987). The molecules form a platform of eight antiparllel β-strands topped by two α-helices. A groove between the α-helices provides a peptide-binding site. Identification of endogenous peptides found within class I peptide-binding grooves has revealed peptides in extended conformations (Madden et al, 1991). Studies with peptide-MHC class II interaction suggest that the bound state of the peptides is conformationally complex (Brown et al, 1988) and it is unlikely that a predictive model as simple as that used by Barzofsky or Rothbard will be highly accurate. Peptides isolated from class

II molecules have had no discernible sequence motifs (Rudensky et al, 1991). As T cells recognise peptides in association with MHC it may be unnecessary to couple peptides to carrier proteins if the peptides are predicted to assume amphipathic helix conformation or contain a Rothbard motif. All of the peptides used for immunization in this project contained Rothbard motifs, hence predicting that each peptide should contain an immunodominant site. A limitation of this technique is that the epitopes from which the algorithms were devised all react with the MHC from in bred mouse strains. The generation of T cell help in rabbits would require T cell epitopes capable of reacting with rabbit MHC molecules. The generation of IgG antibodies against the free peptides indicates that they were not only immunogenic to B cells but also to T cells. The presence of a successful immune response to the peptides did not however ensure that the resulting antisera reacted with native protein.

The results of this section of work seem to confirm the comments of Leist et al (1985) that peptides are a poor method of generating antisera reactive with native IFN-g. The generation of monoclonal antibodies would seem to be a more efficient and successful method. The availability of recombinant protein material makes this possible. This was the direction in which this section of work would have proceeded, but problems with the hybridoma technology meant that three fusions undertaken did not yield any monoclonal antibodies.

Figure 1.

Diagram to show the three-dimensional structure of human interferon-gamma. Alpha helices are represented by cylinders and the non-helical regions by tubes. The amino (N) and carboxyl (C) ends of the dimer are marked. The alpha helices are labelled A-F corresponding with the α-helices indicated on Figure 18. (Amended from Ealick et al, 1991).

Figure 1.

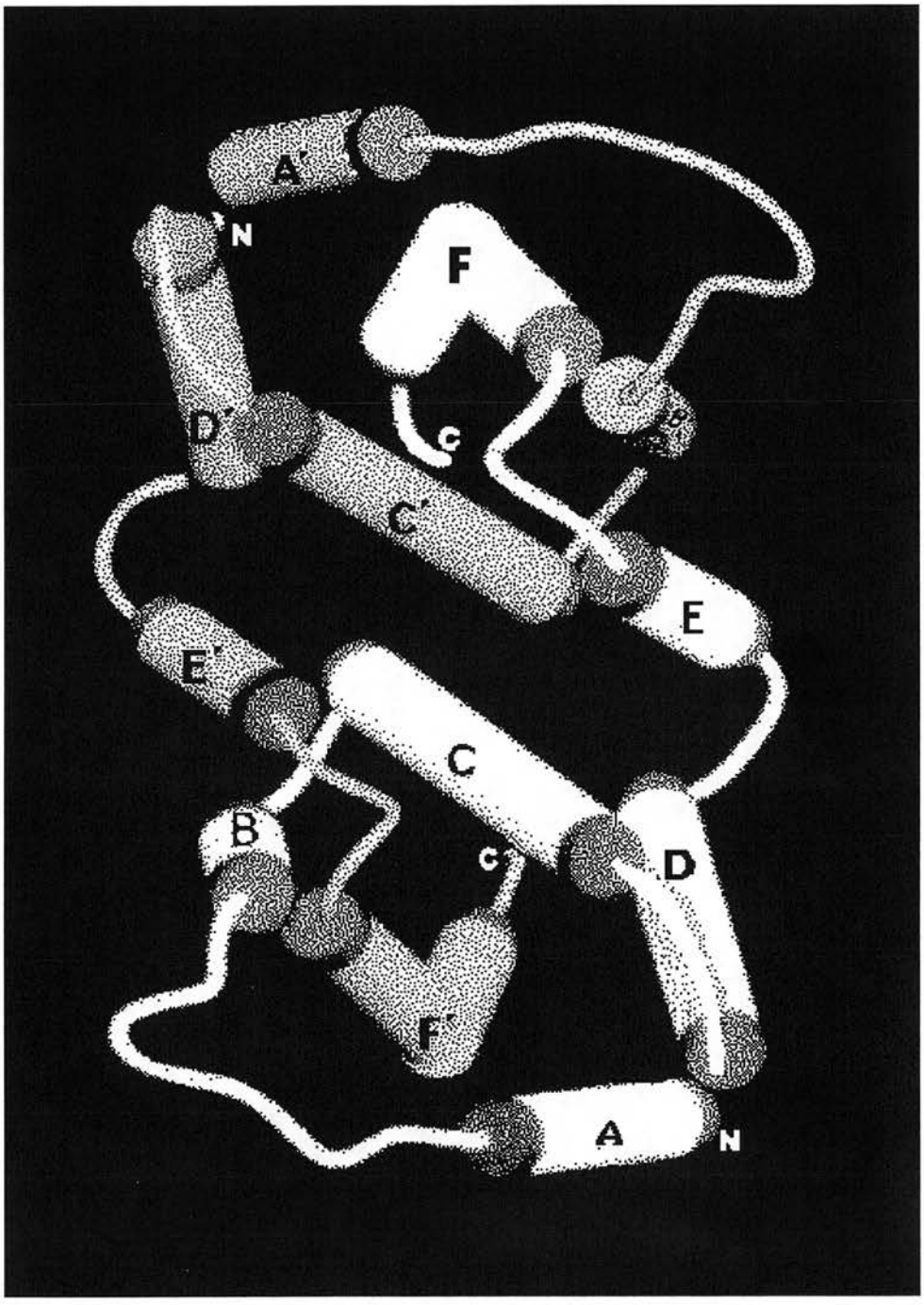


Figure 2.

Amino acid sequences of human (h), bovine (b), murine (m) and rat (r) IFN-g derived from the cDNA sequences. Consensus sequences are marked * when all four sequences are identical, and $\mp$ when three of the four sequences are identical.

Figure 2.

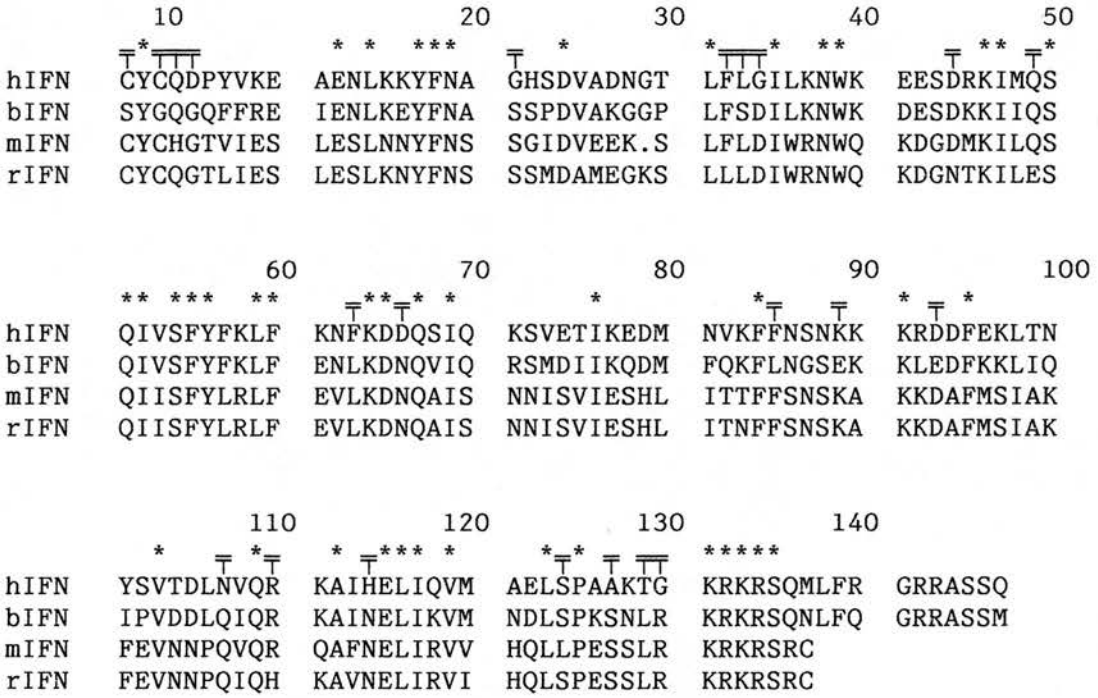

10 20 30 40 50
hIFN CYCQDPYVKE AENLKKYFNA GHSDVADNGT LFLGILKNWK EESDRKIMQS
bIFN SYGQGQFFRE IENLKEYFNA SSPDVAKGGP LFSDILKNWK DESDKKIIQS
mIFN CYCHGTVIES LESLNNYFNS SGIDVEEK.S LFLDIWRNWQ KDGDMKILQS
rIFN CYCQGTLIES LESLKNYFNS SSMDAMEGKS LLLDIWRNWQ KDGNTKILES
60 70 80 90 100
hIFN QIVSFYFKLF KNFKDDQSIQ KSVETIKEDM NVKFFNSNKK KRDDFEKLTN
bIFN QIVSFYFKLF ENLKDNQVIQ RSMDIIKQDM FQKFLNGSEK KLEDFKKLIQ
mIFN QIISFYLRLF EVLKDNQAIS NNISVIESHL ITTFFSNSKA KKDAFMSIAK
rIFN QIISFYLRLF EVLKDNQAIS NNISVIESHL ITNFFSNSKA KKDAFMSIAK
110 120 130 140
hIFN YSVTDLNVQR KAIHELIQVM AELSPAAKTG KRKRSQMLFR GRRASSQ
bIFN IPVDDLQIQR KAINELIKVM NDLSPKSNLR KRKRSQNLFQ GRRASSM
mIFN FEVNNPQVQR QAFNELIRVV HQLLPESSLR KRKRSRC
rIFN FEVNNPQIQH KAVNELIRVI HQLSPESSLR KRKRSRC

Figure 3.

Computer-generated map of bovine IFN-g assessed for secondary structure and antigenicity by the methods of Chou & Fasman (1974) (CF), Garnier et al (1978) (GOR), Kyte & Doolittle (1982) (KD), and Jameson & Wolf (1988). The protein is numbered from 1-166, i.e. includes the 23 amino acid signal peptide, hence the peptides synthesized are now 24-46, 65-91 and 135-160. The plot was generated using the University of Wisconsin GCG7 program.

PLOTSTRUCTURE of: ivbog.p2s September 18, 1991 15:14

PEPTIDESTRUCTURE of: pir1:ivbog.brf Ck: 5502, 23 to: 166

Interferon gamma precursor - Bovine

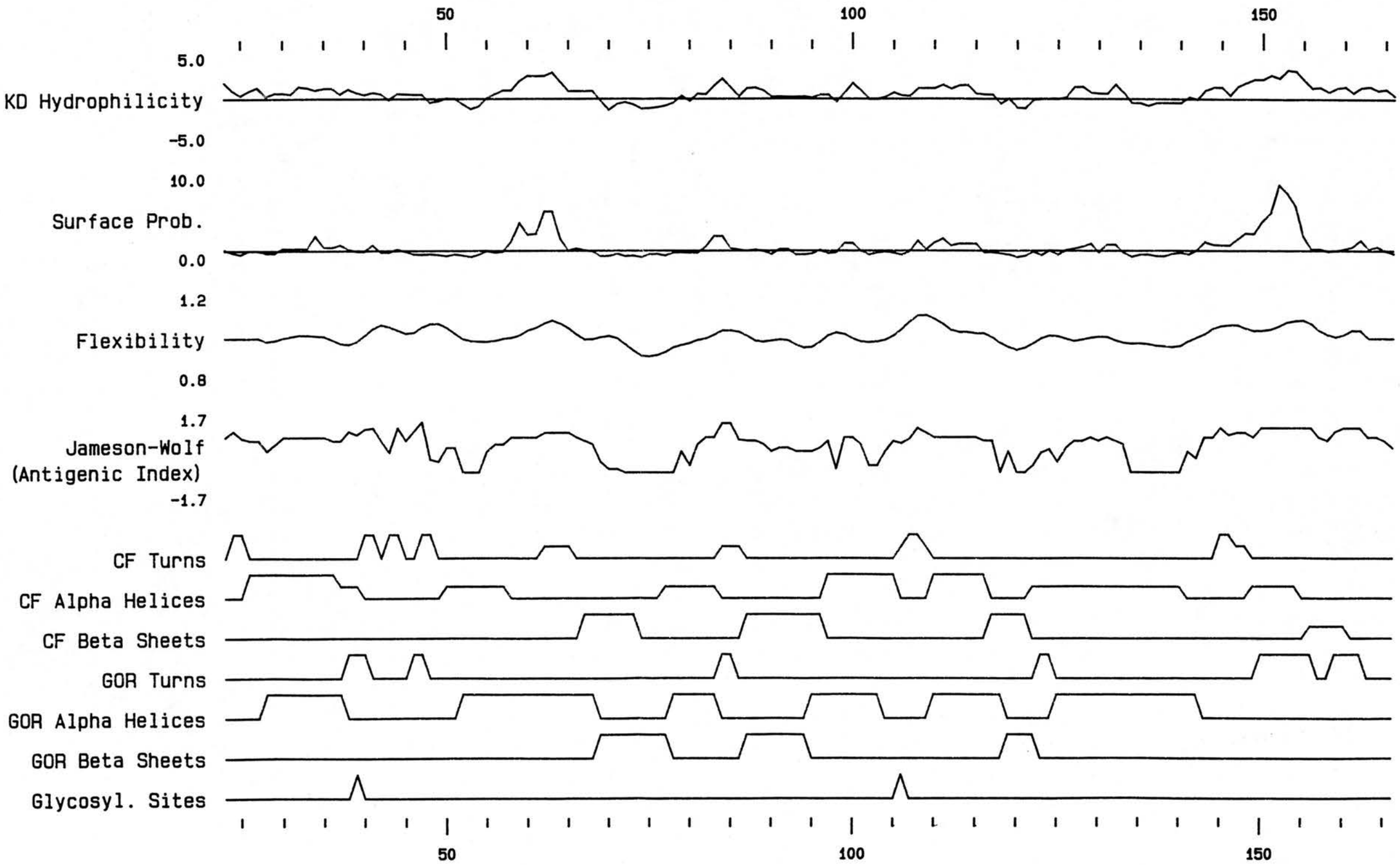


Figure 4.

Chou-Fasman plot of bovine IFN-g with Kyte-Doolittle hydrophobicity-hydrophilicity predictions superimposed. Saw-tooth regions represent beta sheet, wide jagged lines represent alpha helix, and turns represent beta turn regions. The glycosylation sites are marked ρ. Hydrophobic and hydrophilic regions are marked as shown on the diagram.

PLOTSTRUCTURE of: pir1:ivbog.brf ck: 5502

Interferon gamma precursor - Bovine

Chou-Fasman Prediction
September 18, 1991 15:14

KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3

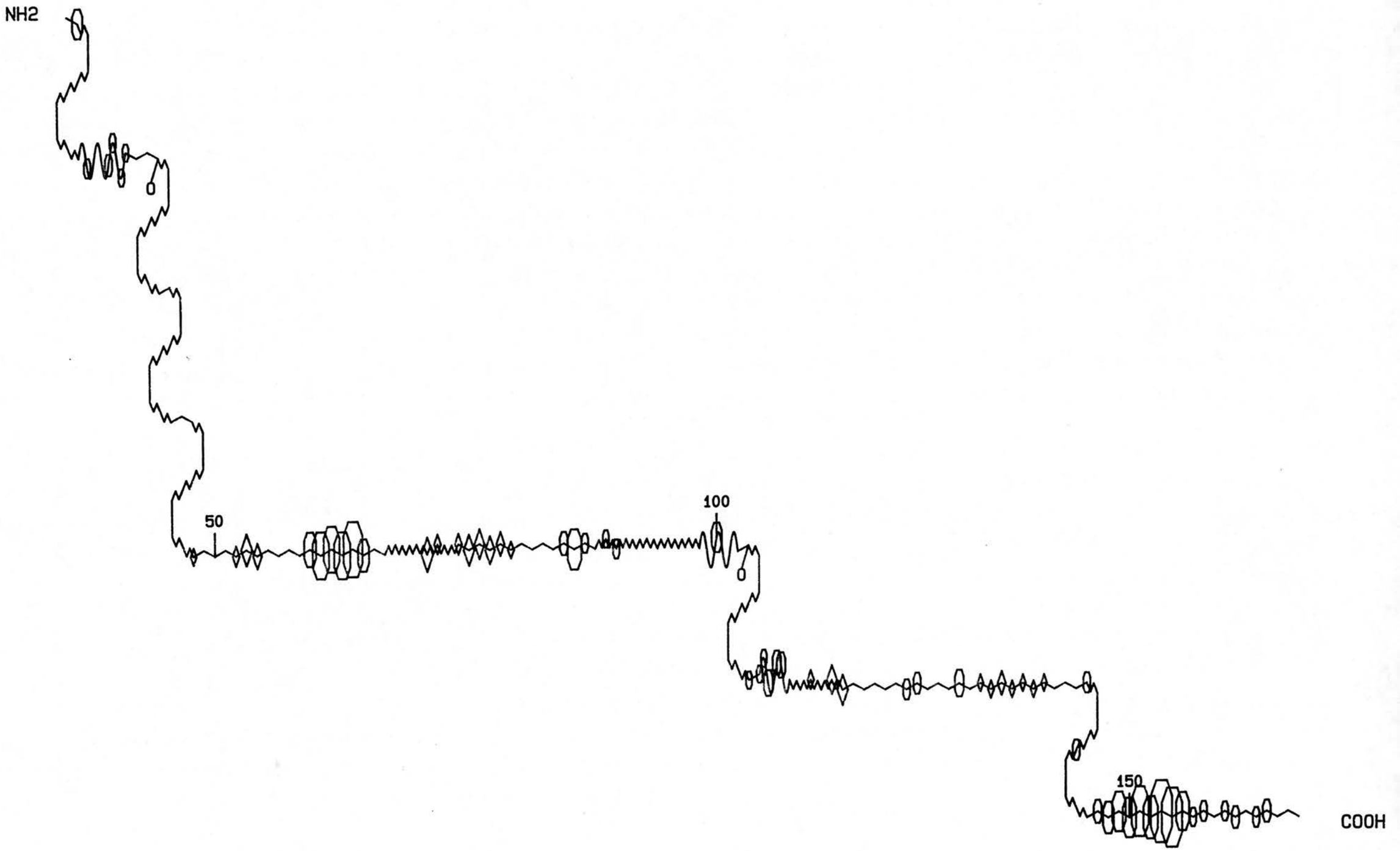


Figure 5.

Bovine IFN-g amino acid sequence with Rothbard motifs marked (underlined).

Figure 5.

Rothbard four amino acid motif

```
1                                                    50
SYGQGQFFRE IENLKEYFNA SSPDVAKGGP LFSDILKNWK DESDKKIIQS

                                                    100
QIVSFYFKLF ENLKDNQVIQ RSMDIIKQDM FQKFLNGSSE KLEDFKKLIQ

                                                    146
IPVDDLQIQR KAINELIKVM NDLSPKSNLR KRKRSQNLFR GRRAST
```

Rothbard five amino acid motif

```
1                                                    50
SYGQGQFFRE IENLKEYFNA SSPDVAKGGP LFSEILKNWK DESDKKIIQS

                                                    100
QIVSFYFKLF ENLKDNQVIQ RSMDIIKQDM FQKFLNGSSE KLEDFKKLIQ

                                                    146
IPVDDLQIQR KAINELIKVM NDLSPKSNLR KRKRSQNLFR GRRAST
```

Figure 6.

Bovine interferon-gamma amino acid sequence marked with the peptides synthesized (underlined). S1-S20 indicates putative signal sequence.

Figure 6.

MKYTSYFLAL LLCGLLGFSG SYGQGQFFRE IENLKEYFNA SSPDVAKGGP

S1 S20|1 30

LFSDILKNWK DESDKKIIQS QIVSFYFKLF ENLKDNQVIQ RSMDIIKQDM

80

FQKFLNGSSE KLEDFKKLIQ IPVDDLQIQR KAINELIKVM NDLSPKSNLR

130

KRKRSQNLFR GRRAST

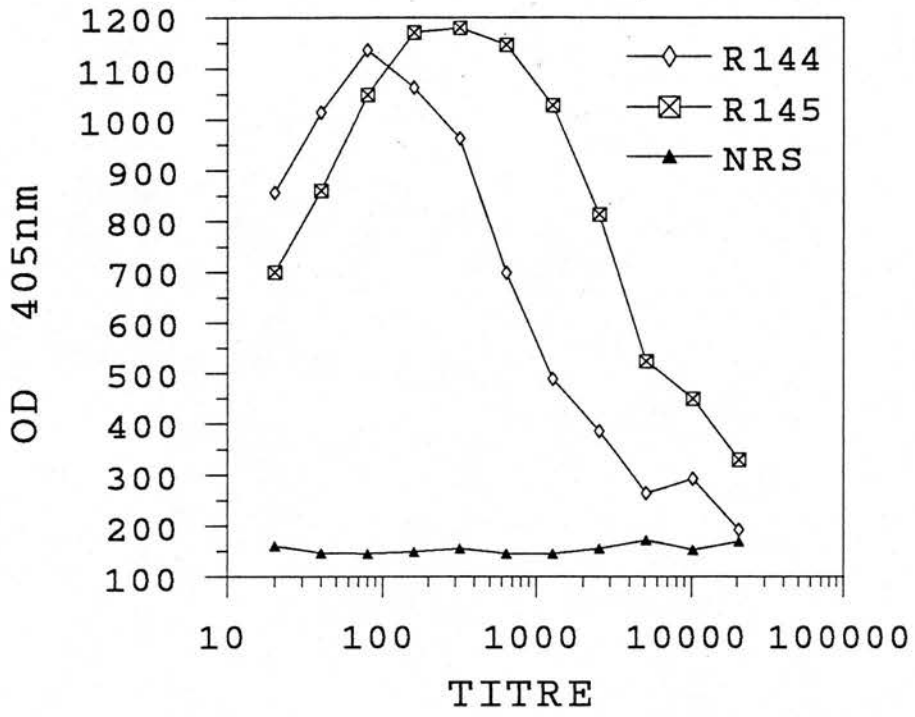
146

Figure 7.

A. Direct ELISA of sera from rabbits immunized with peptide 42-68. Wells coated with peptide 42-68. Doubling dilutions of sera and preimmune sera used.

B. Direct ELISA of sera from rabbits immunized with peptide 112-137. Wells coated with peptide 112-137. Doubling dilutions of sera and preimmune sera used.

Figure 7. A.


1200
1100
1000
900
800
700
600
500
400
300
200
100
OD 405nm
R144
R145
NRS
10
100
1000
10000
100000
TITRE

B.

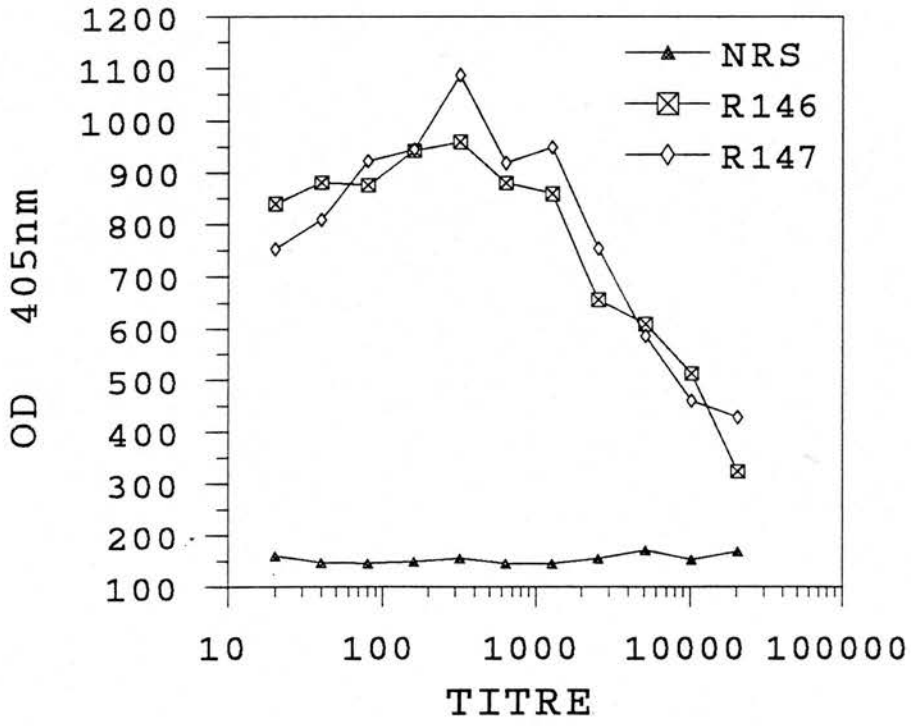
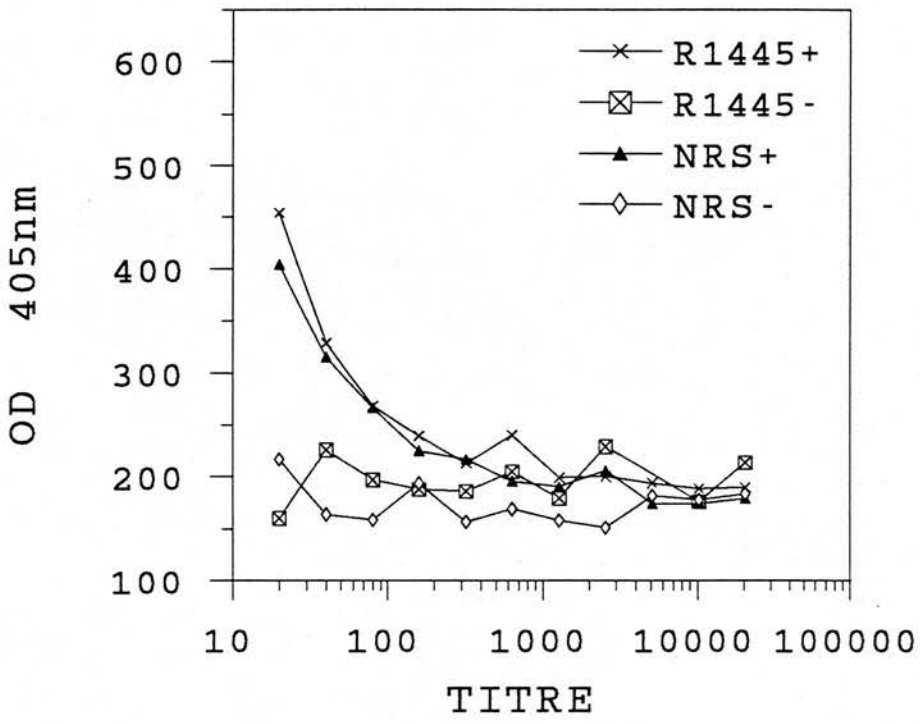

1200
1100
1000
900
800
700
600
500
400
300
200
100
OD 405nm
NRS
R146
R147
10
100
1000
10000
100000
TITRE

Figure 8.

A. Direct ELISA of sera from rabbits immunized with peptide 42-68 (R1445) and preimmune sera (NRS). Wells coated with conditioned medium (+) or control medium (-). Conditioned medium contains IFN-g, control medium does not. Doubling dilutions of sera and preimmune sera used.

B. Direct ELISA of sera from rabbits immunized with peptide 112-137 (R1467) and preimmune sera (NRS). Wells coated with conditioned medium (+) or control medium (-). Conditioned medium contains IFN-g, control medium does not. Doubling dilutions of sera and preimmune sera used.

Figure 8. A.


R1445+
R1445-
NRS+
NRS-
OD 405nm
600
500
400
300
200
100
10
100
1000
10000
100000
TITRE

B.

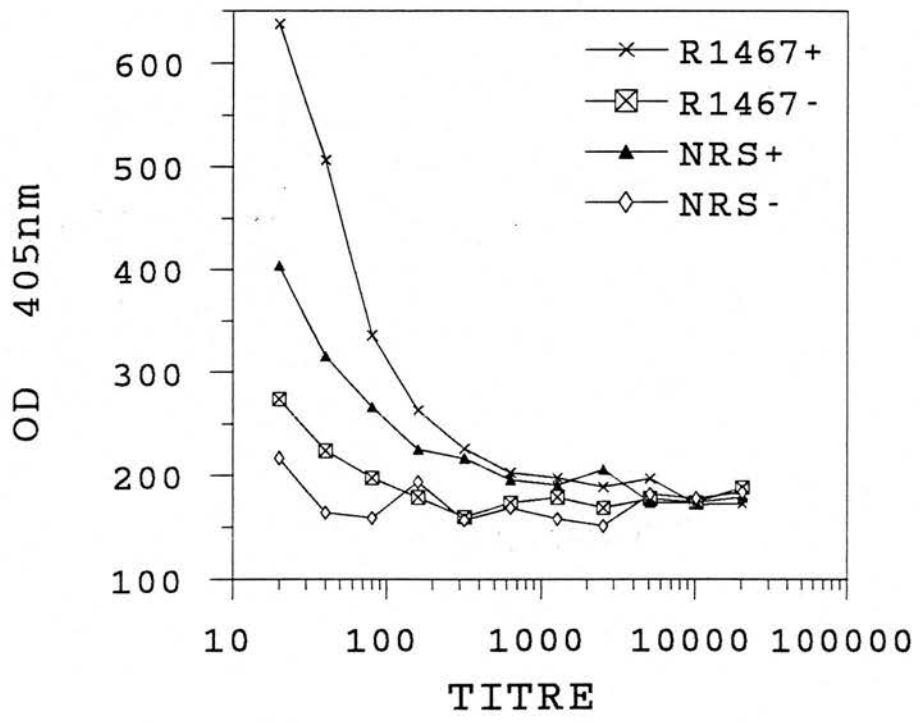

R1467+
R1467-
NRS+
NRS-
OD 405nm
600
500
400
300
200
100
10
100
1000
10000
100000
TITRE

Figure 9.

Direct ELISA of sera from rabbits immunized with peptide 42-68 (R144 and R145), 112-137 (R146 and R147), or preimmune sera (NRS). Wells coated with bovine conditioned medium.

Figure 9.

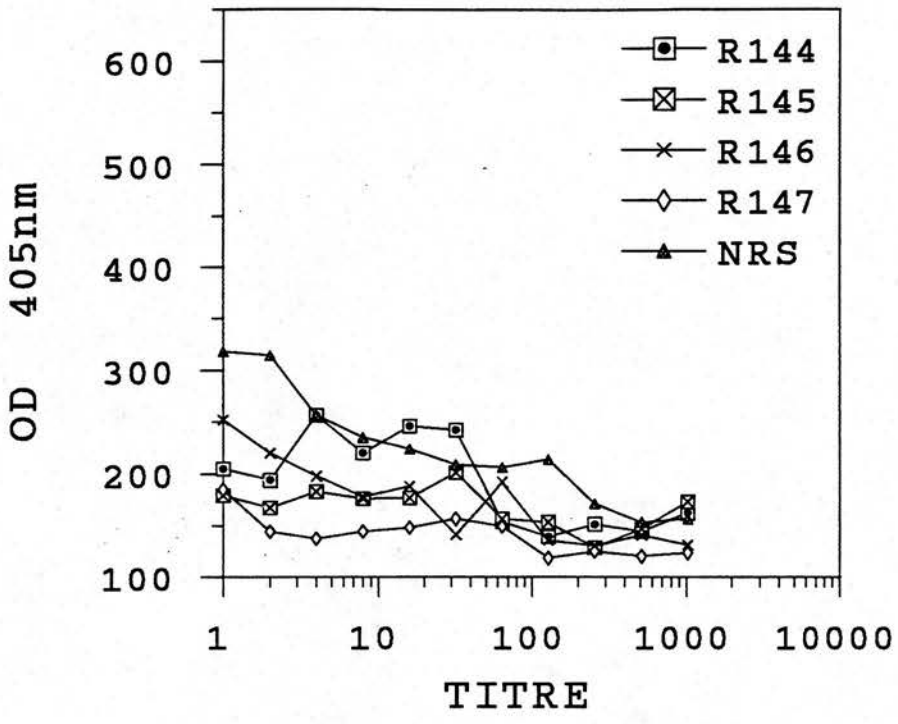

R144
R145
R146
R147
NRS
600
500
400
300
200
100
OD 405nm
1
10
100
1000
10000
TITRE

Figure 10.

Inhibition ELISA to detect ovine IFN-g. Antisera from rabbit R144 at four dilutions preincubated with dilutions of conditioned medium before incubation in wells coated with peptide 42-68.

Figure 10.

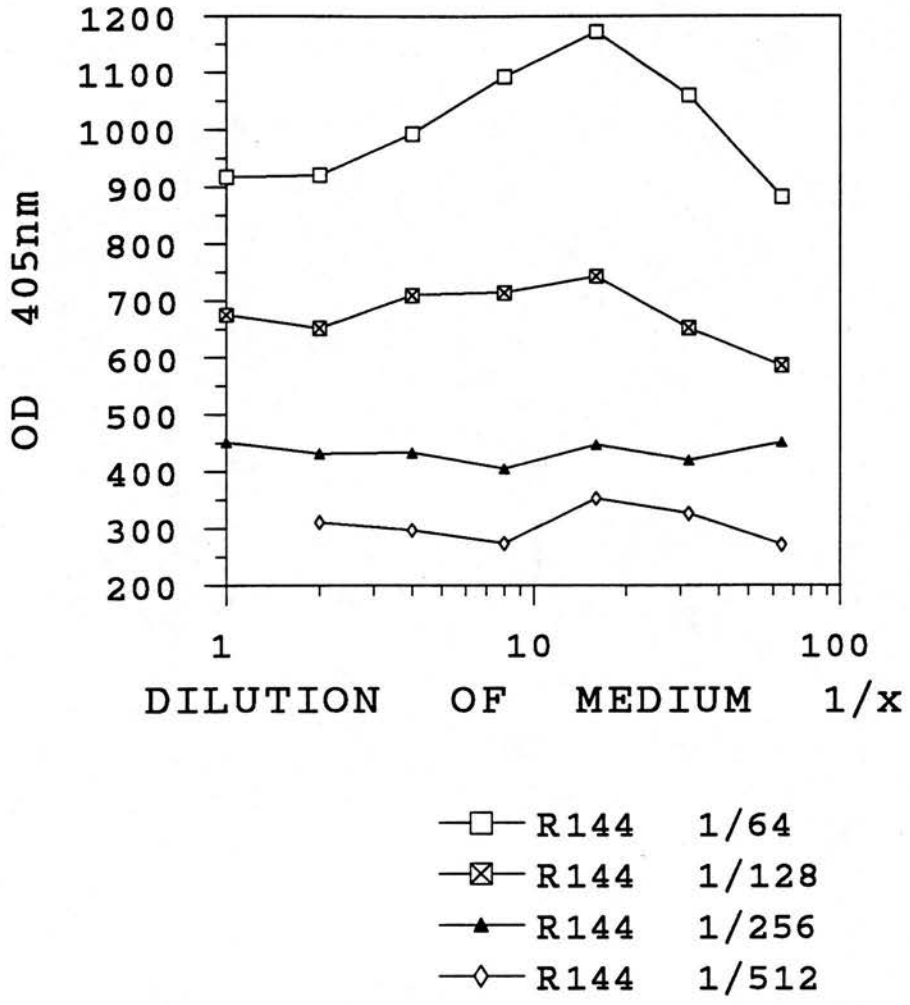

1200
1100
1000
900
800
700
600
500
400
300
200
OD 405nm
1
10
100
DILUTION OF MEDIUM 1/x
R144 1/64
R144 1/128
R144 1/256
R144 1/512

Figure 12.

Representative example of a sandwich ELISA to detect ovine IFN-g in conditioned medium. Wells coated with IgG from rabbits immunized with peptide 112-137. Wells incubated with conditioned medium (+) or control medium (-). Detection by biotinylated IgG from rabbits immunized with peptide 42-68.

Figure 12.

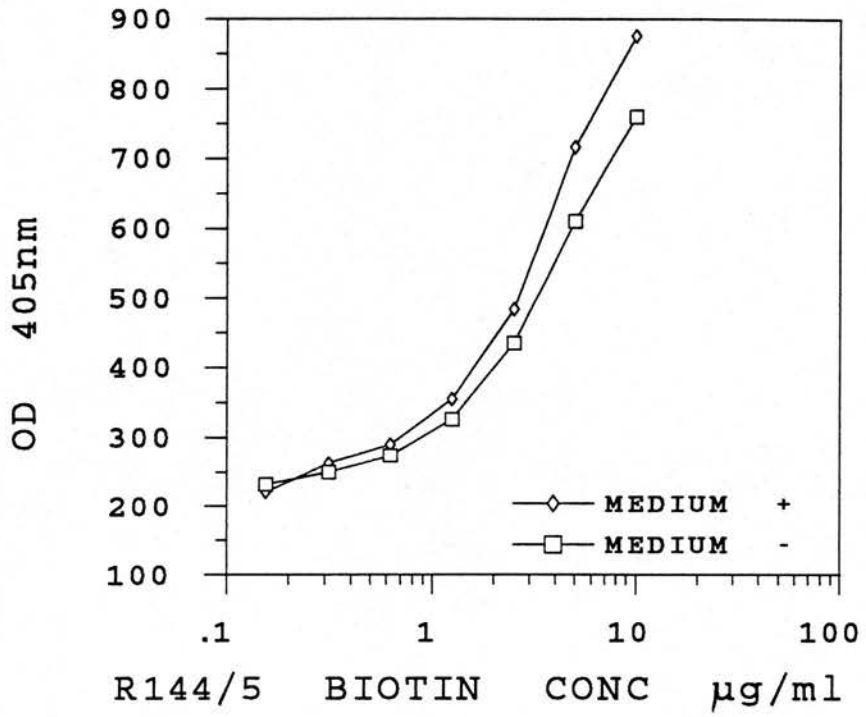

900
800
700
600
500
400
300
200
100
OD 405nm
MEDIUM +
MEDIUM -
.1
1
10
100
R144/5 BIOTIN CONC µg/ml

Figure 13.

SDS-PAGE of bovine recombinant interferon-gamma stained with coomassie blue. Molecular weight markers are shown. Two bands are visible marked ».

Figure 13.

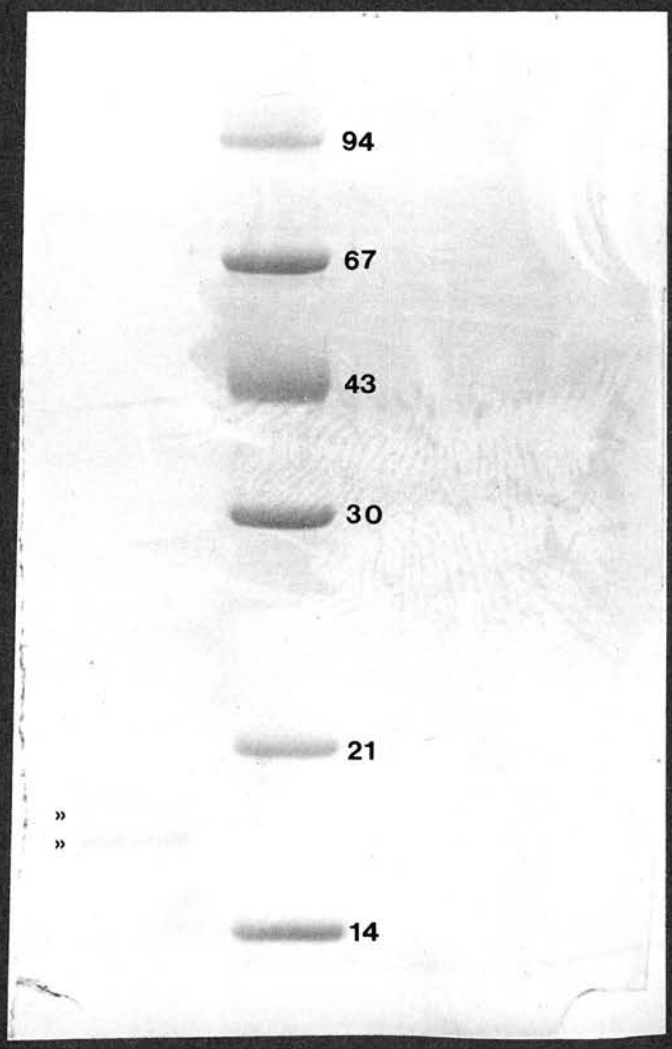


Figure 14.

Western blot of bovine recombinant IFN-g using polyclonal rabbit sera for detection.

Lane	Detecting antibody (immunizing peptide)
A	R146 (112-137)
B	R147 (112-137)
C	R144 (42-68)
D	R145 (42-68)
E	R155 (1-23)
F	R156 (1-23)
G	R161 (20-40)
H	R162 (20-40)
I	R167 (rIFN-g)
J	R168 (rIFN-g)
K	Preimmune rabbit serum

Figure 14.

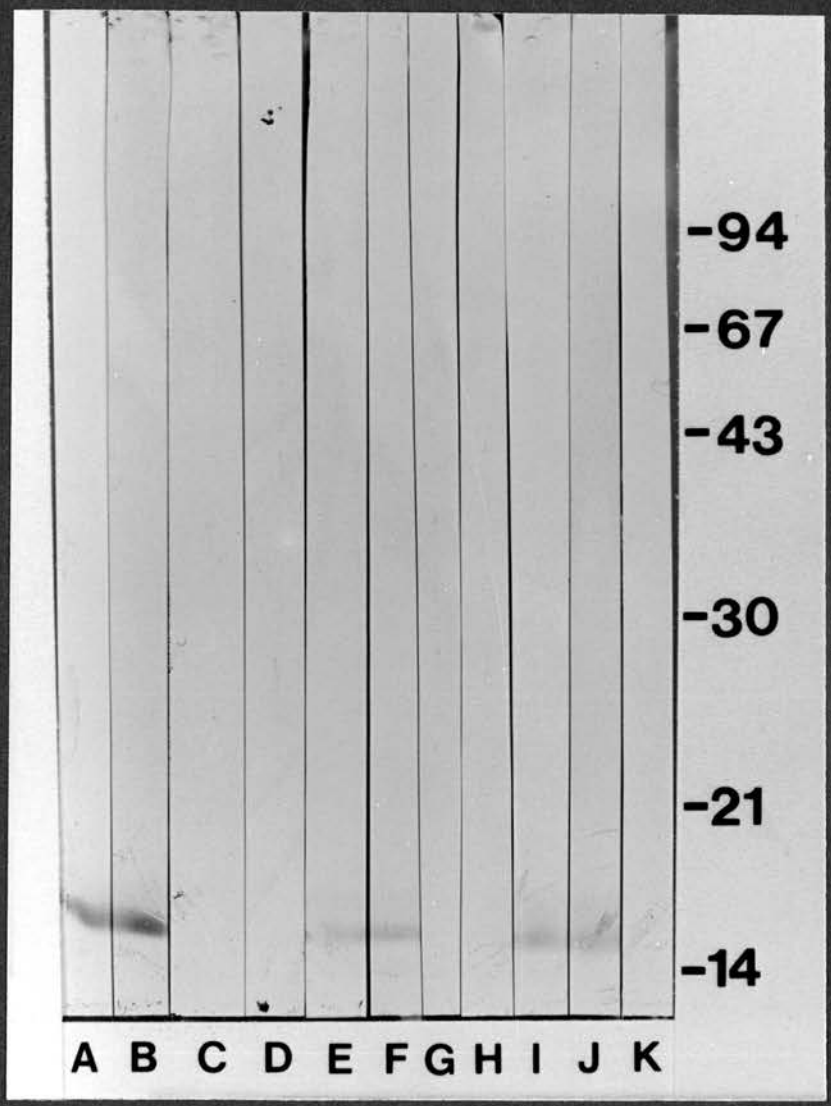


Figure 15.

Western blot of bovine rIFN-g using monoclonal antibody HB 8291 for detection. Molecular weight markers are shown.

Lane	Contents
a	bovine IFN-g 125ng
b	bovine IFN-g 250ng
c	bovine IFN-g 500ng

Figure 15.

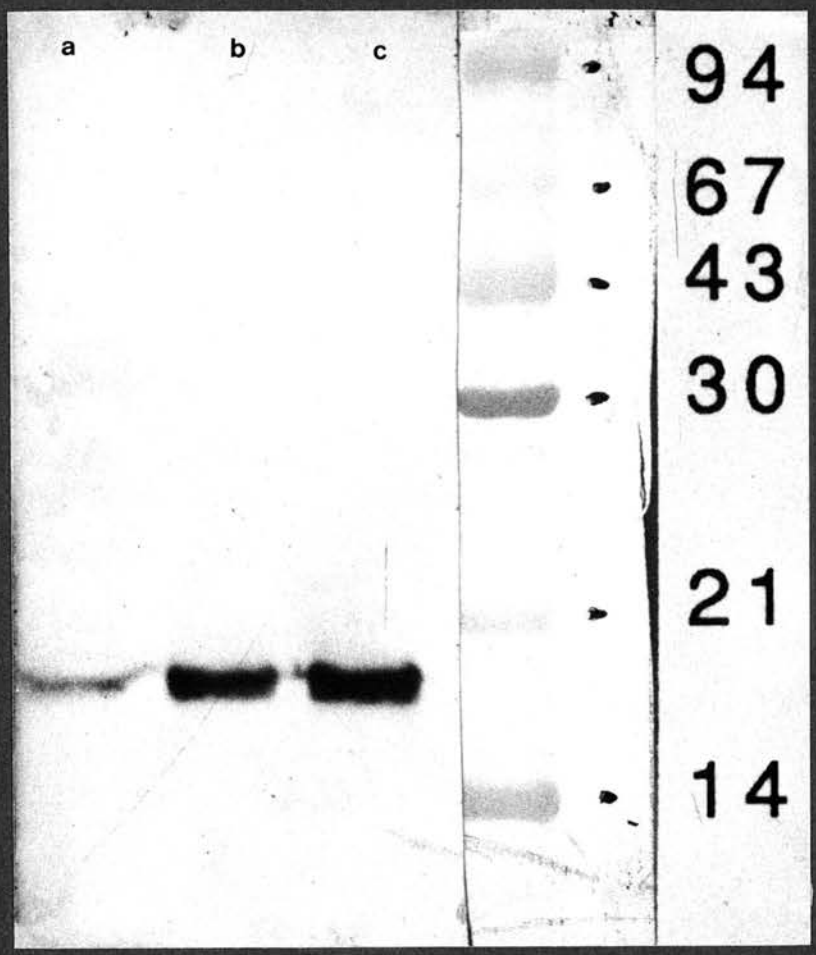


Figure 17.

Sandwich ELISA to detect bovine rIFN-g. Wells coated with sera from rabbits immunized with bovine rIFN-g (R167/8) or with monoclonal antibody HB 8291. Bovine rIFN-g incubated in wells in doubling dilutions series. Detection by biotinylated R167/8.

Figure 17.

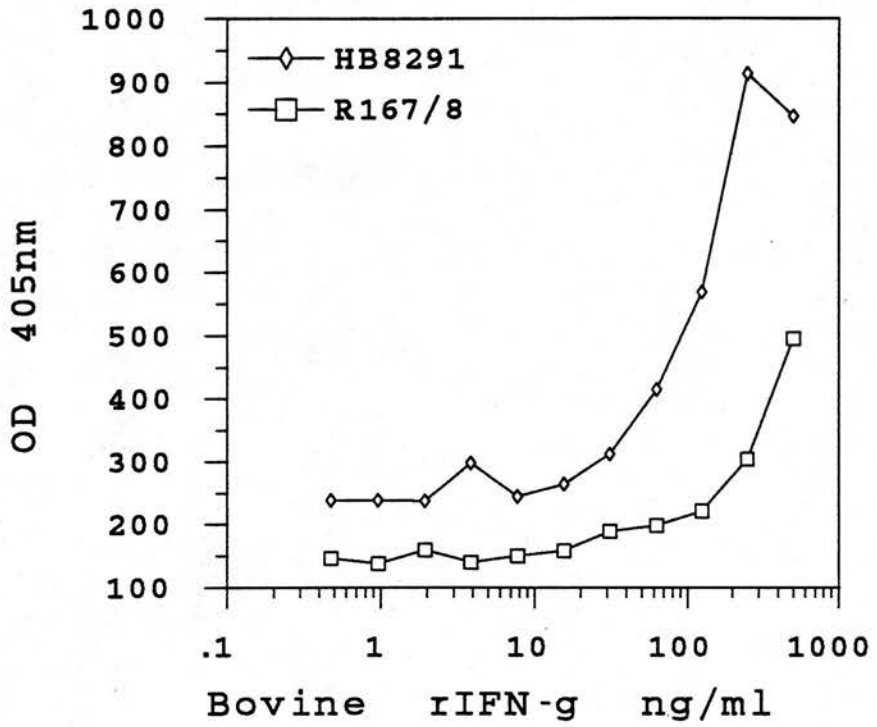
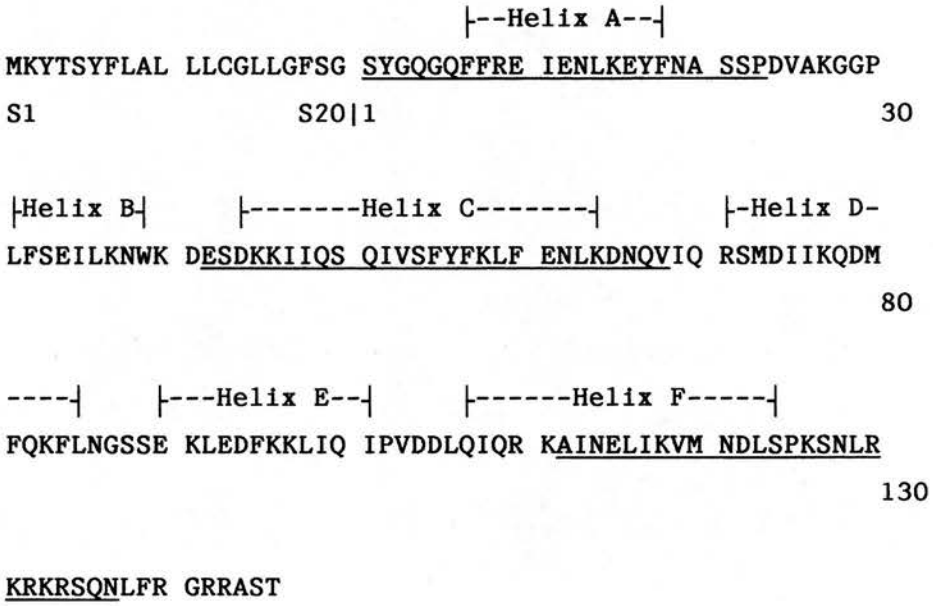

1000
900
800
700
600
500
400
300
200
100
OD 405nm
HB8291
R167/8
.1
1
10
100
1000
Bovine rIFN-g ng/ml

Figure 18.

Bovine interferon-gamma amino acid sequence marked with the predicted tertiary structure. Peptides synthesized are underlined. (Ealick et al, 1991)

Figure 18.


├--Helix A--┤
MKYTSYFLAL LLCGLLGFSG SYGQGQFFRE IENLKEYFNA SSPDVAKGGP
S1 S20|1 30
├Helix B┤ ├-------Helix C-------┤ ├-Helix D-
LFSEILKNWK DESDKKIIQS QIVSFYFKLF ENLKDNQVIQ RSMDIIKQDM
80
----┤ ├---Helix E--┤ ├------Helix F-----┤
FQKFLNGSSE KLEDFKKLIQ IPVDDLQIQR KAINELIKVM NDLSPKSNLR
130
KRKRSQNLFR GRRAST
146

3 THE MAJOR HISTOCOMPATIBILITY COMPLEX.

3.1 INTRODUCTION.

The major histocompatibility complex (MHC) class II antigens are cell membrane glycoproteins consisting of two distinct chains, α and β, which are encoded in the MHC gene region of all vertebrate species examined. They determine the immune response of an animal to a wide range of foreign antigens (Benacerraf, 1981). The class II molecules bind peptide fragments of foreign antigen (Babitt et al, 1985; Buus et al, 1986) and present them to $CD4^+$ T cells. The T cell will only recognise peptides in association with certain restricted allotypes of class II molecules,and this is known as class II antigen restriction. The CD4 molecule and the T cell receptor both bind to the class II heterodimer (Doyle & Strominger, 1987).

The class II proteins were originally discovered in the mouse, and only later in man. The mouse has two types of class II heterodimer named I-A and I-E. The human has three subsets, known as HLA-DR, -DQ and -DP. These subsets have been defined serologically (Tosi et al, 1978), biochemically (Shackelford et al, 1981) and immunogenetically (Shaw et al, 1980). The HLA-DR molecule is the major antigen restricting element in $CD4^+$ cell recognition of peptide. It is primarily responsible for stimulating helper T cells. HLA-DQ restricted T cell clones are relatively rare in immune reactions, but association between specific alleles and autoimmune diseases is strong. This feature has led to the proposal that -DQ molecules may stimulate "suppressor-inducer" cells (Altmann et al, 1991), while -DR molecules stimulate "helper-inducer" cells, and that the HLA-DQ locus may have suppressive influence over the

HLA-DR locus (Hirayama et al, 1987). HLA-DP molecules present antigens similarly to HLA-DR molecules, and no other function has been determined for them to date.

3.1.1 THE MHC OF MAN AND MOUSE.

3.1.1.1 PROTEIN STRUCTURE OF MHC CLASS II.

The MHC class II heterodimer consists of two transmembrane glycoprotein chains. Each chain contains two extracellular domains of 90-100 amino acids, a transmembrane region of 20-25 amino acids and an intracellular region of 8-15 amino acids. The α chain has molecular weight of 34000, and is non-covalently linked to the β chain which has a molecular weight of 29000 (Shackelford et al, 1981). The molecular weights are different mainly due to variable levels of glycosylation (Shackleford & Strominger, 1983). The domain structure of class II molecules is similar to that of MHC class I molecules, but the four domains of class I are arranged as one chain of three domains, and a single domain of β_2-microglobulin. The membrane-proximal domains of class II (β2 and α2) are immunoglobulin-like, with homology to class I α3 and β_2-microglobulin respectively. Comparison of class I with the sequence of class II aligns class I α1 domain with class II α1, and class I α2 with class II β1. The secondary structure of human class I and class II molecules has been examined by Fourier transform infrared spectroscopy and circular dichroism (Gorga et al, 1989). Comparison of HLA-A2, HLA-B7, and HLA-DR1 has revealed the amounts of alpha-helix and beta-sheet to be not significantly different between the three. The α1 and α2 domains of class I HLA-A2 are aligned to form a platform of eight antiparallel β-strands topped by two α-helices

(Bjorkman et al, 1987). A groove between the α-helices provides a binding site for processed foreign antigens. Polymorphic regions of class I are similarly polymorphic in class II, and conserved amino acids give evidence of comparable α-helices. The antigen-binding site of class II has been hypothesized to be structurally homologous to that of class I, with a platform of β-sheets and two α-helices forming the peptide-binding area (Brown et al, 1988). Most of the allelic variation in class II has been localized to the α1 and β1 domains, and domain-shuffling experiments have demonstrated that the polymorphism of the β1 domain of Eβ and Aβ chain genes are the prime determinant in restriction. The allelic variation is concentrated in short "diversity regions", where at least three amino acid allelic substitutions have been detected (Kappes & Strominger, 1988).

The MHC class I and II heterodimers present peptide to T cell receptors. Some peptides have been isolated and microsequenced (Jardetzky et al, 1991). Peptides bound to class I HLA-B27 are nonamers, mainly derived from abundant cytosolic or nuclear proteins such as histone, ribosomal proteins and members of the 90K heat-shock protein family. A motif could be identified within the peptides. Peptides associated with mouse class II I-A^b and I-E^b are found to be 13 amino acids or more in length and to derive from membrane-bound proteins (class II proteins and Murine Leukaemia Virus env protein) and exogenous proteins (BSA) (Rudensky et al, 1991). An invariant chain-derived peptide has also been identified.

3.1.1.2 DNA STRUCTURE OF HUMAN MHC CLASS II.

The domain structure of class II proteins is reflected in the DNA arrangement of exons and introns in human class II genes (Kaufman et al, 1984). The α chain genes contain 5 exons encoding the signal sequence, α1 extracellular domain, α2 extracellular domain, transmembrane and cytoplasmic region, and the 3' untranslated (UT) region. The β chain genes are identical save that the cytoplasmic region is encoded in separate 24bp exon 5, and the 3' UT coding region nominally becomes exon 6.

At the genomic level, a combination of pulse field gel electrophoresis and Southern blotting have shown that all the class II genes are contained in a 1Mb region (Hardy et al, 1986). A map of the MHC class II region is shown in Figure 19.

The DR subregion contains a variable number of β chain genes depending on haplotype, and a single functional α chain gene. The DR3 haplotype has been examined and contains three DRB genes (Rollini et al, 1985). Two of the three genes encode functional proteins with different antigenic properties. The third gene is a pseudogene containing frame shifts in the first and third exons, and deletion of the second exon altogether (Rollini et al, 1987). The DR4 subregion also contains a β chain pseudogene and two functional β chain genes (Spies et al, 1985; Andersson et al, 1987).

The DQ subregion encodes single DQA and DQB genes. Within the same region are two additional genes, the DXA and DXB genes (Jonsson et al, 1987). They are extremely similar to the DQ genes and are thought to have arisen by gene duplication. The DX genes can be considered as DQ pseudogenes. The DQ genes have also been linked to

the DOB gene (Kappes & Strominger, 1988). The DQ genes are the only ones known to produce functional proteins. Although DOB produces no detected protein, its mRNA is found in B cells at low levels (Tonnelle et al, 1985).

The DP subregion contains single DPA and DPB genes, along with two related pseudogenes DPA2 and DPB2 (also known as SXA and SXB). The pseudogenes contain frame shifts and defective splice junctions, along with deletions of a promoter, signal sequence and 3' UT region in DPA2 (Gustafsson et al, 1987; Kappes & Strominger, 1986). Within the DP subregion also lies the DZ(N)A gene, which like DOB is transcribed in B cells but no protein has been detected (Trowsdale & Kelly, 1985). DZA transcription in fibroblasts can be induced by IFN-g, whereas DOB cannot.

Two new class II-like genes have been described which map between the DP and DQ regions. The genes are proposed to be called HLA-DMA and -DMB. The genes encode transcripts found within total B cell RNA, but not in T cell lines. IFN-g treatment of epithelial and fibroblast lines resulted in de novo synthesis of mRNA for HLA-DM (Kelly et al, 1991). The proposed heterodimer would possess a different distal domain structure, as there are several cysteine residues in both proteins which allows prediction of a more rigid structure with restricted peptide binding capacity.

3.1.1.3 INVARIANT CHAIN ASSOCIATION WITH MHC CLASS II.

The MHC class II heterodimer is associated intracellularly with a 31000 MW transmembrane glycoprotein known as the invariant chain (Ii). The name invariant chain is due to its lack of electro-

phoretic polymorphism (Charron et al, 1979). This glycoprotein associates with class II molecules in the endoplasmic reticulum. Initial suggestions that the Ii chain influences the intracellular transport of class II molecules have been questioned with the finding that αβ dimerization can occur in the absence of the invariant chain, although the rate of transport is reduced (Claesson-Welsh & Peterson, 1985). A role for Ii in regulating the endocytosis of class II antigens (Stockinger et al, 1989) has not been confirmed (Peterson & Miller, 1990). It may be that the invariant chain facilitates assembly and/or transport, while not being essential. The Ii chain inhibits binding of endogenous peptides to the class II heterodimer in the endoplasmic reticulum by blocking peptide access to the peptide-binding groove. In the endosomal compartment of cells the Ii chain is dissociated from the class II molecule, and exogenous peptide is able to bind into the groove. The Ii chain is thus thought of as being the factor which distinguishes the endogenous from the exogenous antigen presentation pathway (Teyton et al, 1990).

Invariant chain and class II genes are regulated together during in vitro stimulation of endothelial cells and dermal fibroblasts by IFN-g (Collins et al, 1984). The treatment of mice with recombinant murine IFN-g leads to the induction of Ia antigens and also invariant chain in a variety of tissues. Differential expression is found in bronchial epithelium, kidney tubules and colonic epithelium where Ii is induced but Ia is not (Momburg et al, 1986).

3.1.2 THE MHC OF THE SHEEP.

3.1.2.1 PROTEIN STRUCTURE OF OVINE MHC CLASS II.

MHC class II proteins have been described in sheep and characterized using a number of monoclonal antibodies. Sheep class II α chain is approximately 32000-35000 molecular weight, and the β chain is 26000-29000 (Puri et al, 1985; Hopkins et al, 1986). Both chains are glycosylated with complex, sialated N-linked oligosaccharides (Puri et al, 1987a; Dutia et al, 1990a). The α and β chain oligosaccharides are MW 6000 and 3000 respectively. This is similar to both man and mouse, but in man one of the α chain oligosaccharides is Endoglycosidase H sensitive, implying a non-sialated sugar chain. Monoclonal antibody studies involving sequential immunoprecipitations have shown that sheep class II molecules can be divided into four structurally and serologically distinct subsets (Puri & Brandon, 1987). In addition, one monoclonal antibody could detect as many as 10-12 distinct class II molecules on the lymphocytes of one outbred sheep . This is evidence that the sheep MHC class II region can be divided into two, and possibly three, subregions. The divisions of the sheep class II region have been further confirmed by N-terminal amino acid sequencing of both alpha and beta class II molecules from one sheep. DRα/IEα-like and DQα/IAα-like subunits were identified. The DRα-like chain exhibited very little polymorphism, whilst the DQα-like chain consisted of two closely-related subunits of α polypeptides which may be explained by either allelic variation or the presence of two DQA genes. The sequence from most of the beta chains showed insufficient homology for correlation with human β chain sequences. Affinity purification of the DQα-like chain led to co-purification of a β chain with homology to human DQβ. At least 16 different β polypeptides were

identified from a single sheep, and the sequence data led to the conclusion that at least nine non-allelic β genes exist (Puri et al, 1987c).

Monoclonal antibodies developed in the Department of Veterinary Pathology, University of Edinburgh react with specific ovine MHC class II molecules defined by N-terminal amino acid sequencing, two-dimensional immunoblotting and studies with transfected cell lines expressing sheep DR-like or DQ-like α and β chains (Dutia et al, 1990a; Dutia et al, 1990b).

3.1.2.2 DNA STRUCTURE OF OVINE MHC CLASS II.

Southern hydridizations have been performed on ovine genomic DNA using human cDNA probes for DRA, DRB, DQA, DQB, DPA, DZA, DOB, and a cosmid clone was used to probe for DPB (Scott et al, 1987). The pattern of hybridization suggested that only one gene is present coding for each of DRA, DZA and DOB. No DPA-like gene was detectable. Two DQA genes are known to exist and have been sequenced and shown to be transcribed in resting macrophages (Scott et al, 1991a). One gene is extremely polymorphic whilst the other is both polymorphic and subject to deletion in some sheep haplotypes. The evidence for deletion comes from the absence of a hybridizing band in a southern genomic blot using the gene as the probe. Nucleotide comparison shows that the two ovine DQA genes are relatively divergent (78%) in the α1 domain, but have high degree of identity over the more membrane proximal regions of the molecule. Typical diversity regions are present in the α1 domain in a similar position to those in the human molecule. Comparisons with other

species indicate that ovine DQA genes are most closely related to bovine DQA genes and HLA-DQA1 and -DQA2 genes, than to bovine DRA, HLA-DRA or HLA-DPA1 genes.

The Southern hybridization data of Scott et al (1987) showed extensive cross-hybridization between the β chain genes. This meant that the number of genes in each subregion could not be accurately determined, but it was postulated that there were three or more DQB genes, and at least two DRB genes. The presence of variable number of DQB genes has also been reported in cattle (Andersson & Rask, 1988). Two DPB genes were thought to exist. The presence of hybridizing bands on a Southern blot only yields information about gene presence, but gives no information about their ability to transcribe functional mRNA. Recently the nucleotide sequences of a class II DQB gene and a DRB pseudogene have been reported. No evidence was given about the transcription of the DQB gene. The DQB gene was shown to be more similar to the bovine DQB and the HLA-DQB1/2 genes, than to the DRB genes. The ovine DRB pseudogene possesses premature termination codons in both exons 3 and 4, is lacking a defined exon 2, and the 3' donor splice site of exon 3 is atypical (Scott et al, 1991b).

A brief report has suggested the existence of at least eight distinct α-chain genes and fourteen β-chain genes (Deverson et al, 1988). The cDNA for the sheep class II genes have so far not been cloned and the regulation of the functional molecules has not been studied. In situ hybridization has localized the MHC gene region to chromosome 20 (Mahdy et al, 1989).

There is no evidence for the existence of ovine equivalents to the bovine MhcBota DYA and DYB genes (van der Poel et al, 1990).

The ovine MHC used to be called OLA (Ovine Lymphocyte Antigen), but it has been proposed that in future the Ovine MHC is known as MhcOvar (Klein et al, 1990). The bovine MHC would change from BoLA to MhcBota.

3.1.3 IMPORTANCE OF MHC CLASS II.

The immune response genes were originally discovered in inbred guinea pigs and mice. They determined the immune response to synthetic polypeptides, and were later known to exert their action on the carrier parts of the immunogenic molecule and not on the immunoglobulin epitope (Benacerraf, 1981). The response to pathogens varies among strains of mice, a feature reflecting the contribution made by the immune response genes. Extensive allotyping of human class I and II molecules has allowed the association between disease and both class I and class II phenotypes to be determined for some human autoimmune diseases. There is much evidence that HLA-DQ alles are associated with predisposition to autoimmune diseases such as pemphigus vulgaris (Scharf et al, 1989) and Type 1 diabetes mellitus (Todd,1990). Infectious disease has also been linked to immune response genes in humans. A case-controlled study of over 2000 people from the Gambia showed that distinct class I (HLA-Bw53) and class II (DRB1*1302-DQB1*0501) phenotypes are linked to resistance to trypanosomiasis, and this effect was equivalent to the protection against malaria derived from the sickle cell anaemia gene (Hill et al, 1991). These MHC phenotypes occur more commonly in healthy humans than in children suffering from severe malarial anaemia or cerebral malaria. They are also extremely rare phenotypes outside the malarial regions of Africa. The simplest explanation for these associations is that

they relate to the ability of the HLA molecules to present antigen in either a class I- or class II-restricted manner. Inability to present important epitopes would reduce the cytotoxic T-cell control of the liver stage of malaria (class I), or reduce the antibody defence against malarial anaemia (class II). This study is the first to provide direct evidence for diseases influencing a polymorphic trait in mankind. A similar effect may hold for diseases and breeds of animal. The study of the structure and function of ovine class II may lead to a better understanding of its association with disease in sheep.

The specific immune response state to some pathogens has been linked to ovine MHC. Ovine class I antigens have been linked to the ability to respond to intestinal parasites (Outteridge et al, 1984), Bacteroides nodosus (a cause of footrot) (Outteridge et al, 1989),and Corynebacterium pseudotuberculosis (cause of caseous lymphadenitis) (Millot, 1989). MHC class I haplotypes have been linked to susceptibility to scrapie (Millot et al, 1988; Millot, 1989), but other authors dispute this finding (Cullen et al, 1984; Cullen, 1989). No such disease association with MHC class II in sheep has been documented.

Recent evidence has suggested that the MHC class II heterodimer may be a cellular receptor for maedi-visna virus (Dalziel et al, 1991), although molecules of a different molecular weight have also been found to bind to visna (Crane et al, 1991). The subset of class II molecules involved was not determined, and may be all subsets or a subpopulation. This virus causes disease with only low prevalence, but some breeds (notably Texel sheep from Holland and Border Leicester sheep from the United States) seem more susceptible (Narayan & Clements, 1990). If the cellular receptor is MHC class

II, then the structure of this molecule may play some part in determining disease susceptibility. Factors which increase expression of class II molecules on cells may also play an important part in the pathogenesis of maedi-visna. Lentiferon (discussed in section 1.6), is a leucocyte-derived interferon which is produced when leucocytes contact visna virus- or CAEV-infected monocyte/macrophages. It has been shown to increase class II expression on spleen and lymph node cells (Kennedy et al, 1985). The differential upregulation of class II molecules by IFN-g may also have a role in infection of cells.

In contrast to resting human lymphocytes resting sheep lymphocytes express class II molecules. The CD4+ and T19+ cells express 3-4 times more DR-like than DQ-like molecules, while CD8+ cells have variable DR-like and more DQ-like than the other cell types. It is thought likely that the differential expression is indicative of a difference in function of sheep DR-like and DQ-like molecules, and hence a difference in the function of cells expressing them (Dutia et al, 1990b). The effect of IFN-g on the expression of these two subsets of class II molecules has not been examined before. The induction patterns on different cell types may reveal the role of IFN-g in the potentially different functions of these molecules.

3.2 MATERIALS AND METHODS.

3.2.1 ISOLATION OF SHEEP MHC CLASS II.

The object of this procedure was the purification of sheep MHC class II protein. It was isolated on a Sepharose 4B column coupled to purified monoclonal antibody SW73.2 (Hopkins et al, 1986).

The SW73.2 hybridoma was grown as an ascites tumour in Lewis rats. The antibody was purified from the ascites fluid by 5% caprylic acid precipitation followed by 50% ammonium sulphate precipitation. The antibody was dialysed against 0.1M $NaHCO_3$/ 0.5M NaCl pH8.4 for 24 hours with several changes of buffer. The preparation was assayed for class II reactivity by both ELISA and western blot. The antibody was mixed with CNBr-activated Sepharose 4B which had been washed in 1mM HCl. The suspension was rotated end-over-end for 3 hours at 20°C. The Sepharose 4B was blocked with 1M ethanolamine pH8.0 for 1 hour, and then washed with TEA/DOC pH8.0 and TEA/DOC/NaCl pH11.5. (Appendix 2). The column was stored in PBS/ 0.1% NaN_3.

Sheep splenocytes were prepared by disrupting spleen tissue with an electric blender into a single cell suspension, washing away large particles and then lysing the cells in Lysis Buffer (Appendix 2) at 4°C. The resultant lysate was centrifuged at 1500xg for 15 minutes to remove major debris, and then at 10,000xg for 1 hour to remove nuclei.

The column was equilibrated in Lysis Buffer before the spleen lysate was pumped through at 20ml/hr. The column was washed through with clean Lysis Buffer and then the sheep MHC class II molecules were

released from the column with TEA/DOC/NaCl pH11.5 and collected in aliquots. The aliquots were returned to neutral pH with 1M HCl. Samples from each aliquot were coated onto an ELISA plate and tested for the presence of MHC class II by reaction with SW73.2 antibody in a simple ELISA assay. Positive fractions were pooled and used in the subsequent slot blot assay.

3.2.2 MHC CLASS II INDUCTION SLOT BLOT ASSAY.

Initial experiments using the slot blot apparatus were performed to determine the optimum detergent concentration required for a clean blot and good detection. Dilutions of lymphocytes were lysed in four different detergent solutions: 0.1% sodium dodecyl sulphate (SDS), 0.25% SDS, 0.25% sodium deoxycholate (DOC), and 0.5% DOC, all prepared in 25mM Trizma pH8.0, 500mM NaCl. These experiments revealed that 0.25% DOC gave the sharpest and most defined titration bands of the four.

A sheep intestinal adenocarcinoma cell line ST6 (Norval et al, 1981) was grown in RPMI + 5% FCS + sodium pyruvate (100mM) + L-glutamate (100mM). Cells were harvested and plated out in 96-well tissue culture plates (Nunc, Copenhagen, Denmark) at 1×10^4 per well in 400µl of the above medium. They were grown for 24 hours at 37°C in 5% CO_2 before 100µl of conditioned medium (see 2.2.3 for production) was added to the wells. Cells were incubated for a further 24 hours at 37°C in 5% CO_2 before they were dislodged from the wells with the tip of a pipette and the cells and supernatant harvested. 200µl of 0.02% disodium EDTA was added and any remaining cells collected.

Cells were centrifuged at 400xg for 5 minutes and then resuspended in the remaining culture fluid before lysing them in 400µl of 0.25% sodium deoxycholate in 25mM Tris pH7.5 with 500mM NaCl.

The lysate was applied through a BIORAD SLOT BLOT onto Hybond C (Amersham, Bucks) nitrocellulose, which had been equilibrated in 25mM Tris pH7.5 with 500mM NaCl (TBS). 100µl of TBS was applied to each slot and drawn through by vacuum before 200µl of each lysate was applied to each well and drawn through by vacuum. The samples were washed through with 200µl of TBS. 200µl of a 1 in 10 dilution of the purified ovine class II protein (from section 3.2.1) and doubling dilutions beyond this was was applied to the membrane.

The membrane was removed and blocked with 5% dried milk powder in PBS. It was then incubated in 1/10 dilution of tissue culture supernatant of SW73.2 monoclonal antibody overnight before being washed five times in 1% dried milk powder in PBS over 30 minutes. The membrane was then incubated in goat anti-rat IgG horseradish peroxidase (SAPU, Carluke) at 1/1000 in 1% dried milk powder/PBS for 1 hour. It was washed again prior to development in diaminobenzidine-H_2O_2, including 1:20 v/v of $CoCl_3$ (Hopkins et al, 1986).

3.2.3 MHC CLASS II ELISA ASSAY ON FIXED OVINE CELLS.

Sheep adenocarcinoma cells (ST-6) were grown and treated with conditioned medium as in 3.2.2. Instead of dislodging the cells from the wells they were fixed to the plate with 20% acetone in methanol for 5 minutes. The cells were then rehydrated in PBS and the plate blocked with 2% BSA in PBS for 1 hour. Cells were assayed

against two monoclonal antibodies, VPM16 (anti-class II) and VPM19 (anti-class I) which were available in the Department of Veterinary Pathology, University of Edinburgh. Each well was incubated with 100µl of undiluted monoclonal antibody tissue culture supernatant for 1 hour. The wells were washed with PBS 5 times over 10 minutes. Each well was incubated with 100µl of a 1/1000 anti-mouse Ig horseradish peroxidase (SAPU, Carluke) for 1 hour, and then washed again in PBS. The wells were incubated with o-phenylenediamine (1mg/ml)(Sigma) in 0.1M citrate-phosphate buffer containing 0.006% H_2O_2 for 20 minutes before the reaction was terminated by the addition of 50µl of 2M H_2SO_4. The optical density at 492nm was measured in a Titertek Multiskan plate reader.

3.2.4 MHC CLASS II FACS ASSAY ON OVINE LYMPHOCYTES.

Lymphocytes were collected from an ovine prefemoral efferent lymphatic duct which was chronically cannulated with an indwelling nylon tubing. Cells were counted and 2 x 10^7 lymphocytes were washed twice in ice-cold PBS/ 1% foetal calf serum. They were cultured in two 1" plastic dishes in 2 ml of RPMI 1640 + 15% foetal calf serum + L-glutamine (100mM) + sodium pyruvate (100mM), at 5 x 10^6 cells/ml in 5% CO_2 at 37°C. To one dish was added 10ng (25IU) of bovine recombinant interferon-gamma (Ciba-Geigy, Switzerland); the other dish was not treated. After 3 days the cells were harvested, washed twice in ice-cold buffer [PBS/1% BSA/0.1% NaN_3] before 50µl aliquots were dispensed into Rh tubes at 2 x 10^6/ml. To each tube two monoclonal antibodies were added. Firstly 25µl of VPM36 (an anti-ovine MHC class II DQα specific IgG1 MoAb) or VPM38 (an anti-ovine MHC class II DRα specific IgG1 MoAb) (Dutia et al, 1990b) to stain different class II populations was added, followed

by 25µl of a biotinylated lymphocyte marker. The lymphocyte markers used were either SBU-T4 (α-CD4), SBU-T8 (α-CD8) (Maddox et al, 1985), VPM30 (α-B-cell) (Naessens & Howard, 1991) and CC15 (α-T19) (Clevers et al, 1990). These four monoclonal antibodies mark out cells bearing molecules corresponding to the CD4 and CD8, together with a B cell marker and a marker of $CD4^-$, $CD8^-$ T cells (largely made up of T cells bearing the gamma/delta T cell receptor).

Cells were incubated with tissue culture supernatants of the monoclonal antibodies for one hour at 22°C. Cells were washed twice in buffer. Resuspended cells were mixed with 25µl anti-mouse IgG1 fluoroscein isothiocyanate and 25µl of streptavidin-phycoerythrin (Amersham) and incubated for one hour at 22°C. Cells were washed once in buffer and analysed using a FACScan (Becton Dickinson) with a 488nm argon ion laser. Dead cells were eliminated from analysis by setting a forward scatter threshold. Cells were initially assessed using linear parameters for forward and side scatter. Live gates were set to limit analysis to small lymphocytes and ten thousand cells were analysed per sample. Subsequent analysis used the Becton Dickinson LYSIS programme.

This experiment was repeated with the addition of a 1 in 1000 dilution of rabbit anti-bovine rIFN-g (R167) to the culture medium at the beginning of the incubation.

3.2.5 MHC CLASS II FACS ASSAY ON OTHER OVINE CELL LINES.

The ST-6 cell line used previously (3.2.3), and an ovine skin fibroblast cell line (321), were cultured in T75 flasks in 20ml of RPMI 1640 + 5% foetal calf serum + sodium pyruvate (100mM) + L-glutamine (100mM). When the cells were confluent 5ml of conditioned media was added to the flask and culture continued for 24 or 72 hours. The cells were harvested by trypsinization and washed twice in buffer before being dispensed at 2 x 10^6/ml into Rh tubes, (50µl) and then stained using 50µl of VPMs 16 (anti-DQ-like and DR-like β chain), 19 (anti-class I), 41 (anti-DQ-like β chain), 37 (anti-DR-like β chain) or 5 (anti-IgM) (Appendix 3). Each antibody was added to different tube. Cells were analysed on the FACScan as before with the exception that side scatter amplification was logarithmic due to the complexity of the cells.

3.3 RESULTS.

3.3.1 MHC CLASS II INDUCTION SLOT BLOT ASSAY.

When the ST-6 cells were used the results generated were unreliable. On some occasions every slot on the membrane was strongly positive. Occasionally the results seemed to yield the expected titration series of the class II control and the class II induction profiles corresponding with the known dilution series of the conditioned media (Figures 21 and 22). However, on many occasions the detergent alone gave a positive dark slot on the membrane. The occasions when this occurred were not predictable and modifications to the procedure did not eliminate this feature. Without reliable controls it was felt that the technique could not be used confidently for studies on the action of IFN-g on MHC class II.

3.3.2 MHC CLASS II ELISA ASSAY ON OVINE CELLS.

The method of cell ELISA described in section 3.2.4 used murine MoAbs to sheep class II. At no time did the use of the murine MoAbs produce meaningful results.

Rat MoAbs had been used successfully for a short period at the Moredun Research Institute (G. Entrican, personal communication). Loss of the rat anti-class I monoclonal cell line had forced him to change to the use of the same mouse MoAbs. He also found that the assay became of no value.

3.3.3 MHC CLASS II FACS ASSAY ON OVINE LYMPHOCYTES.

The use of efferent lymphocytes for the study of MHC class II induction gave much better results than either of the previous techniques. This technique allowed the differential increase in MHC class II DR-like (VPM38-reactive) and DQ-like (VPM36-reactive) molecules to be assessed. Collection of 10,000 events on the FACS gave sufficient numbers of $CD4^+$ and $CD8^+$ cells for analysis, but the numbers of $T19^+$ and $VPM30^+$ cells were small as efferent lymph fluid typically contains only 5% of each cell type. This meant that approximately 500 events were collected for each cell type, a figure which allows only limited interpretation. Results from one experiment are presented in Tables 1, 2, and 3, and Figures 23, 24, 25, and 26. Similar results were obtained in two further experiments using efferent lymphocytes from the same sheep.

The results confirm that each subset of unstimulated small efferent lymphocytes expresses DR-like molecules. The expression of DR-like molecules is altered by incubation with rIFN-g but expression of DQ-like molecules is little changed. Only 15-20% of $CD4^+$ and $CD8^+$ T lymphocytes express DR-like molecules before incubation and there is a very variable degree of expression (Table 1 and Figure 23A and 24A). Incubation with rIFN-g increases the number of cells expressing class II DR-like molecules by a factor of two for $CD4^+$ lymphocytes and by a factor of three for $CD8^+$ lymphocytes. Examination of the FACS plots for both lymphocyte subsets shows the degree of expression of class II is still highly variable and that flow cytometry cannot discriminate separate populations of class II positive cells even after incubation. B lymphocytes are relatively homogeneous in their expression of class II, with a discrete population of high expressing cells (Figure 26A). Three-quarters of

B lymphocytes express DR-like molecules prior to incubation with rIFN-g and this is increased to 86% after incubation (Table 1). The discrete population of high expressing cells is maintained. $T19^+$ cells express more DR-like class II (37%) than other T cells (15-20%) but still possess variable degrees of expression. Incubation with rIFN-g increases the number of class II positive cells and DR-like molecules to higher degrees on most $T19^+$ cells (Tables 1, 2, 3, and Figure 25).

Only 4-6% of $CD4^+$ and $CD8^+$ lymphocytes express DQ-like class II molecules both before and after incubation with rIFN-g (Table 1 and Figures 23B and 24B). Two thirds of B cells expressed DQ-like molecules both before and after incubation with rIFN-g. Only small increases in number of cells expressing and degree of expression were noted after incubation with rIFN-g (Table 1, 2, 3, and Figure 26B).

Inclusion of IFN-g neutralizing antibody in the culture medium totally abrogated the upregulation of class II on lymphocytes.

3.3.4 MHC CLASS II FACS ASSAY ON OTHER OVINE CELL LINES.

The ovine cell lines were initially negative for class II. After culture with conditioned medium there was no change in the FACS profile for MHC class II expression on these cells (data not shown).

3.4 DISCUSSION.

3.4.1 THE RELATIONSHIP BETWEEN MHC CLASS II AND INTERFERON-GAMMA.

Regulation of expression of MHC class I and class II plays a critical role in the initiation and development of an immune response. MHC class I molecules are found on most cells in the body and are involved in target recognition by $CD8^+$ (frequently cytotoxic and sometimes suppresssor) T cells. MHC class II antigens are mainly expressed on B cells, thymic epithelium, activated T cells, macrophages and Langerhans cells, and are involved in the restriction of $CD4^+$ (usually helper) T cells (Kappes & Strominger, 1988). Class II antigens have also been found on some fibroblasts, tumour cells, epithelial cells and endothelial cells, amongst others. Quantitative variation of class I and II antigens at the cell surface plays a central role in immuno-regulation (Janeway et al, 1984; Kaufman et al, 1984).

The initial steps in an immune response are the uptake of antigen by accessory cells and its presentation to T cells. The main cells involved in this are macrophages due to their phagocytic capacity and their ability to present antigen to other cells of the immune system. Langerhans cells within the skin and dendritic cells within lymphoid tissue have similar antigen presentating roles. The accessory cells present processed antigen to helper T lymphocytes in association with class II molecules. Efficient presentation depends upon the expression of class II molecules. MHC class II is commonly inducible by IFN-g and only rarely by IFN-α or -β (reviewed by Cresswell, 1987; Pestka et al, 1987; Kappes & Strominger, 1988; Stzein et al, 1984), while class I molecules are upregulated by all three of the interferon species (Pestka et al, 1987).

IFN-g seems to play a major part in the regulation of the immune response by virtue of its effect on antigen presentation by macrophages (Unanue, 1984). Murine macrophages do not express class II constitutively or do so at low levels. Factors which may stimulate class II expression on macrophages would be important in initiating the immune response. Macrophages and melanoma cells were initially shown to express class II in response to IFN-g (King & Jones, 1983; Basham & Merigan, 1983). Many other cell types express no MHC class II molecules under normal circumstances, but may be induced to express them under the influence of IFN-g. IFN-g has been shown to induce the de novo synthesis of class II antigens in many cells including human endothelial cells and dermal fibroblasts (Collins et al, 1984), keratinocytes (Griffiths et al, 1989), thyroid epithelial cells (Todd et al, 1985), and human osteogenic carcinoma, colorectal cancer, and melanoma cells (Shaw et al, 1985). Upregulation or induction of expression of MHC class II antigens by IFN-g can be inhibited by IFN-α and -β on mouse macrophages (Inaba et al, 1986; Ling et al, 1985), and on mouse fibroblasts and glial cells (Morris & Tomkins, 1989), or enhanced by other cytokines such as tumour necrosis factor α (TNF-α) (Pujol-Borrell et al, 1987).

The magnitude of a T helper-cell immune response is a function of the product of antigen concentration and the number of class II molecules expressed on antigen presenting cells (APCs) (Matis et al, 1983). As the concentration of antigen is outwith the control of the immune system, the primary regulation of response lies with the variation in class II molecules on APCs. IFN-g is the main upregulator of class II cell surface concentration, and hence of importance in the immune response.

The section of work described in this chapter has attempted to utilize the ability of IFN-g to increase the number of MHC class II molecules on cell surfaces. Initially this was with the object of assaying IFN-g in tissue fluids during immune responses. The standard method for assaying interferons has been by a viral plaque reduction assay. This method depends upon plaque formation by a standard dose of specific virus on specific susceptible cells, and its interference by dilutions of interferons. This method does not discriminate between the three types of interferon, unless specific anti-interferon sera are included in the assay to confirm the effects are due to those interferons. It has been shown that the IFN-α/βs are more active in the antiviral assay than IFN-g on an activity per molecule basis (Blalock et al, 1980). The activity of fewer molecules of either of the class I interferons may therefore mask the presence of the IFN-g molecules unless suitable anti-interferon sera are available to increase the specificity of the assay. Consequently alternative bioassays and ELISAs have been described to measure human and mouse IFN-g.

Measurement of IFN-g by changes in MHC class II expression has previously been described. Cells used varied from a mouse IL-2-dependent T cell line, a human B lymphoblastoid line, a human chronic erythroid leukaemia cell line (Nouri-Aria et al, 1988), murine macrophage cell line (Le Moal et al, 1989), and mouse intestinal epithelial cells (Whiting & Bland, 1990). Induction of class II is thought to be an IFN-g-specific property using these cell lines and it only detects biologically active IFN-g molecules. Measurement of class II was by radioimmunoassay or by enzyme-linked immunostaining which was assessed by colour change. All bioassays suffer in that they are time-consuming, results generally taking

one-to-two days. Other, as yet undescribed, cytokines may also be active in the assays. As an ELISA assay was not available for ovine IFN-g a class II induction assay was developed.

The poor results obtained using both ELISA and slot blot technology are not uncommon during the development of class II assays. Background readings seem to have been the major problem with the ELISA assay described here. Lysis solution present in the slot without cellular proteins was sufficient to cause a strong positive signal on many occasions. The basis for this non-specific result is unknown, but could involve non-specific binding of the monoclonal antibody, the conjugate or the developing substrate.

The difficulty in obtaining consistent results supports the idea that the quantitation of MHC class II molecules on cell surfaces is difficult using standard techniques (Whiting & Bland, 1990). These authors went to great lengths to eliminate background readings from their assays which were due to nonspecific binding of radiolabelled conjugate to the cells, and eventually solved their background problems by using an inhibition radioimmunoassay incorporating Sephacryl beads as the solid phase.

The cell ELISA using ST-6 cells did not give any meaningful results using murine monoclonal antibodies. This problem was also found at Moredun Research Institute when they attempted to use the same murine antibodies, but rat MoAbs did initially yield results (G. Entrican, personal communication). It is possible that the fixation technique using 20% acetone in methanol altered the epitopes recognised by the murine MoAbs, but not those recognised by the rat MoAbs. A variety of different murine anti-class II MoAbs were tried in the cell ELISA procedure with no success.

Although the ELISA, cell ELISA and slot blot techniques were not useful, the later results show that the differential upregulation of MHC class II subsets by IFN-g on ovine mononuclear cells may be monitored by the use of FACS analysis. The use of bovine rIFN-g on bovine mononuclear cells has already been reported, but only the initial optimization of the IFN-g stimulation of cells was performed (Walrand et al, 1989). The results presented here elucidate the situation in ovine lymphocytes a little further.

FACScan analysis of cell membrane expressed class II gives a good indication of alterations in cellular expression of the molecules. The class II expression profile is found to cover a range of levels from none through to medium levels of expression. Incubation of ovine lymphocytes with rIFN-g causes a generalized increase in the level of MHC class II expression, but does not induce discrete populations of cells with different degrees of class II expression. The effects of IFN-g on discrete populations of cells cannot therefore be assessed using this technique.

Both ST-6 cells and ovine skin fibroblast cells (321) presented a problem in the FACScan analysis because of their tendency to clump during processing. A limited number of experiments were performed using these cells. Neither cell type expressed MHC class II either before or after treatment with Con A-conditioned medium. These results are in agreement with those of Nash et al (1992) who performed similar experiments on primary ovine fibroblasts. Wong et al (1984) found that IFN-g did not influence class II expression on murine fibroblast lines.

Lymphocytes cannot be used to measure IFN-g concentrations by class II induction assays because the lymphocytes themselves may be induced to produce lymphokines which affect class II expression. Polyclonal T cell activators such as plant lectins and anti-CD3 monoclonal antibodies release lymphokines from T cells including IFN-g, IL-4, IL-10 and IL-6 (IFN-β2). These are all known to increase class II expression on some cells. IFN-g can act on natural killer cells to upregulate its own synthesis (Hardy & Sawada, 1989). Class II expression on T cells is also subject to antagonistic influences, such as IL-4, and synergistic influences such as TNF-α. These could influence class II expression and would render the assay meaningless as a measure of IFN-g. However the effect of rIFN-g on lymphocyte class II expression can be assessed using the FACScan technique. Blockage of the effect on expression by IFN-g neutralizing antibodies confirms the role of this cytokine on class II regulation.

3.4.2 EFFECTS OF rIFN-g ON OVINE LYMPHOCYTE CLASS II EXPRESSION.

The effects of recombinant bovine or ovine IFN-g on ovine T cells have not previously been studied, nor have the subsets of MHC class II which are upregulated, or the mononuclear cell subsets which express class II been examined. It was of interest to determine the effect of rIFN-g on MHC class II molecules and its effect on the different subgroups of these molecules. It would then be possible to assess the contribution of IFN-g on the regulation of ovine class II which is found on small resting T lymphocytes in contrast to class II expression on human T lymphocytes.

Resting sheep T cells express MHC class II molecules to a greater extent than human T cells (Dutia et al, 1990b). Secondary challenge of antigen-primed sheep results in efferent lymph containing twice as many DR^+, and four times as many DQ^+ small T lymphocytes. Each cell expressed a profound increase in class II expression, increasing from $2x10^4$ molecules per cell found on resting T cells to four times this number on the T cells draining an active lymph node (Hopkins et al, in preparation). The factor(s) responsible for this increase in expression have not been identified.

Lentiferon, produced during the interaction of lentivirus-infected macrophages and lymphocytes (section 1.6), increases MHC class II expression (measured using a pan-specific monoclonal antibody) on cultured sheep macrophages (Kennedy et al, 1985). Lentiferon has not been purified or cloned, and the effects descibed could be due to the presence of a mixture of cytokines in the supernatant. If it truly exists it may however be involved in the regulation of class II on T lymphocytes.

The induction of MHC class II DR-like molecules by rIFN-g described here demonstrates that sheep T cells express IFN-g receptors and that IFN-g can influence class II expression. The DR-like increase may be influenced by IFN-g induced secretion of a second factor from other cells in the lymph, but only IFN-g, IL-4 and IL-10 are known to induce MHC class II molecules in other animal systems. From work involving murine T cell clones, it has been demonstrated that IFN-g is antagonistic towards the proliferation of cells responsible for the synthesis and release of both IL-4 and IL-10 (Mosmann & Moore, 1991). Consequently it is unlikely that either of these two cytokines have a significant role in the expression of class II on T

cells. Although DR-like molecules were upregulated by IFN-g, the results show there was little effect on DQ-like molecule expression and this suggests that the regulation of DQ-like molecules differs from that of DR-like molecules in sheep T lymphocytes as in other species and cell types. The results are in contrast to those found after incubation of ovine alveolar macrophages with bovine rIFN-g. Ovine DR-like, DQ-like and DP-like molecules were all significantly upregulated by rIFN-g which suggests that in this cell type all three class II subsets are subject to the same major regulatory factors (Nash et al, 1992).

MHC class II molecules are found in low numbers on resting human T cells (Engelman et al, 1980) and are upregulated during activation following antigenic or mitogenic stimulation. Some exchange of class II molecules occurs between responder and stimulator cells during a mixed lymphocyte reaction (Yu et al, 1980), but Ia molecules are known to be actively synthesized by T cells. IFN-g augments the expression of HLA-DR on pokeweed mitogen-stimulated human T cells (Miyawaki et al, 1984).

3.4.3 EFFECTS OF IFN-g ON HUMAN T LYMPHOCYTES.

The effect of IFN-g on human T lymphocytes has been assessed previously with respect to various parameters (Landolfo et al, 1988). They found that human T cells possess specific receptors for IFN-g comparable to B cells in affinity and number of binding sites. IFN-g does not induce an antiviral state in human T cells, nor induce (2'-5')oligoadenylate synthetase. (2'-5')oligoadenylate synthetase is responsible for the activation of an endoribonuclease which cleaves both viral and cellular RNA (Dougherty et al, 1980),

and hence is important in the regulation of viral and cellular macromolecular synthesis. IFN-g does, however, induce MHC class I and enhances T cell proliferation to mitogens. IFN-α and -β both induce an antiviral state and the synthesis of (2'-5')oligoadenylate synthetase in human T cells. The authors deduced that the transduction signal of IFN-g from the receptor into the intracellular compartment proceeds along metabolic pathways different from those of IFN-α/β. IFN-g has been demonstrated to cause enhanced production of IL-2 by T cells (Frasca et al, 1985) and increased expression of IL-2 receptor (Webb & Goeddel, 1987). IFN-g is also an essential factor for the proliferation and differentiation of cytotoxic lymphocytes (Landolfo et al, 1985).

3.4.3 THE ROLE OF MHC CLASS II ON T CELLS.

The role of MHC class II on T lymphocytes is unclear. Activated ovine T cells can stimulate allogeneic T cells in the mixed lymphocyte reaction (MLR), but resting T cells do not possess this ability. This is probably due to the low numbers of class II molecules on these cells (Hopkins et al, in preparation). The magnitude of T cell proliferation is related to the number of class II molecules presented on cell surfaces, along with the antigen concentration (Matis et al, 1983). Proliferation of allogeneic T cells induced by activated sheep T cells can be blocked by pre-incubation with an anti-class II monoclonal antibody in a dose-dependent manner. (Hopkins et al, in press). T cells may stimulate allogeneic T cells in MLR, but antigen presentation to autologous T cells has only been demonstrated in a few instances in vitro. Antigenic peptides associated with class II in isolated lipid membranes are sufficient to stimulate murine T cell hybridomas, but

normal resting T cells are unaffected (Watts et al, 1984; Beverley et al, 1986). Viral peptides were presented to T cell clones in a class II-restricted manner, but whole inactivated virus could not be presented, indicating that T cell clones lack the ability to endocytose or process antigen efficiently (Hewitt & Feldman, 1989). Similar results were obtained with presentation of autoantigen peptide and whole protein by T cell clones (LaSalle et al, 1991). T cells have been demonstrated to present denatured protein in some reports (Treibel et al, 1986), but the purity of the T cell populations was not fully assessed in others reporting the same (Gerrard et al, 1985). Native antigen targeted to T cell surface molecules, such as HIV gp120 or a mouse monoclonal antibody to the transferrin receptor, can be processed and presented in a class II-restricted manner (Lanzavecchia et al, 1988a,b). This implies that T cells are capable of normal processing and presentation of antigens but are limited by their ability to capture antigen.

The differential expression of class II on resting lymphocytes is indicative of a difference in function between the DR-like and DQ-like molecules, and the effect of IFN-g on the expression of these molecules further enhances this hypothesis. If DQ-like molecules are merely acting as antigen presenting molecules for stimulator cells it would be expected that both DR and DQ would be regulated similarly. DQ-restricted precursor $CD4^+$ T cells are relatively frequent in humans, but the proliferative response they induce in autologous or allogeneic MLR is small compared with that against DR molecules (Fujisawa et al, 1991). HLA-DR, -DQ, and -DP molecules are differentially expressed on human T cells after activation (Oshima & Eckels, 1990).

A greater expression of class II molecules on T cells is likely to lead to increased interaction between T cells in which enhancement or suppression of function may be forthcoming. DQ molecules are proposed to be the products of dominant immune suppression (Is) genes and are responsible for stimulating "suppressor-inducer" cells (Altmann et al, 1991). The Is genes would then determine whether, and to what extent, an immune system can produce active suppression to a specific antigen. $CD4^+$, DQ-restricted "suppressor-inducer" cells would be involved in activating an MHC class I-resticted, $CD8^+$ suppressor cell which would in turn act on $CD4^+$, DR-restricted helper T cell.

3.4.4 EFFECTS OF rIFN-g ON MHC CLASS II ON B LYMPHOCYTES.

Class II expression on ovine B cells was at a high level before culture with IFN-g. The expression was increased slightly both in cell numbers expressing class II, and the level of expression by cells. These findings are contrary to those found with human and murine B cells. Class II molecules on human and murine B cells are under a different control mechanism to other cells. They are upregulated by interleukin 4 (IL-4) (Roehm et al, 1984; Noelle et al, 1984; Rousset et al, 1988) and interleukin 10 (IL-10) (Go et al, 1990), but down-regulated by IFN-g (Mond et al, 1986). It can be postulated that during an immune response B cells bind specific antigen via surface immunoglobulin, and subsequently endocytose it. The antigen may then be processed and presented on the cell surface in association with MHC class II. T cells with the appropriate TCR will be induced to secrete cytokines. If the T cell secretes IL-4 or IL-10 it will upregulate the expression of class II and increase the antigen presenting capabilities of the B cell. If however the T

cell secretes IFN-g, it will downregulate class II and hence decrease the antigen presenting ability of the B cell. These cytokines may thus stimulate B cell proliferation or suppression. More work is required to further determine whether IFN-g upregulates class II on ovine B cells, or whether this is an aberant result.

3.4.5 MECHANISMS OF REGULATION OF MHC CLASS II.

Class II genes are a family of related genes encoding proteins of similar function. They are commonly regulated in a coordinate manner with respect to tissue distribution and induction. Typically B cells express all class II genes, and macrophages increase the expression of all class II molecules when stimulated by IFN-g. Cells from patients with Bare Lymphocyte Syndrome typically have loss of expression of all class II genes, which suggests that a defective *trans*-acting factor is required for the expression of all the class II genes (Glimcher & Kara, 1992). However examples of non-coordinate regulation do occur. There are a range of responses to IFN-g even within the clones of a single tumour. Only one of twenty-one clones from a human metastatic melanoma expressed high levels of HLA-DQ after treatment with human recombinant IFN-g, whilst all the clones expressed high levels of -DR and -DP antigens (Anichini *et al*, 1988). Human dermal fibroblasts are differentially affected by IFN-g. Both HLA-DR and -DP-restricted T cell clones were induced to proliferate by IFN-g treated dermal fibroblasts, while -DQ-restricted clones failed to proliferate. Although the expression of DQ was low on these fibroblasts the stimulation of the T cells did not correlate with the quantity of -DR or -DP antigens present (Maurer *et al*, 1987). HLA-DQ expression is commonly not found on haematopoietic precursor cells that express HLA-DR and -DP

molecules (Radka et al, 1986), even though -DQ is expressed with -DR and -DP molecules on mature B cells (Gonwa et al, 1983). A B lymphoblastoid line has been identified which expresses HLA-DQ, but not HLA-DR or HLA-DP (Ono et al, 1991). This clone can be induced to express the other class II genes by cellular fusion with a murine B lymphoma, indicating the defect lies with a trans-acting factor.

The up-regulation of class II DR molecules by IFN-g has been shown to be due to increased transcription (Rosa & Fellous, 1988; Fertsch-Ruggio et al, 1988). Evidence for post-transcriptional control was surmised from data that some cell lines produce mRNA for class II, but no class II antigen is found on the cell surface, and that increases in class II mRNA are twenty-fold the increases in transcription as assayed by nuclear run off (Rosa & Fellous, 1988). Others argue that this effect is due to the long half-life of class II mRNA, but the rapid decline in transcription on removal of IFN-g (Kern et al, 1989).

The marked induction of DR-like molecules and the small effect on DQ-like molecules suggests that the two classes of molecule are under different transcriptional control. The gene promoter sequences of ovine class II genes are unknown, but the human and mouse class II genes have been examined extensively. The 5' upstream flanking regions of most of the functional class II genes contain several cis-acting elements (Figure 20). Two highly conserved enhancer regions upstream of the transcription initiation site are thought to be the major control elements in the class II promoter region. These regions are known as the X and Y boxes (Mathis et al, 1983) and are found between -61 and -108. The Y box is a 10bp motif found approximately 40 to 90 base pairs upstream of the transcription start site, and contains an inverted CCAAT

sequence which is known to be a general transcriptional activator. The X box is located a conserved distance (19 to 20 bases pairs) upstream of the Y box. The X box is composed of 14 base pairs. At the 3' end of the X box and overlapping the 5' interspace region is a seven to eight base pair stretch, known as the X_2 box. Deletion of the X and Y boxes abolishes promoter and enhancer activity in cells and in in vitro transcription systems. The binding of nuclear protein to the Y box of the HLA-DQA gene is markedly reduced compared its binding to other class II gene promoters (Nishimura et al, 1990). A further region, the S box motif, 15 to 17 base pairs upstream from the X box, has an important role. An IFN-g consensus sequence (ATAAGTCAG) at -264 to -256 is found in the 5' regions of MHC class I, class II and β_2-microglobulin (Basta et al, 1987). Enhancer regions are found in the first intron of the HLA-DRA gene which are similar to essential enhancers found in SV40 and polyoma viral enhancers (Sullivan et al, 1987). A "TATA" box is found upstream of the cap site at -30, and is important in accurately initiating transcription (Breathnach & Chambon, 1981). A further control element has been described, the immunoglobulin octamer ATTTGCAT, (at -45 to -52) which was originally found in the promoters of immunoglobulin light and heavy genes, and is known to enhance reporter genes (Parslow et al, 1984). This is found only in HLA-DRA.

A model has been proposed which explains the inducible nature of class II genes in different tissues (Sullivan et al, 1987). Cells with high levels of constitutive class II expression, such as B cells, have active tissue-specific enhancers, active promoters and an absence of repressor factors. In human T cells, which do not have constitutive expression of class II but may be induced to express class II, the promoter region is blocked by repressors but

the enhancer region is functional. The repressors can be bypassed by cell activation, or by viral trans-activators. In tissues with no constitutive expresson of class II, such as antigen presenting cells, the enhancer and promoter regions are inactive but the repressors acting on the promoter can be modified by IFN-g-induced factors, allowing transcription to proceed. In non-inducible cells the promoters and enhancers are non-functional and IFN-g-induced factors cannot remove the repression.

3.4.6 FUTURE WORK.

It may be postulated that ovine class II expression on lymphocytes is acting under a complicated network of enhancers, promoters and trans-acting factors. T lymphocyte expression of class II may be subject to gene-specific factors which upregulate the DR-like gene or downregulate the DQ-like gene. Consequently differential expression of these two gene products is seen upon IFN-g stimulation. The gene control boxes of sheep class II genes have not been sequenced, but future work in the department is intended to pursue this. The effects of IFN-g on this region, either itself or by a second intracellular messenger, would be a useful aspect of this work to follow. There may be an interaction of visna virus with the class II genes if the class II molecules are the cellular receptors for the virus. The mechanism of upregulation of class II expression by lentiferon would be a interesting subject for examination. This does depend upon the existence of lentiferon to be confirmed by gene cloning first.

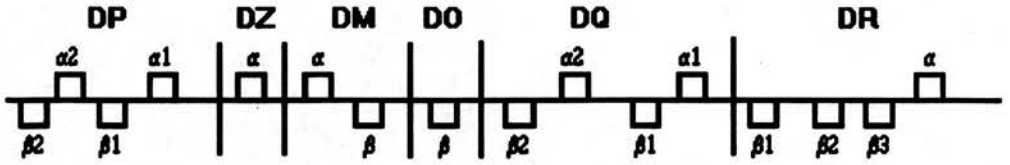


Figure 19. The genetic organization of the human MHC.

Figure 20.

Promoter region of HLA-DR gene 5' to exon 1. The X, Y and Z boxes are marked along with the "TATA" box (T) and the immunoglobulin octomer (O). The numbers refer to the number of nucleotide base pairs upstream of the exon.

Figure 20.

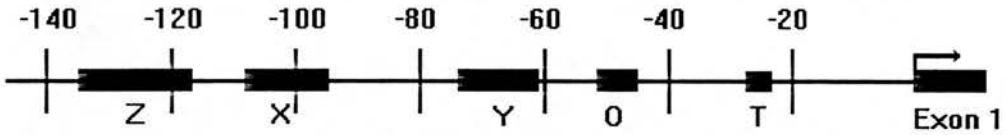


Figure 21.

Induction of MHC class II molecules on ovine ST-6 cells after 24 hours stimulation with lymphocyte conditioned media.

1 Negative control medium

2 Mesenteric lymph node conditioned medium (IL-2 and concanavalin A for 24 hours)

3 Spleen conditioned medium (concanavalin A for 48 hours)

4 Medium cultured with resting spleen cells for 48 hours and then concanavalin A added after harvest

5 Immunopurified ovine class II protein in doubling dilution

Figure 22.

Induction of MHC class II molecules on ovine ST-6 cells after 24 hours stimulation with lymphocyte conditioned media.

1 ST-6 cells stimulated with medium alone

2 Immunopurified ovine class II protein in doubling dilution

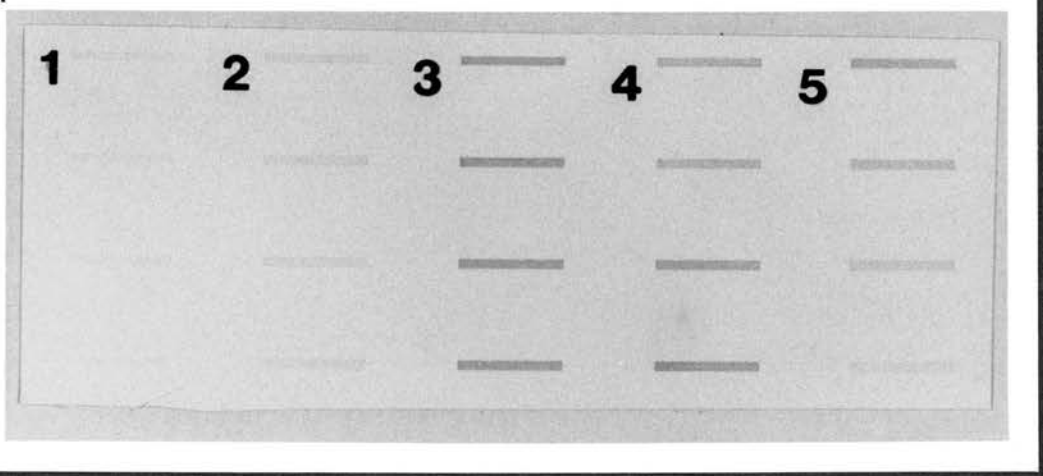
3 Medium cultured with resting mesenteric lymph node cells for 48 hours and then IL-2 and conanavalin A added

4 Mesenteric lymph node conditioned medium (48 hours with IL-2 and concanavalin A)

5 Upper two- spleen conditioned medium
Lower two- as above after blocking by R144 sera

6 Upper two- as above after blocking by R145 sera
Lower two- as above after blocking by preimmune rabbit sera

21



22

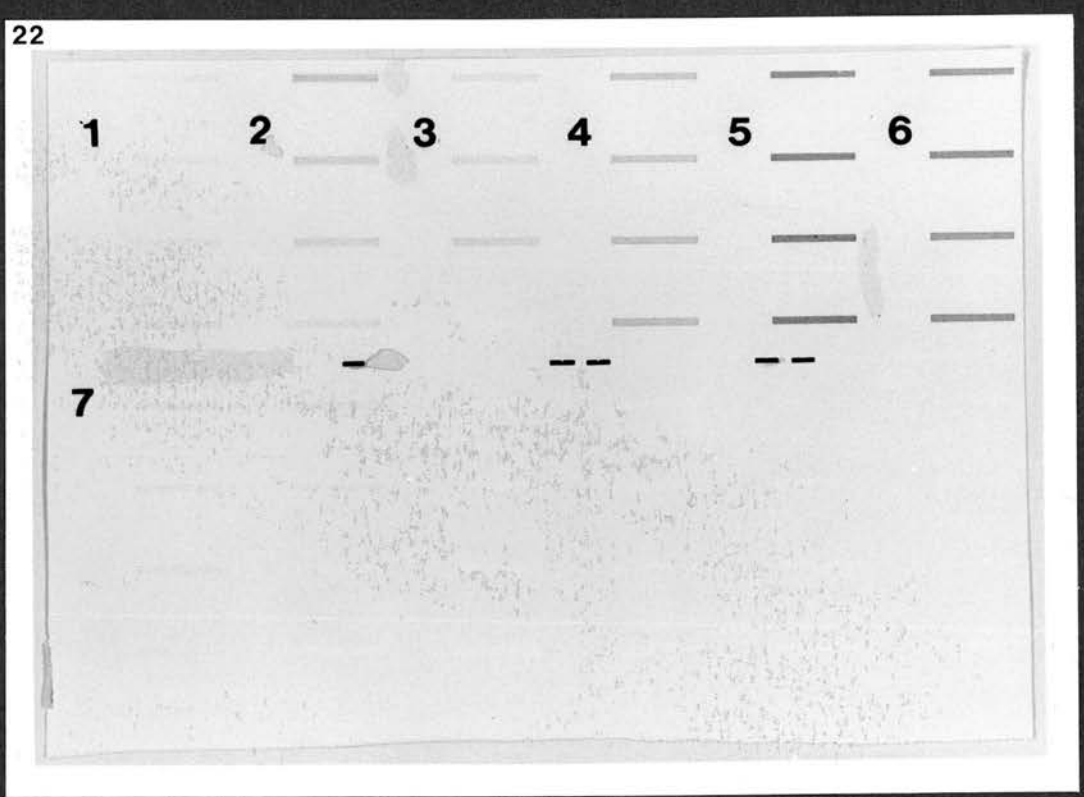
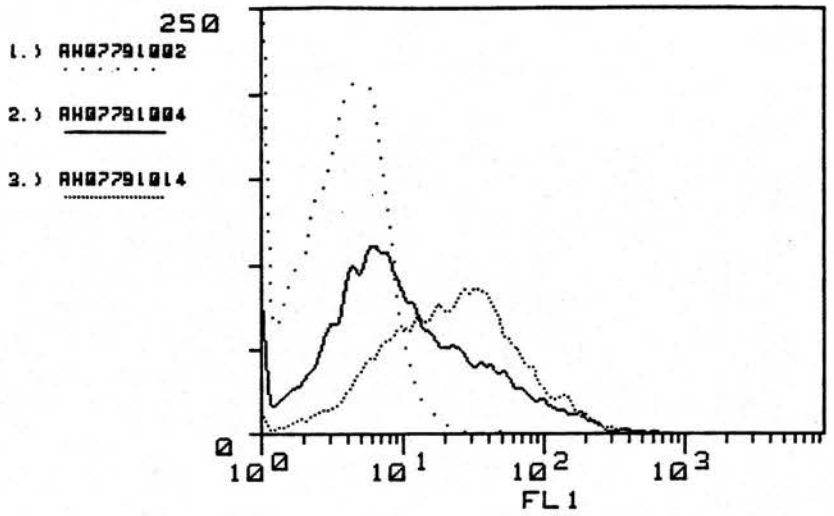


Figure 23.

A. FACS profile $CD4^+$ cells stained for MHC class II DR after incubation for 72 hours with or without bovine IFN-g.
AH07791004 - incubation in absence of bovine IFN-g
AH07791014 - incubation in presence of bovine IFN-g
AH07791002 - cells stained with irrelevant monoclonal antibody

B. FACS profile $CD4^+$ cells stained for MHC class II DQ after incubation for 72 hours with or without bovine IFN-g.
AH07791003 - incubation in absence of bovine IFN-g
AH07791013 - incubation in presence of bovine IFN-g
AH07791002 - cells stained with irrelevant monoclonal antibody

Figure 23. A.


250
1.) AH027910002
2.) AH027910004
3.) AH027910014
0
10^0
10^1
10^2
10^3
FL1

B.

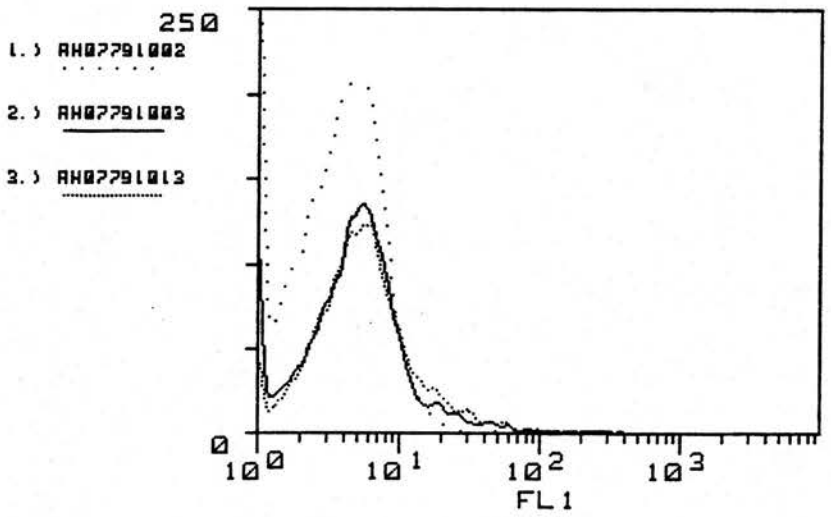
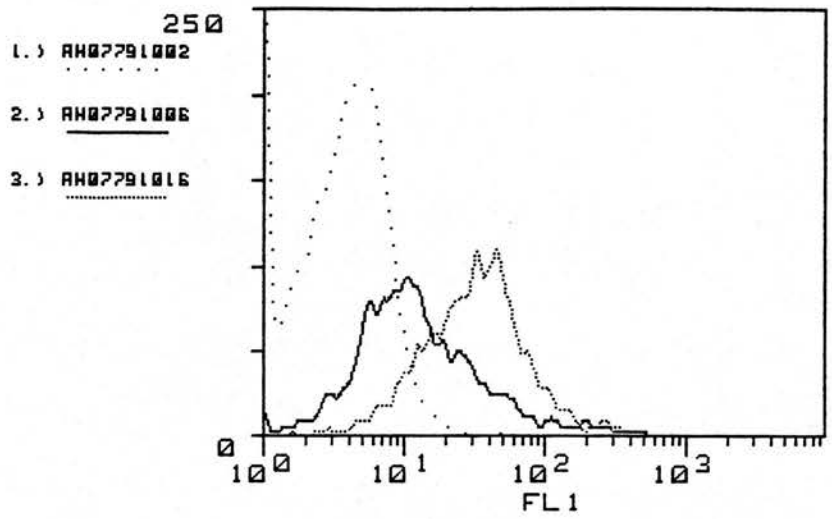

250
1.) AH027910002
2.) AH027910003
3.) AH027910013
0
10^0
10^1
10^2
10^3
FL1

Figure 24.

A. FACS profile $CD8^+$ cells stained for MHC class II DR after incubation for 72 hours with or without bovine IFN-g.
AH07791006 - incubation in absence of bovine IFN-g
AH07791016 - incubation in presence of bovine IFN-g
AH07791002 - cells stained with irrelevant monoclonal antibody

B. FACS profile $CD8^+$ cells stained for MHC class II DQ after incubation for 72 hours with or without bovine IFN-g.
AH07791005 - incubation in absence of bovine IFN-g
AH07791015 - incubation in presence of bovine IFN-g
AH07791002 - cells stained with irrelevant monoclonal antibody

Figure 24. A.


250
1.) AH02791002
2.) AH02791006
3.) AH02791016
0
10^0
10^1
10^2
10^3
FL 1

B.

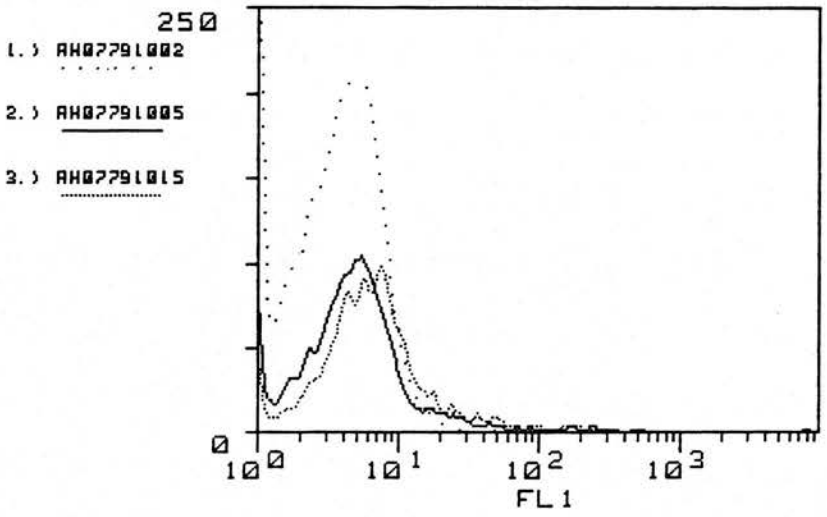

250
1.) AH02791002
2.) AH02791005
3.) AH02791015
0
10^0
10^1
10^2
10^3
FL 1

Figure 25.

FACS profile $T19^{+}$ cells stained for MHC class II DR after incubation for 72 hours with or without bovine IFN-g.

AH07791008 - incubation in absence of bovine IFN-g

AH07791018 - incubation in presence of bovine IFN-g

AH07791002 - cells stained with irrelevant monoclonal antibody

Figure 25.

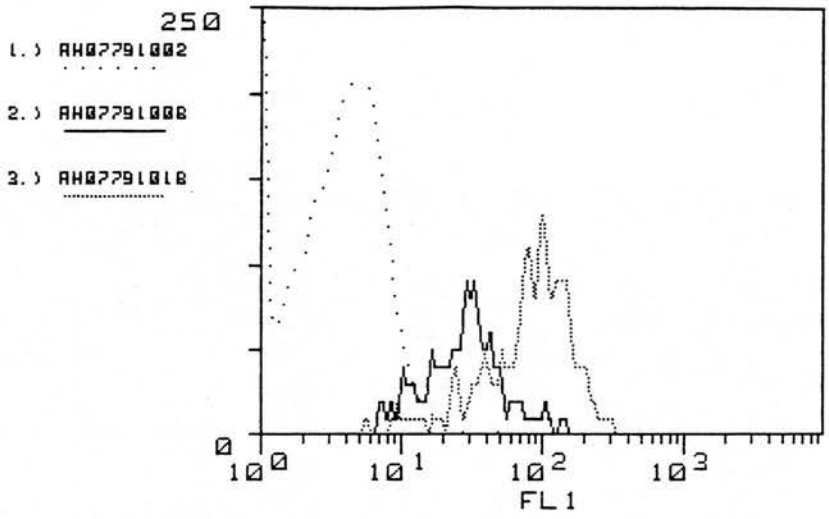
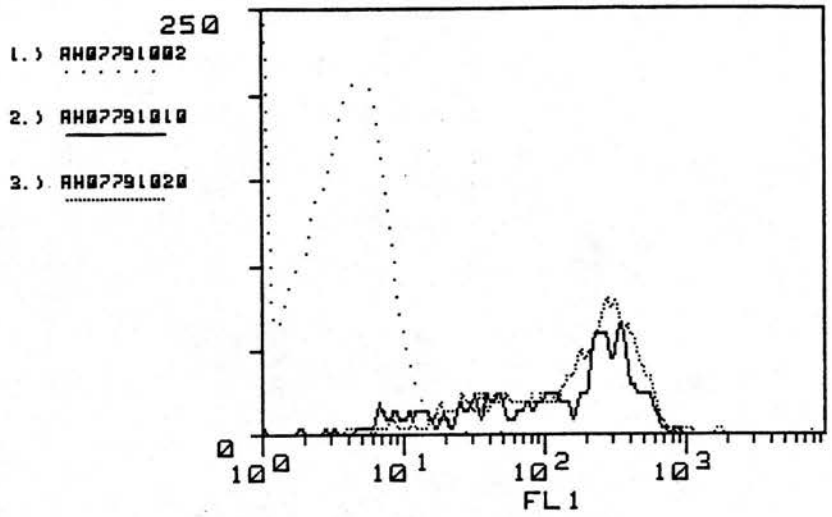

250
1.) AH0279100Z
2.) AH0279100B
3.) AH0279101B
0
10^0
10^1
10^2
10^3
FL1

Figure 26.

FACS profile VPM30^{+} cells stained for MHC class II DR after incubation for 72 hours with or wihtout bovine IFN-g.
AH07791010 - incubation in absence of bovine IFN-g
AH07791020 - incubation in presence of bovine IFN-g
AH07791002 - cells stained with irrelevant monoclonal antibody

B. FACS profile VPM30^{+} cells stained for MHC class II DQ after incubation for 72 hours with or without bovine IFN-g.
AH07791009 - incubation in absence of bovine IFN-g
AH07791019 - incubation in presence of bovine IFN-g
AH07791002 - cells stained with irrelevant monoclonal antibody

Figure 26. A.


250
1.) AH02791002
2.) AH02791010
3.) AH02791020
0
10^0
10^1
10^2
10^3
FL1

B.

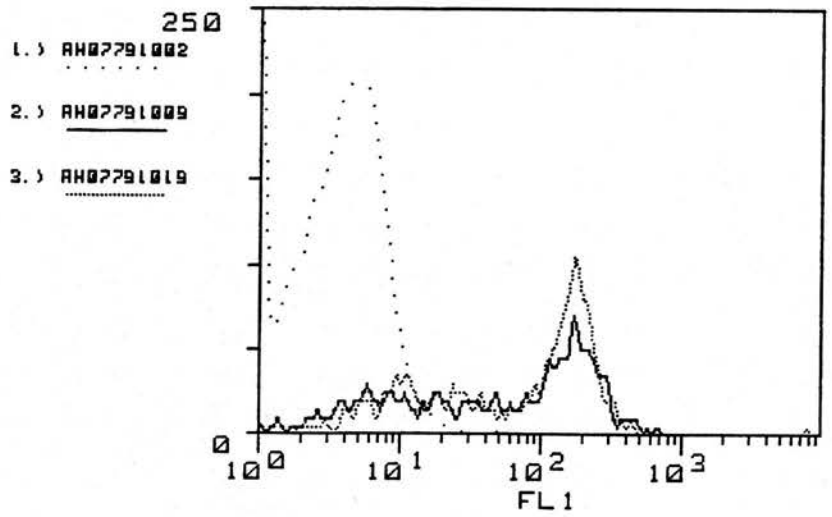

250
1.) AH02791002
2.) AH02791009
3.) AH02791019
0
10^0
10^1
10^2
10^3
FL1

Table 1. Figures from one experiment showing the percentage of each lymphocyte subset positive by FACS for MHC class II DR or DQ molecules before and after incubation with bovine rIFN-g.

Table 2. Figures from the same experiment showing the modal channel number of each lymphocyte subset by FACS for MHC class II DR or DQ molecules before and after incubation with bovine rIFN-g.

Table 3. Figures from the same experiment showing the mean channel number of each lymphocyte subset by FACS for MHC class II DR or DQ molecules before and after incubation with bovine rIFN-g.

Table 1.

PERCENTAGE POSITIVE CELLS

	DRα		DQα	
IFN-g	-	+	-	+
$CD4^+$	17.9	35.5	4.1	5.6
$CD8^+$	16.8	49.8	6.3	7.4
$VPM30^+$	76.0	86.5	59.1	66.1
$T19^+$	37.0	85.6	ND	ND

Table 2.

MODAL CHANNEL NUMBER

	DRα		DQα	
IFN-g	-	+	-	+
$CD4^+$	5.46	12.53	5.50	5.46
$CD8^+$	10.85	34.46	4.73	7.29
$VPM30^+$	270.01	279.94	168.83	181.48
$T19^+$	28.76	105.57	ND	ND

CONTROL MODAL VALUE = 5.46

Table 3.

MEAN CHANNEL NUMBER- VALUES LESS CONTROL

	DRα		DQα	
IFN-g	-	+	-	+
$CD4^+$	21.11	32.50	6.10	8.47
$CD8^+$	23.69	58.67	37.11	14.01
$VPM30^+$	192.57	255.23	120.16	147.84
$T19^+$	78.53	127.10	ND	ND

4 MOLECULAR BIOLOGICAL STUDIES ON OVINE INTERFERON-GAMMA.

4.1 INTRODUCTION.

Early attempts to study the biology of IFN-g were significantly hampered by the lack of a source of pure IFN-g uncontaminated by other cytokines. This was a problem common to most cytokine studies in the early 1980s. Only with production of recombinant IFN-g could the biology of this cytokine be more accurately defined. A number of strategies have been used to clone the IFN-g gene, and in some species the IFN-g gene has been isolated using human IFN-g cDNA as a probe.

4.1.1 CLONING OF HUMAN INTERFERON-GAMMA.

The first isolation and characterization of a cDNA sequence encoding IFN-g was described by Gray et al (1982). They prepared polyadenylated RNA from human peripheral blood lymphocytes that had been stimulated with Staphylococcal enterotoxin B and desacetylthymosin α1 for 48 hours. IFN-g activity was determined by translation of RNA in Xenopus laevis oöcytes and subsequent assay in a standard antiviral assay. RNA from the most active fraction was converted to cDNA and cloned into pBR322. Transformed bacterial clones were transfered onto nitrocellulose filters and replicate filters were screened with one of two probes. One filter was screened with ^{32}P-labelled cDNA prepared from the RNA of the stimulated lymphocytes, the other filter was screened using a similar probe derived from unstimulated lymphocytes producing no detectable interferon. Positive clones were isolated. The longest open-reading frame found was for a protein of 166 amino acid

predicted length. The first 20 amino acids were hydrophobic and thought to constitute a signal sequence similar to those of the IFN-α. The cDNA obtained in the initial isolation was subsequently used to assess the structure of the IFN-g gene (Gray & Goeddel, 1982).

At the same time Devos et al (1982), isolated a very similar clone coding for the identical protein. One further isolation of human IFN-g cDNA is reported, in which the isolate was identical in amino acid sequence except for the presence of a glutamine residue instead of a lysine residue at position 9. This was expressed and found to be active (Nishi et al, 1985).

Other papers have reported the construction of synthetic genes for human IFN-g (Alton et al, 1983; Tanaka et al, 1983) which have been used for recombinant protein synthesis.

Analysis of all the sequences for human IFN-g describe a mature protein length of 146 amino acids with a hydrophobic signal sequence of 20 amino acids, but amino-terminal sequencing of the natural mature protein showed it to begin with a pyroglutamate residue and not the predicted cysteine (Rinderknecht et al, 1984). This was thought to occur by cleavage of the translated protein at residue 24, a glutamine, and subsequent cyclization of this to pyroglutamate. Furthermore, DNA constructs which code for synthesis of IFN-g containing the Cys-Tyr-Cys residues at the amino-terminal end produce protein which has only 10% of the antiviral activity of recombinant protein lacking these residues (Burton et al, 1985). The carboxyl-terminus of natural IFN-g seems to be quite variable and at least six different termini have been observed.

4.1.2 CLONING INTERFERON-GAMMA GENES OF OTHER SPECIES.

The cDNA isolated for human IFN-g has been used as a probe to isolate murine IFN-g from a genomic phage library (Gray & Goeddel, 1983), rat IFN-g from a genomic phage library (Dijkema et al, 1985) and bovine IFN-g from a cDNA phage library (Cerretti et al, 1986).

Comparison of the species sequences shows all of them to code for proteins containing two potential N-glycosylation sites, although not in conserved sites. This has been demonstrated in human IFN-g and explains the two molecular species resolved on SDS-PAGE gels. The larger species of 25,000 Da has both sites glycosylated, whilst the smaller species of 20,000 Da has only the first site glycosylated (Rinderknecht et al, 1984). Only a small amount of nonglycosylated protein (15,500 Da) has been detected (Kelker et al. 1984). The sequences of the various species of IFN-g thus far cloned have a relatively low degree of identity. Human and bovine IFN-g have 63% identity (Cerretti et al, 1986), while human and rodent IFN-gs have only 40% identity (Gray & Goeddel, 1983; Dijkema et al, 1985). These low degrees of identity explain the species specificity of IFN-g action on cells.

4.1.3 GENOMIC STRUCTURE OF INTERFERON-GAMMA.

The human cDNA isolated by Gray et al (1982) was used as a probe in a Southern hybridization of human genomic DNA. The hybridization revealed that the IFN-g gene was either present as a single gene copy or as a tandem gene. Hybridization to a 3' untranslated region of the gene showed only one band in all the hybridizations, hence supporting the theory that IFN-g is a single gene (Gray & Goeddel,

1982). The cDNA has been used to isolate clones from genomic libraries and determine the structure of the gene. Human IFN-g was shown to have three introns by both sequencing of clones (Gray & Goeddel, 1982) and also by electron microscopy of DNA-cDNA heteroduplexes (Taya et al, 1982). The smallest intron was found in a region conserved between the three interferon species (α,β & g), although both α and β have no introns. Murine and rat IFN-g genes have similar structure. The human gene was found to be on chromosome 12 (Naylor et al, 1983; Trent et al, 1982), whilst the genes for IFN-α and -β are on chromosome 9 (Owerbach et al, 1981; Shows et al, 1982; Trent et al, 1982). IFN-g has been shown by Southern blotting to be a single-copy gene in human, mouse, rat and cattle. This is in marked contrast to the interferon-α family of genes of which a dozen members have been found to date (Capon et al, 1985).

4.1.4 SOME OBJECTIVES WITHIN THE THESIS.

In order to dissect cytokine functions, it is now becoming virtually essential to know the cytokine gene sequence. This allows synthesis of recombinant cytokine uncontaminated by other proteins. Recombinant protein allows experiments to define the role of that particular cytokine in immune phenomena. The production of large quantities of a recombinant cytokine allows development of monoclonal antibodies to it which may be used to neutralize its effects or to develop a sensitive and specific assay for native protein. Very large quantities of recombinant cytokines may have therapeutic uses (eg IFN-g as a vaccine adjuvant).

The isolated gene, once cloned, can itself be used to study the

structure of the genomic gene(s) and their activation. It can also be used to assess mRNA levels on a variety of cells and various states of activation. It also allows analysis of the cytokine-receptor interactions by altering the functional regions of the cytokine using site-directed mutagenesis of the gene.

The essential for all these potential experiments is having the gene cloned, sequenced and available in ready quantities. Unfortunately ovine IFN-g had not been cloned at the beginning of this project. A strategy to obtain the cDNA for part of this gene was therefore devised.

4.1.5 CLONING STRATEGIES AVAILABLE.

Several methods are available to clone a gene when the gene has been sequenced in other species. Commonly used methods have involved synthesizing double-stranded DNA from poly-adenylated RNA and inserting this into a lambda phage vector, thus creating a phage library which can be screened with:

1) a closely-related labelled DNA sequence (ie bovine, human, murine or rat IFN-g);

2) a labelled oligonucleotide corresponding to an assumed conserved region of the gene;

3) a mixed oligonucleotide derived from amino acid sequence data which contains all the possible codons for the amino acids;

4) an antibody to the desired protein (i.e. antisera to recombinant bovine IFN-g) in order to detect recombinant protein sequences expressed as fusion proteins in appropriate phage vectors. Antibodies can be used to screen similar libraries in mammalian or insect cell expression vectors.

An alternative is to clone the species genome into a phage vector (genomic library) and screen with labelled DNA or oligonucleotide. This method does not enrich the desired sequence prior to screeing, and consequently many more phage must be screened before it is likely that the desired clone is found. When the nucleotide sequence of the gene itself or a related-species gene is known the polymerase chain reaction may be used.

4.1.5.1 NUCLEIC ACID HYBRIDIZATION.

The isolation of a cDNA clone for a gene by a nucleic acid hybridization protocol is a very specific method in which the degree of specificity can be altered by changing the washing conditions. The cDNA clone will yield data on the gene sequence and will allow manipulation of the gene for expression experiments.

4.1.5.2 ANTIBODY SCREENING OF EXPRESSION VECTOR.

Isolation of a clone by the antibody screening method has the advantage that the gene is in frame and expressed already by the bacteria. λgt11 (Young & Davis, 1983b) and λZAP (Short et al, 1988) are phage vectors which contain cloning sites within the lacZ gene and are under a strong E.coli promoter. A gene sequence cloned within this site, if in the correct orientation and reading frame, will be transcribed and translated with the β-galactosidase gene to form a fusion protein. Fusion of a foreign protein into a stable E. coli protein can enhance the stability of the foreign protein and this has been demonatrated in several cases (Kupper et al, 1981; Stanley, 1983; Davis et al, 1981). The infected bacteria may be grown up in large quantities for the generation of fusion protein, which may represent from 0.1% up to 4% of the total E.coli protein

(Young & Davis, 1983b; Snyder et al, 1987). Isolation of genes is best performed with affinity-purified antisera to the protein, or with a pool of monoclonal antibodies (Young & Davis, 1983a). Polyclonal antisera commonly contain antibodies reactive to E. coli proteins which must be removed prior to screening. Monoclonal antibodies may react with similar epitopes present on unrelated proteins, hence single monoclonal antibodies should not be used for screenings. The success of a library screening is largely dependent on the quality of the library and the probe.

4.1.5.3 POLYMERASE CHAIN REACTION.

More recently the very powerful technique of polymerase chain reaction (PCR) has allowed the rapid and relatively easy cloning of many genes for which the sequence was known for one species (Saiki et al, 1985). Oligonucleotide primers are chosen from the known sequence which may be expected to bind to the same gene in another species. An oligonucleotide identical to a 5' region and an oligonucleotide complementary to a 3' region are used. Employing these primers the PCR specifically amplifies the sequence of the gene between the primers. A single amplified product of roughly known size is expected. The power of the technique is such that a very small quantity of template DNA is required (down to femtomole levels). Each round of extension theoretically doubles the quantity of amplified DNA present. A PCR of 30 cycles will generate 2^{28} copies of the gene product, assuming 100% efficiency. Thus beginning with 1ng of template (the region between the primer sites), 268µg of product could be generated. Realistically however substrates become limiting before this level and efficiencies are initially only 80-90%.

The polymerase chain reaction depends for its initiation on the annealing of oligonucleotide primers to the template DNA. Primers should be complementary to the template strands of DNA. Perfectly complementary primers are ideal but mismatches can be overcome by a lower annealing temperature. This does, however, allow promiscuous binding to similar sequences in unrelated genes present in the DNA. Consequently spurious bands or smearing may be seen on a gel.

The PCR technique is a rapid technique for isolating DNA sequences, and was the first choice for the cloning of a section of the ovine IFN-g cDNA as the bovine sequence was already known.

4.1.6 EXPRESSION OF RECOMBINANT INTERFERON-GAMMA.

Recombinant IFN-g has been expressed in a number of cell types. Gray et al (1982) were the first to express the human protein, both in E. coli and in monkey COS-7 cells. E. coli lysates had activity of 250 units/ml, whilst COS-7 cells produced 50-100 units/ml. This indicated that glycosylation was not essential for IFN-g activity. Devos et al (1982) transformed AP8 monkey cells with their cDNA isolate to yield a clone producing 100 units/ml. Chinese hamster ovary cells have been transformed amd found to produce IFN-g at high levels (20,000-100,000 units/ml) (Haynes & Weissmann, 1983; Scahill et al, 1983). Yeast cells have also been used in production of recombinant IFN-g (Derynck et al, 1983) but relatively low expression was achieved. Tanaka et al (1983) synthesized the recombinant protein using the novel technique of chemically synthesizing the gene from 62 oligonucleotides incorporating codons known to be used by genes highly expressed in E.coli. Despite this, the expression attained was poor. This was thought to be due

to inefficient promoter function. Codon usage is known to be different between eukaryotic and prokaryotic cells, and it is thought that this may be a significant reason for the poor yields of IFN-g obtained from bacterial and yeast cultures. Non-glycosylation of the protein was initially considered to be a potential problem as the sugar residues were of unknown relevance to the functioning of the protein, however bacterial recombinant protein is active to a comparable level.

Bovine IFN-g has been expressed in E.coli to a level of 115,300 units/ml in an antiviral assay (Cerretti et al, 1986). It has also been expressed in E. coli by Ciba Geigy to a purity of 2.5×10^6 units/mg of protein (Data Sheet, Ciba Geigy).

4.2 MATERIALS AND METHODS.

4.2.1 ISOLATION OF RNA FOR USE AS TEMLATE FOR PCR.

Mesenteric lymph nodes were obtained from a freshly killed sheep. Lymphocytes were teased from the node under sterile conditions using forceps and scissors into ice-cold Hanks Buffered Salt Solution. Debris was allowed to settle for five minutes before cells were pipetted from the debris and centrifuged three times at 400xg for 5 minutes, resuspending in RPMI 1640 medium containing 5% foetal calf serum after each spin. The lymphocytes were resuspended in 10ml of medium and counted. They were then cultured at 5 x 10^6 cells/ml in RPMI 1640 medium containing 10% foetal calf serum, 100mM L-glutamine and 100mM sodium pyruvate in a 5% CO_2 atmosphere. Concanavalin A (Sigma) (5µg/ml) was added at the beginning of the incubation period. The cells were cultured for eight hours before being washed twice in ice-cold PBS before lysis in 20ml of 4M guanidinium thiocyanate (GTC) (Appendix 4).

RNA was isolated by the method of Chirgwin et al (1979). 10ml of the GTC lysate was layered above 2ml of 5.7M caesium chloride in a Beckman SW40Ti polyallomer ultracentrifuge tube. The tubes were centrifuged in a Beckman L70 ultracentrifuge at 28000 RPM for 16 hours at 4°C. The GTC and $CsCl_2$ were removed and the pellet containing RNA washed with 70% ethanol. The pellet was resuspended in 500µl of diethylpyrocarbonate (DEPC)-treated distilled water and the RNA was precipitated with 2 volumes of ethanol at -70°C overnight before centrifugation at 12000xg for 10 minutes. At this stage the pellet was resuspended in water and then extracted in an equal volume of phenol/chloroform and then chloroform to remove cellular protein. It was then precipitated with 2 volumes of 100%

ethanol before the pellet was finally resuspended in water and the concentration of RNA was estimated from spectrophotometry at 260nm and 280nm.

Single-stranded cDNA was synthesized from the isolated RNA using oligo dT-primed reverse transcription of 1.28µg of total RNA (Amersham cDNA synthesis kit) (Gubler & Hoffman, 1983). Incorporation of α-^{32}P-dCTP in this reaction allowed the yield of cDNA to be estimated. Stock cDNA at 1.7ng/µl was used in polymerase chain reaction experiments to clone part of the ovine interferon-gamma gene.

4.2.2 POLYMERASE CHAIN REACTION TO CLONE PART OF THE OVINE INTERFERON-GAMMA GENE.

4.2.2.1 POLYMERASE CHAIN REACTION.

Primers used for PCR were designed from the bovine interferon-gamma sequence (Cerretti et al, 1986). Primers contained 20 or 21 deoxynucleotides. The 5' primer was identical and the 3' primers were complementary to the coding strand of bovine interferon-gamma.

PRIMER	SEQUENCE	NUCLEOTIDES
343E (5')	5'-GGGCCTCTCTTCTCAGAAATT-3'	238-258
474F (3')	5'-GAGGTTAGATTTTGGTGACAG-3'	520-540
344E (3')	5'-TGACTTCTCTTCCGCTTTCT-3'	541-560

Primers were synthesized by the Oswel DNA Service (University of Edinburgh), purified by HPLC, and dissolved in sterile distilled water. Each oligonucleotide was supplied with the 5' terminus hydroxylated.

PCR was carried out in 100µl reaction volumes containing 0.4µM primers, 250µM deoxyribonucleotides (Pharmacia), 6.7mM $MgCl_2$, 170µg/ml bovine serum albumin, 67mM Trizma pH 8.8, 1.7ng single-stranded cDNA, and 1.5U of Taq polymerase (Boehringer Mannheim, Gmbh, Germany). Automatic thermal cycling was performed on a Techne PHC-1 machine, using temperatures of 95°C for 0.6 minutes, 53°C for 0.6 minutes and 75°C for 1.5 minutes. Thirty cycles were performed. A final extension at 75°C for 3 minutes was employed to complete synthesis of most products. 10µl of the reaction was run on a 2% agarose gel to detect products.

4.2.2.2 CLONING OF THE PCR PRODUCT.

The DNA was to be cloned into the blunt-ended Sma I site of pTZ18R (Pharmacia), consequently the PCR products were modified prior to gel purification to ensure that most strands had blunt ends and that 5' ends were phosphorylated and could be ligated.

The PCR reaction mix was cleaned to remove protein and deoxyribonucleotides. It was extracted with an equal volume of phenol/chloroform, twice with equal volumes of chloroform, and finally precipitated by the addition of 1/10 volume of 3M sodium acetate and two volumes of 100% ethanol. The DNA was held at -20°C for 1 hour to allow precipitation and then centrifuged at 12000xg for 10 minutes. The pellet was washed with 70% ethanol and vacuum dried. DNA was resuspended in distilled water before treatment with

1 Unit of Klenow fragment of DNA polymerase (NBL, UK) in T4 kinase buffer (Appendix 4) for 30 minutes at 30°C to ensure the PCR product had blunt ends. The reaction was then treated with 1mM ATP and 1 Unit of T4 kinase (NBL, UK) for 1 hour at 37°C to phosphorylate the 5' termini.

The PCR product was gel purified on a 1.5% Seaplaque (FMC) low-melting temperature agarose gel in 1 x TAE (Appendix 4) and containing 0.5µg/ml ethidium bromide, run at 4V/cm for 3 hours. The band was visualized and cut out of the gel whilst viewing under UV light.

4.2.2.3 PREPARATION OF pTZ18R VECTOR.

5µg of pTZ18R plasmid were digested by Sma I restriction endonuclease (NBL, UK) to linearize the phagemid and leave blunt ends. 1.5µg of phagemid was then dephosphorylated at its 5' termini using calf intestinal phosphatase (CIP, Boehringer Mannhein, Gmbh, Germany). Treatment was with 1 Unit for 15 minutes at 37°C, followed by 1 unit for 45 minutes at 56°C. This was to prevent self-ligation by recircularization of the DNA. The DNA was purified by phenol/chloroform extraction, chloroform extraction, ether extraction and ethanol precipitation.

4.2.2.4 LIGATION OF PCR PRODUCT INTO pTZ18R.

Ligation was performed in agarose using the method of Struhl (1985). PCR product in Seaplaque agarose was heated to melt the agarose (70°C for 10 minutes). 200ng of linearized, CIP-treated pTZ18R was

combined with 0ng, 200ng or 400ng of the PCR product in ligation buffer (Appendix 4) with 5 Units of T4 ligase, and incubated at 15°C for 16 hours.

4.2.2.5 BACTERIAL CELL TRANSFORMATION AND ISOLATION OF PLASMID DNA.

Escherichia coli, strain JM101 (Yannish-Perron et al, 1985), was the recipient bacteria for recombinant plasmids derived from pTZ18/19. It has the following genetic characteristics:

F' traΔ36 proA$^+$ proB$^+$ lacI^q lacZΔM15/supE thi Δ(lac-proAB)

Competent stock was prepared according to the method of Chung et al (1989). Overnight cultures of JM101 cells were centrifuged at 1500xg for 10 minutes and resuspended in 1/10 volume of 1 x TSS (Appendix 4). Cells were frozen at -70°C in 100µl aliquots until required. When cells were to be transformed, an aliquot was thawed on ice, plasmid added, and kept on ice for 20 minutes to allow DNA uptake. 0.9ml of LB medium (Appendix 4) was then added and the suspension shaken at 37°C for 60 minutes to allow expression of the antibiotic resistance gene. Cells were plated out on LB agar plates containing ampicillin, X-GAL and IPTG (Appendix 4) to select for recombinant plasmids by colour change. Plates were incubated at 37°C overnight. Bacteria containing plasmid without an insert ligated in the lacZ gene expressed functional β-galactosidase activity and converted X-GAL to a blue colour, but bacteria containing plasmids with inserts were defective for β-galactisidase enzyme and were white.

White colonies which were picked for analysis. The miniprep method of Serghini et al (1989) was used. 1.5ml of an overnight culture of transformed bacteria was pelleted, resuspended in 50µl TNE (Appendix 4) and vortexed with 50µl phenol/chloroform/isoamyl alcohol (25/24/1) to release plasmid DNA into the aqueous phase. The microtube was centrifuged at 12000xg for 5 minutes and the aqueous phase collected. The DNA was precipitated by addition of ammonium acetate to 2M final concentration and then 2 volumes of 100% ethanol.

Plasmid DNA was prepared in a larger quantity by the alkaline lysis technique (Birnboim & Doly, 1979). 250ml overnight cultures of transfected bacteria were pelleted and resuspended in 2ml of GTE buffer (Appendix 4). 0.5ml of GTE with 25mg/ml hen egg white lysozyme (Sigma) was added to break down the bacterial cell wall and the mixture incubated for 10 minutes at room temperature. 5ml of fresh 0.2M NaOH/1% SDS was stirred into the mixture and incubated for 10 minutes on ice, and a further 10 minutes on ice was allowed after addition of 3.75ml of 3M potassium acetate pH5.5. This step precipitated the bacterial chromosomal DNA but left the plasmid DNA in solution. The supernatant was collected after centrifugation at 15000xg for 10 minutes in a Beckman JA-20, and the plasmid DNA was precipitated with 0.6 of a volume of isopropanol for 20 minutes at 20°C. The pellet was washed with 70% ethanol before being dissolved in 2ml of TE (Appendix 4). RNA was removed by RNase A digestion (20µg/ml at 37°C for 20 minutes). The solution was extracted with phenol once, and with phenol/chloroform once before precipitation of the DNA by addition of an equal volume of 4M ammonium acetate and two volumes of ethanol. Further purification of plasmid DNA was

obtained by addition of 0.4 volumes of a solution containing 30% PEG 8000 and 1.6M NaCl, incubation at 4°C overnight and subsequent centrifugation at 12000xg for 10 minutes (Lis, 1980).

The plasmid containing the ovine IFN-g sequence was designated pOVifn.

The insert was excised from the plasmid by EcoRI and HindIII digestion, and was gel purified twice on Seaplaque low-melting agarose to remove any plasmid DNA. This fragment was used as a probe in hybridization experiments.

4.2.3 SEQUENCING PLASMID INSERT.

4.2.3.1 PREPARATION OF SINGLE-STRANDED DNA TEMPLATE FOR SEQUENCING.

The method of production of ssDNA from pTZ18R has been described (Zoller & Smith, 1983). E. coli strain JM101 containing the relevant plasmid was grown in 2xYT (Appendix 4) + 150µg/ml ampicillin to an OD_{660nm} of 0.5-0.8. 400µl of this was infected with M13K07 helper phage at a moiety of infection (MOI) of 10 (10 pfu/cell). This was shaken for 1 hour at 37°C, and then 10ml of 2xYT with kanamycin (70µg/ml) and ampicillin (150µg/ml) added. This culture was shaken at 37°C overnight.

Purification of the ssDNA was achieved by firstly removing all cells by repeated centrifugation and then a final filtration through a 0.22µm filter. The phage particles were precipitated on ice for 30 minutes with 1/4 volume of 20% PEG 8000 in 3.5M NaCl.

Centrifugation at 12000xg for 30 minutes at 4°C yielded a pellet which was resuspended in NTE prior to phenol/ chloroform extraction and ethanol precipitation.

4.2.3.2 SEQUENCING OF PLASMID INSERT.

DNA sequence was obtained using the Sanger dideoxynucleotide chain termination method (Sanger et al, 1977). A BIORAD Sequi-Gen Nucleic Acid Sequencing Cell was used to run a 6% acrylamide/7.5M urea/0.5xTBE gel. Polymerization was initiated with 1µl/ml 6.6M TEMED (BIORAD) and 1µl/ml 25% ammonium persulphate.

Labelling and chain termination was performed using a USB Sequenase kit to incorporate ^{35}S-dCTP. The gel was run at 2500V for 4 hours. Samples were loaded at 0 or 2 hours to allow different separation of parts of the sequence. The gel was dried onto 3M filter paper for 2 hours at 80°C and autoradiographed on Kodak X-AR film overnight.

4.2.4 OVINE INTERFERON-GAMMA CLONING FROM LAMBDA PHAGE LIBRARY.

4.2.4.1 LAMBDA PHAGE LIBRARIES USED -- λgt11 AND λZAP.

Two lambda phage cDNA libraries were obtained from Dr. Wayne Hein, Basel Institute for Immunology. T lymphocytes activated with Concanavalin A for 24 hours or 72 hours were the source of RNA. The cDNA inserted was synthesized from a mix of this RNA. The initial generation of the λgt11 library has been described (Hein et al,

1989). Both \gt11 (Young & Davis, 1983b) and \ZAP I (Short et al, 1988) libraries were obtained in amplified form. The initial titres of the unamplified libraries were unknown.

4.2.4.2 BACTERIAL STRAINS USED IN PHAGE WORK.

A number of strains of E. coli were used during screening of lambda phage, and also during the production of lysogens. (Young et al, 1985; Short et al, 1988)

STRAIN	GENOTYPE	PHAGE
Y1090	Δ lacU169 proA+ Δ lon araD139 strA supF [trpC22::Tn10] (pMC9)	λgt11
Y1089	lacU169 proA+ Δ lon araD139 strA hflA150 [chr::Tn10] (pMC9)	λgt11
BB4	supF58 supE44 hsdR514 galK2 (rk-, mk+) galT22 trpR55 metB1 tonA lambda- Δ(arg-lac)U169 [F' proAB $lacI^{q}ZΔM15$ Tn10(tet^{R})]	λZAP I

4.2.4.3 SCREENING A BACTERIOPHAGE LIBRARY.

The λZAP library was screened on E. coli strain BB4. The λgt11 library was screened on E. coli strain Y1090. Initial infection of the bacteria was the same for both λ strains (Current Protocols in Molecular Biology, eds: Ausubel FM et al).

The specific cell strain was grown to saturation in LB broth with 0.2% maltose. 0.6ml of this culture was added to phage stock containing approximately 10000 phage and the mix incubated at room temperature for 20 minutes to allow phage adsorption. The tubes were then incubated at 37°C for a further 10 minutes to allow phage DNA injection. 7.5ml of warm LB top agarose was added to the cells, mixed and poured on a 150mm bacterial plate containing solid LB bottom agar (Young et al, 1985). At this point the screening method determined the incubation regime and subsequent treatment. Two main methods of screening were used: (i) DNA hybridization with a labelled probe, (ii) detection of fusion proteins with labelled antibodies.

4.2.4.4 SCREENING λZAP LIBRARY BY OLIGONUCLEOTIDE HYBRIDIZATION.

The plates prepared in 4.2.4.3 were incubated at 37°C overnight and then allowed to cool for 30 minutes. Nitrocellulose filters (Millipore, HATF13750) were applied sequentially to each plate for 2 minutes, marked with a needle for orientation later and then air-dried. The filters were alkali denatured for 2 minutes, neutralized for 2 minutes and equilibrated for 2 minutes before baking at 80°C in a vacuum oven for 2 hours. (Appendix 4 for solutions).

A complementary oligonucleotide was designed from the bovine interferon-gamma sequence. This oligonucleotide was used in the PCR but subsequently discarded. It had the following sequence from the 3' end of the cDNA:

PRIMER	SEQUENCE	NUCLEOTIDE
344E (3')	5'-TGACTTCTCTTCCGCTTTCT-3'	540-560

The oligonucleotide was labelled by 5' end-labelling using T4 kinase to incorporate ^{32}P-ATP (400Ci/mmol) (Appendix 4).

The filters were washed three times in 3xSSC/0.1%SDS at 20°C, and once at 65°C in the same solution for 90 minutes to remove bacterial debris. They were then prehybridized in prehybridization solution (Appendix 4) for 1 hour at 37°C. Filters were then hybridized with labelled oligonucleotide probe for 16 hours at 42°C in hybridization solution (Appendix 4), washed in 6xSSC/0.05% sodium pyrophosphate at 20°C for 75 minutes with 5 changes of solution, and then in the same wash buffer at 55°C for 30 minutes. The filters were exposed to radiographic film (Kodak X-AR) at -70°C for 18 hours to register areas where specific hybridization had occurred.

4.2.4.5 SCREENING λZAP WITH THE PCR PRODUCT.

4.2.4.5.1 LABELLING pOVifn FOR USE IN SOUTHERN HYBRIDIZATIONS.

The insert from pOvifn was labelled to high specificity with α-^{32}P-dCTP (400 Ci/mmol or 3000 Ci/mmol, Amersham). 25-100ng of DNA was heat denatured and annealed to random hexanucleotides. It was

extended by Klenow fragment of E. coli DNA polymerase (NBL, UK) to incorporate the labelled nucleotide. Incorporation of label was assessed by trichloroacetic acid precipitation on glass filters, and Cherenkow counting. DNA was labelled to between 5 x 10^8 and 1 x 10^9 cpm/µg.

4.2.4.5.2 HYBRIDIZATION PROTOCOL.

Lambda phage were plated out as above. The filters were prehybridized in Prehybridization solution 1 for 3 hours (Appendix 4). The probe was generated by random-primed radiolabelling (Feinberg & Vogelstein, 1983) of the PCR product cut from the plasmid, to a specificity of 3.7 x 10^7 cpm/µg. The filters were hybridized for 3 days at 42°C in Hybridization solution 1 (Appendix 4), before washing with 2 x SSC/ 0.1% SDS four times of 10 minutes, and 0.2 x SSC/ 0.1% SDS four times of 20 minutes at 65°C. The dried filters were autoradiographed against Kodak X-AR film.

4.2.4.6 ANALYSING λZAP BY PCR.

The λZAP library was assessed for the presence of interferon-gamma sequence by PCR (modified from Saiki et al, 1988). One microlitre of dilutions of the library representing 10^5 pfu/µl and ten-fold dilutions were added to PCR using primers 343E and 474F and the original conditions were used (see section 4.2.2.1). After 30 cycles 10µl of the reaction products were separated by electrophoresis on a 1% agarose gel and the gel viewed under UV light.

4.2.4.7 ANTIBODY SCREENING OF λgt 11.

4.2.4.7.1 PRETREATING SERA TO REMOVE ANTI-E. COLI ANTIBODIES.

To remove the E. coli reactivity contained in the polyclonal rabbit sera it was incubated with E. coli proteins (Young & Davis, 1983a).

Briefly E. coli Y1090 cells were grown to saturation, pelleted and resuspended in 1/30 volume of TE. The cells were freeze-thawed three times to lyse them, and then sonicated on ice. The supernatant was coated onto Hybond C (Amersham) nitrocellulose and the antisera incubated with this coated nitrocellulose for 24 hours at 4°C. This was repeated.

Test strips of Y1090 protein spotted on Hybond C were processed as for phage filters. Serum was only used when Y1090 reactivity had decreased at 1/500 dilution.

4.2.4.7.2 SCREENING λgt11 WITH ANTI-BOVINE INTERFERON-GAMMA RABBIT SERA.

The λgt11 library was plated out at 20000 pfu per 150mm bacterial plate in 0.7% LB agarose onto 1.5% LB agarose containing ampicillin. Ten plates were used. They were incubated at 42°C for 3.5 hours, before a dry nitrocellulose filter (Millipore, HAHY 13750), previously soaked in 10mM IPTG (Appendix 4), was laid on the top agar. The plates were incubated for 3 hours at 37°C. The filters were removed and replaced with similar ones for a further 3 hours.

All the filters were then treated in a similar manner. and all procedures were carried out at 4°C. Filters were initially washed in TNT (Appendix 4) for 10 minutes and then blocked in 5% dried cow's milk (Marvel, Cadburys) in TNT buffer overnight. Filters were incubated for 2.5 hours in pre-absorbed rabbit antisera at 1/500 in blocking buffer. They were washed in blocking buffer three times over 30 minutes. Filters were then incubated in 1/2500 anti-rabbit IgG biotin (Sigma) in blocking buffer for 2 hours before washing again. Incubation in 1/4000 Extravidin Alkaline Phosphatase (Sigma) in blocking buffer for 1 hour was followed by development with nitroblue tetrazolium and bromo-chloro indolyl phosphate in 0.1M Trizma pH9.0, 3.6mM $MgCl_2$ (Leary et al, 1983).

Phage plaques corresponding to positive spots were picked within a core of material and released into SM media (Appendix 4). The phage stock were titrated and plated out for repeated screening. This regime was followed until a pure phage stock was obtained. The phage stock was designated λ1.14.

4.2.4.8 ISOLATION OF LAMBDA PHAGE DNA.

The next stage in the procedure was to isolate phage DNA from plate lysates (Sambrook et al, 1989). In brief, five tubes of Y1090 bacteria (100µl) were infected with 10^5 pfu of phage and allowed to stand for 10 minutes at 20°C. The cultures were then incubated at 37°C for 20 minutes to allow DNA inoculation of the bacteria before the cultures were plated out as above and incubated at 42°C for 16 hours. 3ml of TM solution (Appendix 4) was added to each plate whcih was then rotated for 2 hours to elute the phage. The resulting phage suspension was centrifuged at 4000xg for 10 minutes to remove bacterial debris. Bacterial nucleic acids were digested

with RNAase (1µg/ml) and DNAase I (1µg/ml) for 15 minutes at 37°C. The phage were precipitated by incorporation of PEG 8000 to 10% and NaCl to 1M, and left for 1 hour on ice before centrifugation at 10000xg for 10 minutes at 4°C. The phage were then resuspended in 0.5ml of TE and disrupted with 0.1% SDS for 5 minutes at 68°C, and the protein removed by phenol/chloroform extraction, followed by chloroform extraction. The DNA was precipitated with an equal volume of isopropanol at -70°C resuspended in TE prior to analysis.

4.2.4.9 PRODUCTION OF LYSOGEN FROM PHAGE.

A large quantity of fusion-protein was produced from extracts of lysogenic E. coli infected with λ1.14. The method used has been described (Young & Davis, 1983a). The essential details are described.

E. coli Y1089 was grown in LB/Ampicillin/0.2% maltose overnight at 37°C. 50µl of the culture was diluted into 2ml of LB/10mM $MgSO_4$, and 100µl aliquots were dispensed to four tubes. These were inoculated with 10^7, 5 x 10^7 and 2 x 10^8 pfu of λ1.14 phage. The fourth tube received no phage. The tubes were kept at room temperature for 20 minutes to allow phage DNA inoculation, and then 10µl of the mix was diluted in 10ml of LB/10mM $MgSO_4$. 100µl of this was plated onto LB/10mM $MgSO_4$/Ampicillin plates. The plates were incubated at 30°C for 24 hours to allow growth of individual lysogenic colonies. Individual colonies from each plate were picked and replica-plated onto two plates. One was incubated at 30°C, the other at 42°C. Colonies which grew at 30°C but not at 42°C had been infected with λ1.14 phage which is lytic at 42°C. They were grown in liquid culture (LB/Ampicillin) overnight at 30°C, and 125µl

aliquot of this was diluted into 10ml of media and grown at 30°C with shaking to an OD_{600nm} of 0.45. The culture tube was transfered to a 43°C water bath for 20 minutes to inactivate the λgt11 encoded temperature-sensitive repressor. The culture was induced to produce protein with IPTG at a final concentration of 10mM, and further cultured for 2 hours at 37°C. A similar culture did not have IPTG added to induce protein production. 1.5ml of each culture was centrifuged (12000xg for 30 minutes) and then vortexed with 100µl of extraction buffer (Appendix 4). The resulting suspension was freeze-thawed twice in liquid nitrogen to disrupt the bacteria prior to analysis.

4.2.4.10 ATTEMPTING TO OBTAIN λ1.14 INSERT BY PCR USING λGT11-SPECIFIC PRIMERS.

Primers specific to the flanking region of the λgt11 EcoRI site were synthesized by the Oswel DNA Service. As the λgt11 sequence has not been published the primers were taken from the lacZ gene which contains the EcoRI site of λgt11.

PRIMER	SEQUENCE	NUCLEOTIDES
λGT11 (5')	5'-GCACGCGGAAGAAGGC-3'	2928-2943
λGT11 (3')	5'-GACACCAGACCAACTGG-3'	3048-3064

The template was prepared similarly to the assessment of the λZAP library. The reaction was performed using the following cycling conditions:

Denaturing	95°C	1.5mins
Annealing	50°C	1.5mins
Extension	72°C	3.0mins

for 35 cycles.

4.3 RESULTS.

4.3.1 POLYMERASE CHAIN REACTION TO CLONE PART OF THE OVINE INTERFERON-GAMMA GENE.

4.3.1.1 PCR USING PRIMERS 343E AND 344E.

Analysis of initial polymerase chain reactions on 1.5% agarose gels showed a reaction tube contained a discrete product of approximately 300bp (Figure 27). Attempts to clone this were unsuccessful. Subsequent PCRs gave a fainter band of 300bp amongst a smear of DNA. The band was purified and cloned as described (4.2.2.2-4.2.2.5) into pTZ18R. White colonies were subsequently cultured overnight in medium and the plasmid DNA was then isolated. Restriction enzyme digestion with EcoRI and Hind III was used to release inserts from the plasmids. Electrophoresis on a 2% agarose gel did not yield the expected single band of 322bp so to analyse the true nature of the inserts single-stranded DNA was produced as template for sequencing. Two representative sequences are shown in Figure 28. They both contain multiple copies of the primers. The junction between the primers show a region of complementarity which has aided the production of these "primer-dimers".

```
343E  5' GGGCCTCTCTTCTCAGAAATT 3'
                          |||||
                   3' TCTTTGGCCTTCTCTTCAGT 5'      344E
```

Excess primer concentrations have produced concatamers of primers. The band at 320bp probably did represent the desired PCR product but contaminating DNA was cloned. The obvious way of avoiding the

primer-dimer formation was to change one of the primers such that complementary 3' ends were avoided. Therefore primer 474F was subsequently used in place of 344E.

4.3.1.2 PCR USING PRIMERS 343E AND 474F.

The new primer 474F and the original primer 343E were used in a polymerase chain reaction. This reaction succeeded in synthesizing a product of approximately 300bp in length. The product was cloned successfully into pTZ18R and grown in E. coli JM101.

Four of the plasmids were sequenced. Sequence autoradiographs from two of the plasmids are shown (Figure 29). All four isolates were found to contain the same insert. The plasmid containing the ovine sequence described here is subsequently referred to as pOVifn. The section of the ovine IFN-g sequence present in the plasmid is known as OVifn.

The nucleotide and amino acid sequence of the inserts compared to the bovine sequence are shown in Figures 30. Comparison with bovine IFN-g showed a high degree of identity at the amino acid level (98%). Comparisons with known IFN-g sequences from other species showed less identity with human (63%), and even less with the rodent sequences (mouse 41%, rat 40%). Subsequent descriptions of both ovine (McInnes et al, 1990; Radford et al, 1991) and porcine IFN-g (Dijkmans et al, 1990) further confirmed the identity of the sequence cloned. The porcine sequence was 86% identical at the amino acid level.

4.3.2 OVINE INTERFERON-GAMMA CLONING FROM LAMBDA PHAGE LIBRARIES.

4.3.2.1 SCREENING OF λZAP LIBRARY BY OLIGONUCLEOTIDE.

The use of oligonucleotide 344E to screen the phage library was unsuccessful. No positive plaques were isolated using the method described.

4.3.2.2 SCREENING OF λZAP LIBRARY USING Ovifn AS PROBE.

The probe was generated by random-primed labelling of Ovifn, incorporating ^{32}P-dCTP in the reaction. The probe was at an activity of 3.7 x 10^7 cpm/µg. The probing of the \ZAP library did not yield any positive plaques.

4.3.2.3 ANALYSING λZAP LIBRARY BY PCR.

PCRs were performed using 343E and 474F primers and 10^5pfu or ten fold dilutions of λZAP phage as template DNA. Analysis by electrophoresis on 2% agarose gel showed that a band of 302bp was not synthesized. The positive control IFN-g PCR product was synthesized when pOVifn was used as template.

4.3.2.4 SCREENING λgt11 LIBRARY WITH ANTI-BOVINE INTERERON-GAMMA RABBIT SERA.

The lack of success in attempting to isolate a phage clone of ovine IFN-g by DNA hybridization techniques, and the subsequent doubts over the presence of such a clone in the λZAP library, prompted the use of another method for screening the remaining λgt11 library.

The availability of the rabbit antisera to the bovine recombinant IFN-g allowed the screening of the λgt11 for a clone which encoded ovine IFN-g within a fusion protein.

When the antisera, preabsorbed against E. coli Y1090 bacterial proteins to remove antibodies reactive with E. coli, was used to screen the library, one plaque (λ1.1) gave a positive signal on duplicate filters. This plaque was taken through secondary and tertiary screens, and eventually to purity. It then became known as λ1.14.

Attempts to isolate the cDNA insert from the phage failed despite repeated phage preparations and different restriction enzyme digestions.

4.3.2.5 ANALYSIS OF λ1.14 CLONE BY COOMASSIE STAINING AND WESTERN BLOTTING OF LYSOGEN CULTURES.

4.3.2.5.1 ANALYSIS OF PROTEINS FROM LYSOGENIC CULTURES.

It was decided that the preparation of lysogens may give a clearer idea if the phage clone was truly of ovine IFN-g. Preparations of lysogenic cultures were either induced with IPTG (I), or not induced (NI). Proteins from both induced and non-induced cultures were analysed on an 8% SDS-PAGE followed by Coomassie staining, and also by immunoblotting using both the anti-bovine interferon-gamma rabbit polyclonal sera (R167/8), and the monoclonal antibody to human interferon-gamma carboxyl peptide, HB8291. An anti-CD3 polyclonal rabbit antiseum (R181) and an anti-sheep MHC class II monoclonal antibody (VPM37) were used as negative control antibodies. The Coomassie stained gel (Figure 32) shows that a number of additional

protein bands are produced in induced λ1.14 lysogen cultures, principally at molecular weights of ≈110k, 67k, 39k and 26k. The protein at 110k is clearly of the same mass as native β-galactosidase.

The western blots of the induced and uninduced lysogen cultures (Figure 33) show that R167/8 (anti-bovine rIFN-g) sera reacts with a protein of approximately 39k molecular weight in both cultures, but primarily the induced culture. The R181 (anti-sheep CD3) sera reacts with many proteins in both cultures, but not with the 39k protein. This sera had not been preabsorbed against E. coli proteins. The monoclonal antibodies HB8291 (anti human IFN-g carboxy peptide) and VPM37 (anti-sheep MHC class II) also react faintly with the 39k protein. Although this is difficult to see in the figure, it was more obvious in the original blot. These results suggest that phage λ1.14 encodes a protein which reacts non-specifically with immunoglobulin of both mouse and rabbit origin.

4.3.2.6 ATTEMPTING TO OBTAIN THE λ1.14 CLONE INSERT BY PCR.

The phage λ1.14 was used as template in a PCR as described in the section 4.2.5.10, using the λgt11 primers. The agarose gel showed that the PCR had generated a fragment of between 100bp and 200bp for λ1.14 which was the same size as that generated using a known phage without an insert. The primers used were best estimates using the known sequence of the lacZ gene.

The initial isolate from the primary screening of λgt11 (λ1.1) was analysed by performing PCR on phage DNA with primers 343E and 474F. Control PCRs on a negative phage isolate, and also on plasmid pOVifn were also included in the experiment. This PCR on λ1.1 failed to give a band of 322bp on a 2% agarose gel.

The result above and the failure to obtain the DNA insert from the phage led to the discontinuation of the work with λ1.14. The western blots enhanced the belief that the phage probably did not encode an ovine IFN-g protein, but the reaction with VPM37 would tend to suggest that it produced a protein which merely reacts with immunoglobulin.

In retrospect it would have been useful to have analysed the λgt11 library by PCR for IFN-g sequences using the oligonucleotide primers 343E and 474F. This may have yielded information on the presence or absence of an ovine IFN-g cDNA clone, and hence saved some time.

4.4 DISCUSSION.

The cloning of part of the ovine IFN-g gene using PCR oligonucleotides based on the bovine sequence demostrates that cytokine cloning across species using PCR is possible. This has been confirmed by other reports on the PCR cloning of ovine IFN-g (McInnes et al, 1990; Radford et al, 1991), IL-2 (Goodall et al, 1990), IL1-β (Fiskerstrand & Sargan, 1990), and TNF-α (Green & Sargan, 1991). Each ovine sequence was cloned using bovine-derived primers in the PCR. Ovine TNF-α has been cloned by PCR using consensus primers derived by comparison of human, murine and rabbit TNF-α sequences (Green & Sargan, 1991) Sequences which are highly species-specific, such as IL-4 (typically 40% amino acid identity between species), are likely to be difficult to clone by PCR unless species are closely related (P Wood, personal communication).

The complete ovine IFN-g amino acid sequence of McInnes et al (1990) has 96% identity with bovine IFN-g. This is comparable with other cytokine genes cloned in both species. Ovine interleukin-2 has 96% identity with the bovine IL-2 (Goodall et al, 1990), and ovine IL-1β has 84.9% identity with bovine IL-1β (Fiskerstrand & Sargan, 1990). Comparison of ovine TNF-α with bovine TNF-α is not possible as bovine TNF-α has not been cloned and sequenced.

Radford et al (1991) cloned ovine IFN-g by PCR and derived two different sequences from two of the clones. The two sequences varied at three nucleotides, of which two fall within the region of OVifn. McInnes et al (1990) also cloned ovine IFN-g by PCR but only reported one sequence. The two nucleotide changes in one of Radford et al's clones have identity with the described sequence here and are at variance with the McInnes et al sequence, whilst in the other

clone the converse is true. At one further position (369 on Figure 31) the three ovine sequences vary. OVifn has a T, but McInnes et al and Radford et al record a C, which is also the nucleotide present in the bovine sequence. None of the three nucleotide variations alters the amino acid sequence.

The variable nucleotides between Radford et al's clones were assumed to be due to variations between mRNA transcripts or reverse transcription reaction products and it is possible that the changes are reverse transcription or PCR-generated errors. Radford et al also reported a nucleotide deletion and insertion at the 5' end of the gene which altered five amino acids. They commented that they believed this sequence region to be incorrect. This could possibly be a reverse transcription or PCR-generated error. All four sequences were derived from cloned PCR-generated DNA and it is known that Taq polymerase enzyme used in these reactions has a relatively high error rate with no proof-reading ability. The three common variations would be consistent with the mutational base pair substitutions incorporated by Taq polymerase described in other papers. Tindall & Kunkel (1988) described that of 23 base pair transitions detected in a Forward Mutational Assay (detects loss of α-complementation in β-galactosidase when a 390bp single-stranded gap is filled by Taq polymerase), 18 were of the T -> C type. Similarly Keohavong & Thilly (1989) described twelve mutations detected by denaturing gradient gel electrophoresis after PCR using Taq polymerase, all of which were of the A·T to G·C type. Saiki et al (1988) found ten of thirteen transitional errors were A·T to G·C. It would however be unlikely that independently derived sequences would contain the same errors. The finding of the common sites of nucleotide changes in the four described sequences makes it unlikely that these are reverse transcription errors. The changes are more

likely to be mRNA encoded, and hence to represent allelic variation in the sheep IFN-g genomic DNA sequence. There would be little selection against these variations as they are silent nucleotide substitutions. Similar polymorphic variation occurs in ovine TNF-α (Green & Sargan, 1991).

Analysis of the sequences described for human IFN-g (Gray et al, 1982; Gray & Goeddel, 1982; Devos et al, 1982; Taya et al, 1982) reveals only one nucleotide variation. This occurs in the Gray et al sequence at the carboxyl terminus where an A replaces a G, in turn altering the amino acid from glutamine to arginine. Gray & Goeddel (1982) report that sequencing of six cDNA clones from four individuals revealed only this one change in one clone. They suggested from this, and an absence of restriction length polymorphisms, that the IFN-g gene and surrounding region are relatively conserved. The two variants of ovine IFN-g and similar variants of ovine TNF-α suggest that limited polymorphism of cytokines may occur in sheep.

Much effort was expended in attempting to clone ovine IFN-g from phage libraries. Two cDNA libraries were used and a variety of detection methods. The failure to clone the ovine sequence could be for many reasons, but there are two major requirements for success in colony hybridization experiments. Firstly, the library must contain recombinant phage encoding the desired sequence. If the desired mRNA is an unstable species or is found at low frequency in cells, the conversion to cDNA may not yield sufficient clones for detection later. A high number of individual recombinant phage formed during the creation of the library may offset the low initial proportion of desired recombinants. This is conveniently measured

as the titre of the library before amplification. Unfortunately the titres were not known for the libraries used, and only amplified libraries were used.

During preparation of the library the input mRNA should be enriched for the desired sequences by appropriate stimulation of the cells before lysis to boost the transcription of the desired mRNA. Both libraries used contained cDNA derived from a mixture of 24 and 72 hour concanavalin A-stimulated lymphocytes. These cells are still actively proliferating at these times and should contain mRNA for IFN-g (Efrat et al, 1982). Indeed, bovine IFN-g was cloned from a phage library containing cDNA derived from lymphocytes stimulated with concanavalin A for 17 hours (Cerretti et al, 1986).

The second requirement is to have a sensitive probe that will detect the small quantities of either DNA or protein in a phage plaque. Both random-primed ^{32}P-labelled Ovifn and ^{32}P-end-labelled oligonucleotide probes were used to detect IFN-g DNA sequences in the libraries. Both were calculated to be labelled at more than 1 x 10^7 cpm/µg which is a commonly quoted minimum activity required (Sambrook et al, 1989). The lack of success using these probes could therefore be due to absence of a clone in the library or excess washing of the filters. Standard conditions for the hybridization and washing of membranes hybridized with the oligonucleotide probe were used (Sambrook et al, 1989). The washing conditions for the Ovifn probe may have been a problem when the melting temperature for the probe-insert duplex is calculated.

With a wash solution containing 0.2 x SSC (i.e. 0.033M Na^+), the melting temperature can be calculated according to the equation of Bolton & McCarthy (1962):

$$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%\ G+C) - (600/n)$$

The % G+C is 32 for OVifn, and the probe length (n) is 302. This gives a T_m of 67°C. The wash temperature used of 65°C could have removed a significant amount of the probe from the filters.

Further experiments to demonstrate the presence or absence of an IFN-g-specific sequence in the library by using the oligonucleotides already used to clone Ovifn in a PCR with phage DNA as template showed that no clones were detectable by this sensitive method. This is much the more likely explanation for the repeated failure to clone by this method.

Screening the λgt11 library using the antisera against recombinant bovine IFN-g was as frustrating as screening the λZAP library with the DNA probes. Although antibody screening is useful when a specific antisera or monoclonal antibody is available, it theoretically has less chance of success than using a DNA hybridization technique. Any phage containing the desired cDNA sequence must also have it in the correct orientation for expression. It must also be in the correct translational reading frame. Consequently only one in six phage containing the desired DNA are likely to be expressed correctly. The library must be relatively enriched for the cDNA desired. λgt11 libraries suffer from the fact that nonabundant mRNAs are represented very rarely, if at all, and often encode only a 3'-terminal portion of the gene (Snyder et al, 1987). Also λgt11 libraries contain non-recombinant

phage as there is no elimination step during amplification of the library. A further problem with expression screening of libraries is that the antibody probe may be directed against a contaminant in the original antigen preparation, or that other proteins may share epitopes with the protein of interest (Young & Davis, 1983a). The clones thus isolated have to be taken to the sequencing stage before they can be discarded in confidence.

The isolation of an immunoreactive phage led to much work to verify its true nature. The production of lysogenic colonies and subsequent culture to obtain protein for SDS-PAGE immunoblotting did not resolve the potential of the phage clone, and its subcloning and sequencing were a major problem which could not be resolved. A larger β-galactosidase fusion protein should be seen on an SDS-PAGE after protein synthesis by a lysogenic culture, but its absence does not eliminate the possibility of this phage clone expressing ovine IFN-g sequences unfused to β-gal. The phenomenon of has been reported, especially when using genomic libraries, and may be due to degradation or anomolous translation initiation (Young & Davis, 1983). Unfused proteins may still be under the influence of the lacZ promoter (Snyder et al, 1987).

Restriction endonuclease digestion of phage DNA did not release a fragment which could be subcloned. This may be due to loss of restriction sites during the library production as other workers have suffered similar problems (C. Fiskerstrand, personal communication). This is a not-uncommon finding with λgt11, where positive appearing phage plaques cannot be subcloned as the restriction digest sites appear to have been disrupted during the

library generation. It was for this reason that λZAP was originally devised, with its easy recovery of the insert from within the Bluescript phagemid (Short et al, 1988).

Figure 27.

Agarose gel of products of polymerase chain reaction utilizing IFN-g-specific primers. A 300bp DNA band is marked.

Figure 27.

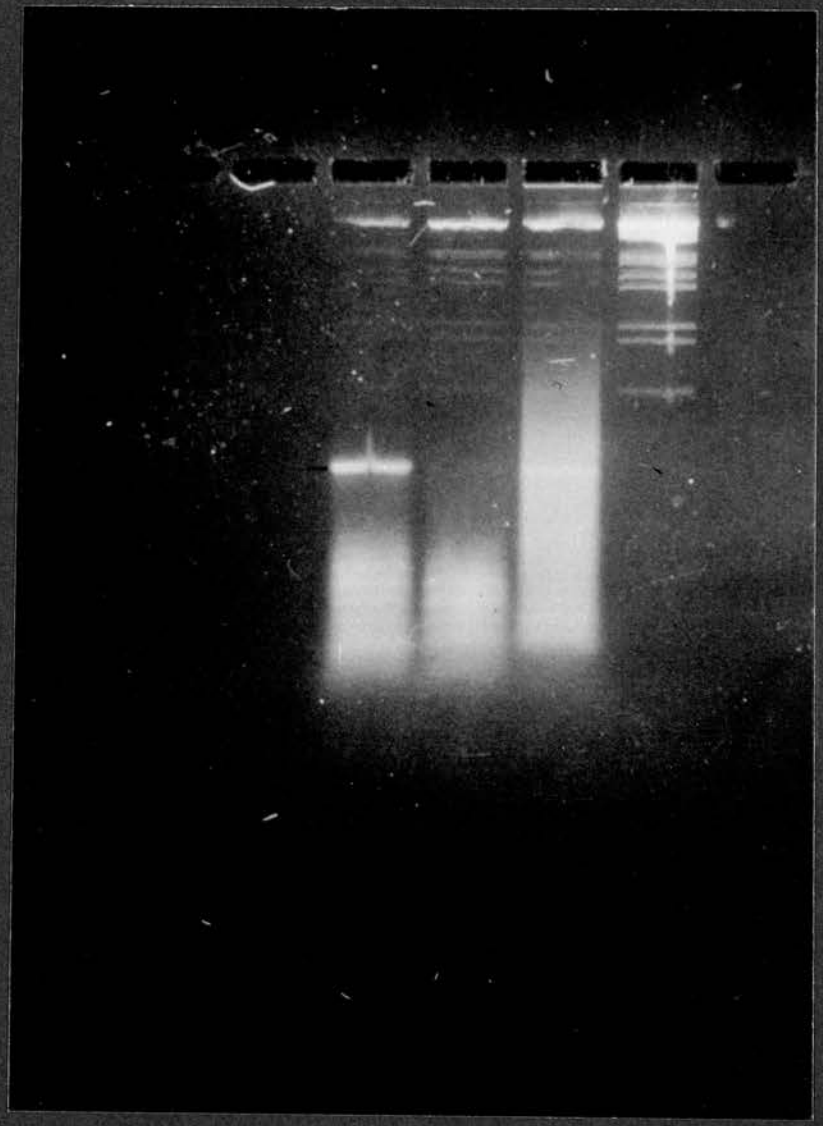


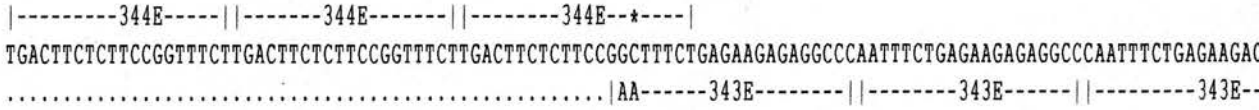
Figure 28.

Primers 343E and 344E that were used in initial PCR experiments are shown. Plasmid 14 and plasmid 19 were synthesized by PCR and their sequences are shown. The origin of the "primer-dimers" are indicated.

Figure 28.

343E (5') 5'-GGGCCTCTCTTCTCAGAAATT-3' 238-258
344E (3') 5'-TGACTTCTCTTCCGGTTTCT-3' 541-560

Plasmid 14:



Plasmid 19:

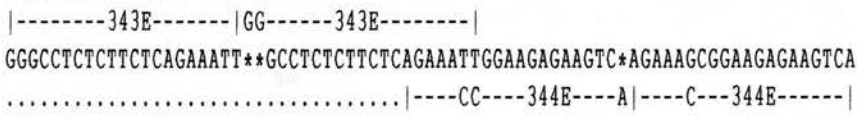


Figure 29.

Autoradiograph of sequencing gel of pOVifn. Clones 4 and 5 are shown side by side. The eight tracks in lane P were run for 4 hours, and those in lane S for 2 hours. The first four tracks in each lane reading from the left are adenine (A), cytosine (C), guanine (G) and thymine (T).

Figure 29.

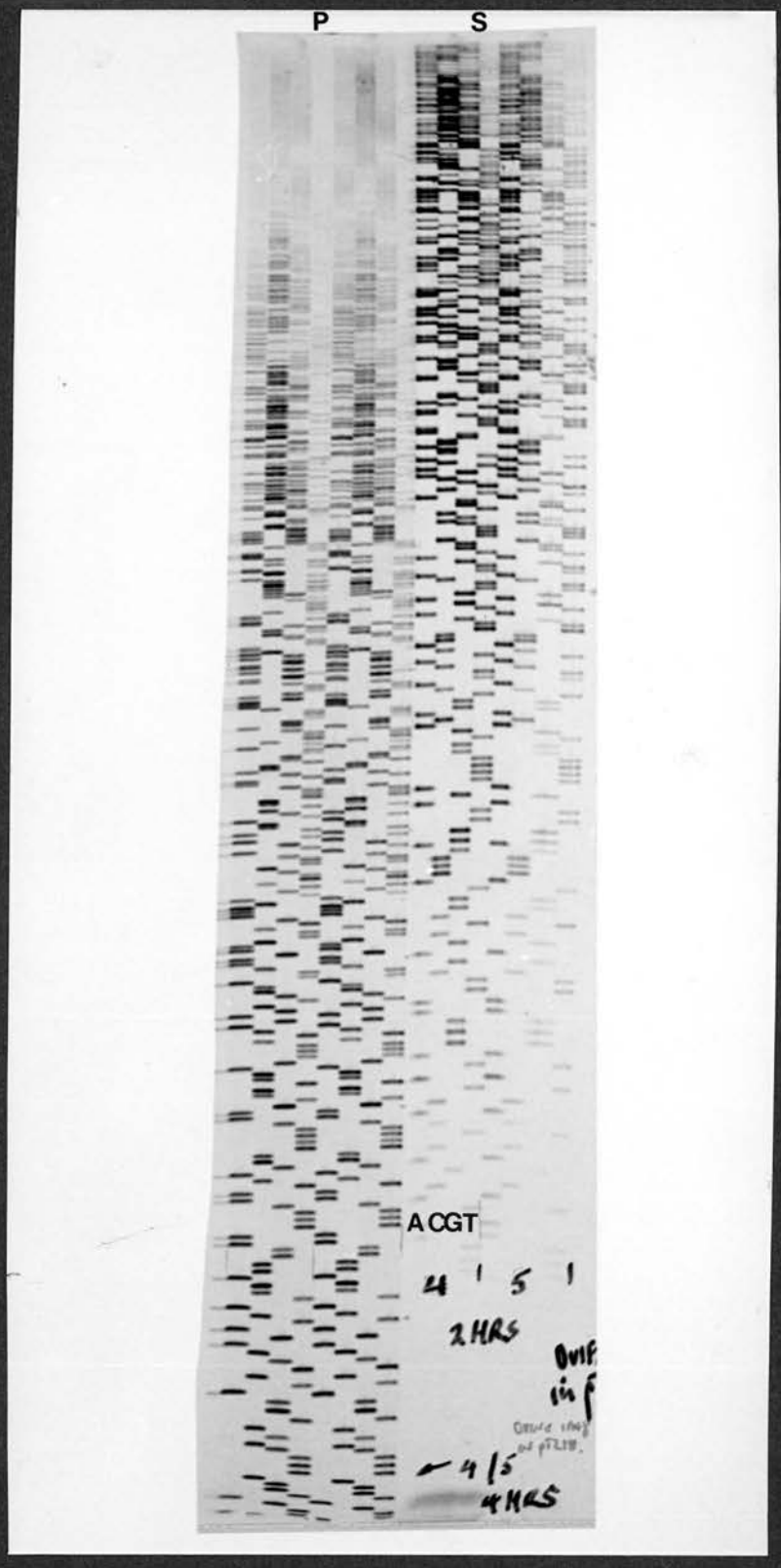


Figure 30.

Comparison of amino acid sequences of bovine IFN-g (BOV) and that predicted for ovine IFN-g from the nucleotide sequence of plasmid OVifn (OVifn). Identity is indicated | and non-identity by X.

Figure 30.

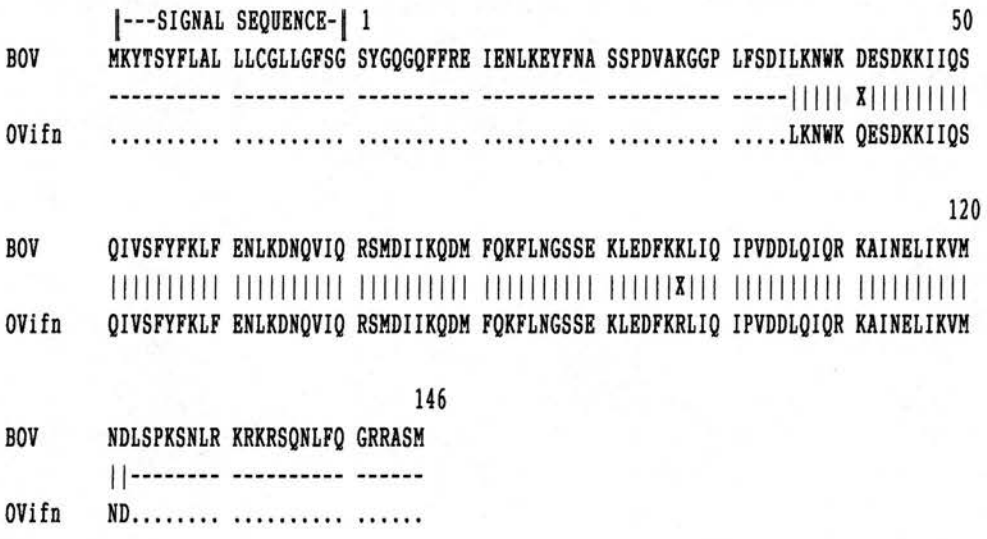

|---SIGNAL SEQUENCE-| 1 50
BOV MKYTSYFLAL LLCGLLGFSG SYGQGQFFRE IENLKEYFNA SSPDVAKGGP LFSDILKNWK DESDKKIIQS
OVifn ....LKNWK QESDKKIIQS
120
BOV QIVSFYFKLF ENLKDNQVIQ RSMDIIKQDM FQKFLNGSSE KLEDFKKLIQ IPVDDLQIQR KAINELIKVM
OVifn QIVSFYFKLF ENLKDNQVIQ RSMDIIKQDM FQKFLNGSSE KLEDFKRLIQ IPVDDLQIQR KAINELIKVM
146
BOV NDLSPKSNLR KRKRSQNLFQ GRRASM
OVifn ND..... ....

Figure 31.

Comparison between the nucleotide sequeneces of OVifn, ovine IFN-g derived by McInnes et al (1990) (OVmc), ovine INF-g derived by Radford et al (1991) (OVra), and bovine IFN-g (Cerretti et al, 1986) (BOV). Differences between sequences are marked. Where two nucleotides have been proposed by the same group they are both marked, e.g. $_{C}{}^{T}$.

Figure 31.

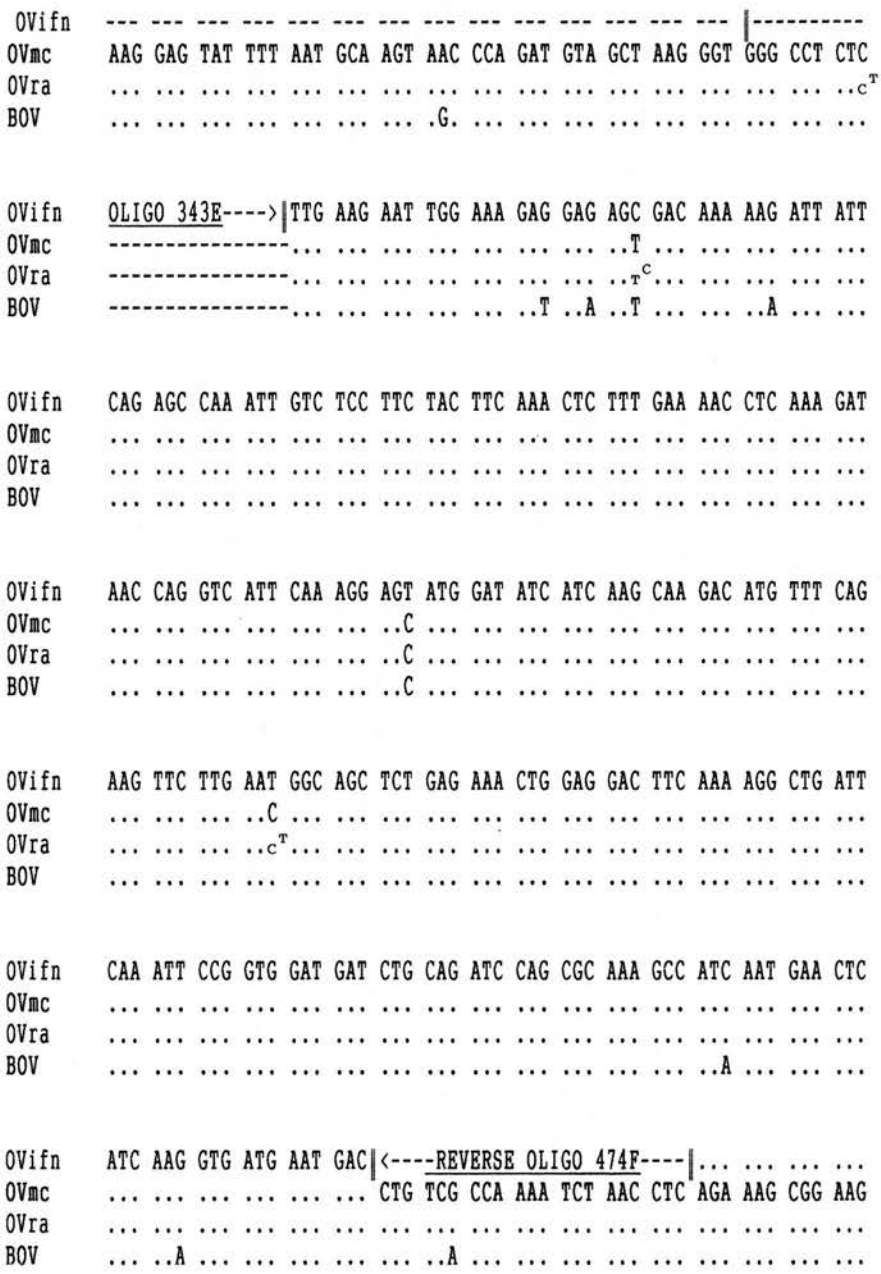

OVifn --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
OVmc AAG GAG TAT TTT AAT GCA AGT AAC CCA GAT GTA GCT AAG GGT GGG CCT CTC
OVra ... ...cT
BOV ... ... .G. ... ...
OVifn OLIGO 343E----> TTG AAG AAT TGG AAA GAG GAG AGC GAC AAA AAG ATT ATT
OVmc ---------- ... ...T ... ...
OVra ---------- ... ...TC ... ...
BOV ---------- ... ...T ..A ..T ... .A ... ...
OVifn CAG AGC CAA ATT GTC TCC TTC TAC TTC AAA CTC TTT GAA AAC CTC AAA GAT
OVmc ... ...
OVra ... ...
BOV ... ...
OVifn AAC CAG GTC ATT CAA AGG AGT ATG GAT ATC ATC AAG CAA GAC ATG TTT CAG
OVmc ... ...C ... ...
OVra ... ...C ... ...
BOV ... ...C ... ...
OVifn AAG TTC TTG AAT GGC AGC TCT GAG AAA CTG GAG GAC TTC AAA AGG CTG ATT
OVmc ... ...C ... ...
OVra ... ...cT ... ...
BOV ... ...
OVifn CAA ATT CCG GTG GAT GAT CTG CAG ATC CAG CGC AAA GCC ATC AAT GAA CTC
OVmc ... ...
OVra ... ...
BOV ... ...A ... ...
OVifn ATC AAG GTG ATG AAT GAC <----REVERSE OLIGO 474F---- ... ...
OVmc ... ... CTG TCG CCA AAA TCT AAC CTC AGA AAG CGG AAG
OVra ... ...
BOV ... ..A ... ...A ... ...

Figure 32.

7-15% gradient SDS-PAGE gel of lysogenic cultures of λ1.14 stained with coomassie blue. Cultures were induced or non-induced with IPTG.

Lane	
L	Low MW markers (kDa)
B	β-galactosidase
I	Induced lysogen
NI	Non-induced lysogen
H	High MW markers

Figure 32.

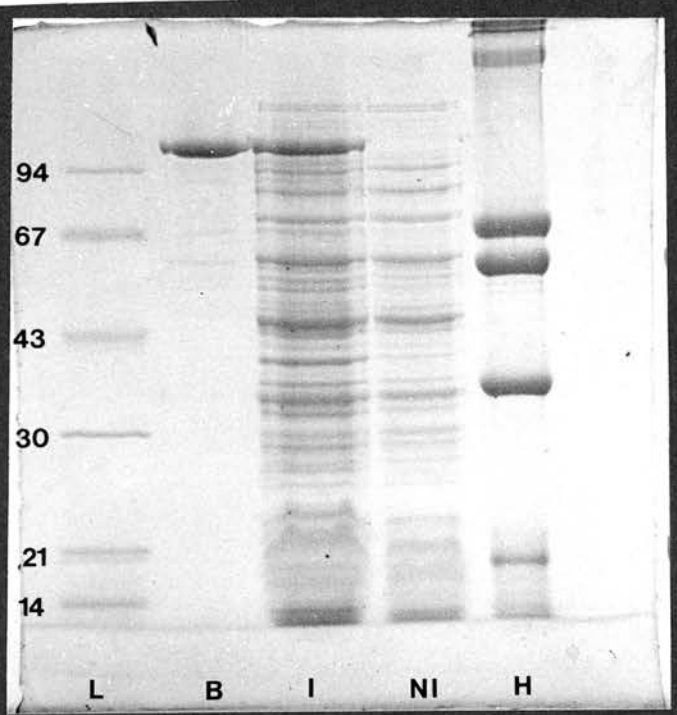
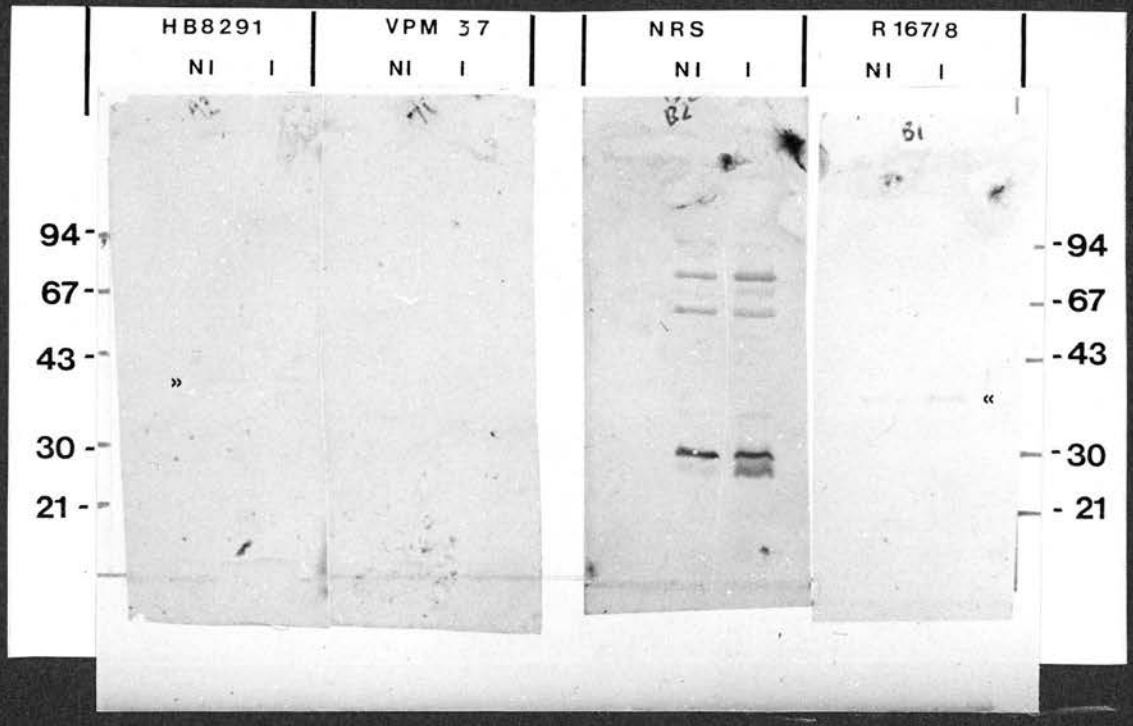


Figure 33.

Immunoblot of lysogenic cultures either induced (I) or non-induced (NI) with IPTG to express recombinant protein. Blots were run under non-reducing conditions. Blots developed to show reactivity with HB 8291 (anti-human IFN-g peptide), VPM37 (anti-ovine class II), NRS (preimmune rabbit sera), or R167/8 (anti-recombinant bovine IFN-g). Specific bands seen only in HB 8291 and R167/8 are marked «. Molecular weights are indicated down the sides.

Figure 33.


HB8291
NI I
VPM 37
NI I
NRS
NI I
R 167/8
NI I
94
67
43
30
21
94
67
43
30
21

5 CYTOKINE mRNA LEVELS IN ACTIVATED LYMPHOCYTES.

5.1 INTRODUCTION.

5.1.1 CELL SUBSETS INVOLVED IN IFN-g PRODUCTION IN VITRO.

Most cells involved in the immune response are capable of secreting a number of cytokines. T cells, B cells, NK cells, monocytes and macrophages can all produce several cytokines following activation (Arai et al, 1990). These cytokines are produced by cells which have been induced from the resting state to the G1 phase of the cell cycle (Stadler et al, 1981). Several cytokines may be produced by a cell following a single stimulus, but different stimuli may induce different combinations of cytokines (Gauchat et al, 1991).

Examination of T cell subsets for the production of cytokines, including IFN-g, has concentrated on the CD4/CD8 division. Intracellular immunofluorescent staining of human mononuclear cells freshly isolated from blood has demonstrated that in vitro anti-CD3 stimulation induces IFN-g production by cells expressing T cell antigens CD2, CD3 and CD4 or CD8 or natural killer cell markers CD16 (Andersson et al, 1988). Staining for CD45R isoforms indicated that production was predominantly by $CD45RO^+$ cells, i.e. memory cells. The overall picture that emerges is that early IFN-g production is by memory $CD8^+$ and $CD4^+$ cells, and later production is supplemented by virgin T cells, and an unknown group of cells, probably NK cells. Similar studies in mice by the same authors confirm the role of $CD8^+$ cells in IFN-g production during a mitogen response, where 90-95% of IFN-g-producing cells were of this phenotype (Sander et al, 1991). Human $CD4^+$ T cell clones stimulated by Concanavalin A, or ionophore

and phorbol esters nearly all produced IL-2, IL-4 and IFN-g (Palliard et al, 1988). All $CD8^+$ T cell clones produced IL-2 and IFN-g, but only a third to a quarter of them produced IL-4.

Stimulation of T helper cells by specific antigen also results in synthesis of IFN-g (Landolfo et al, 1982; Conta et al, 1983; Kasahara et al, 1983). Human rabies vaccine recipients have rabies virus-specific $CD4^+$, class II restricted T cells, and $CD8^+$ T cells, which both produce IFN-g when exposed to rabies antigens (Celis et al, 1986). Patients immunized against influenza virus have $CD8^+$ cells which secrete IFN-g on re-exposure to influenza antigens (Ennis & Meager, 1981; Yamada et al, 1986).

Much recent work has been prompted by the observation that isolated murine $CD4^+$ T cell clones can be categorized into two major groups, known as T helper 1 and 2 (T_H1 and T_H2) (Mosmann & Coffman, 1989). These two groupings synthesize different cytokines during activation. T_H1 cells synthesize IFN-g, IL-2, LT (lymphotoxin), GM-CSF (granulocyte-macrophage colony stimulating factor), TNF and IL-3, and are involved in promoting delayed type hypersensitivity (DTH) and immunoglobulin isotype class-switching to IgG2a. T_H2 cells synthesize IL-3, IL-4, IL-5, IL-6, IL-10, and lesser amounts of TNF-α and GM-CSF. They are involved in B cell growth and differentiation, and isotype class-switching to IgM, IgG1, IgA and IgE. Mouse $CD8^+$ T cell clones mainly produce the T_H1-cell cytokine pattern, although IL-2 production is commonly low or absent (Fong & Mosmann, 1990). Two further T helper groups have been identified from mouse T cell clone cytokine secretion profiles, the T_{HP} group and the T_{HO} group (Mosmann & Moore, 1991). The T_HP group produce only IL-2 and are thought to be T cells that have not been

stimulated recently, whilst the T_H0 group produce IL-2, IFN-g, GM-CSF, IL-3, IL-4, IL-5 and IL-10. The role of these two groups is unclear at present.

The different cytokine profiles have been used to predict the expected roles of the two subsets in normal responses to immunogens. The T_H1 response is expected to result in the enhancement of various cytotoxic mechanisms. IFN-g enhances the synthesis of Fc receptors for IgG2a antibodies on macrophages, and also causes class-switching to IgG2a antibodies. The macrophages are activated by both IFN-g and LT to kill intracellular parasites and tumour cells. The combination of these responses would enhance the antibody-mediated macrophage cytotoxicity. T_H1 cells are expected to cause DTH responses, and in the absence of a T_H2 response would give a DTH in the presence of little or no antibody. IFN-g acts to reduce the proliferation and activation of T_H2 cells.

T_H2 cell responses are primarily expected to help in the synthesis of a good antibody response to an antigen. Most of the cytokines synthesized by T_H2 cells are primarily active on B cells. Antibody class-switching to IgE mediated by IL-4 is accompanied by increased levels of Fc receptors for IgE on B cells (Rousset et al, 1988), and IL-3 and IL-4 act synergistically as growth factors for mucosal mast cells, which also possess Fc receptors for IgE. IL-5 induces the proliferation and differentiation of eosinophils. Elevated IgE concentrations, high numbers of mucosal mast cells and circulating eosinophils are all present in nematode responses.

The presence of similar helper T cell subsets in humans is still the subject of debate, and they have not been demonstrated for any other species.

The differential synthesis of some cytokines has been related to the presence/absence of CD45R (Leukocyte Common Antigen) isoforms on $CD4^+$ cells. CD45 antigen has a number of molecular isoforms which are dependent on the exons used in the mRNA synthesis and the different isoforms are believed to define cells involved in cell-mediated or humoral immune responses. CD45RA isoform contains the A exon in the molecule, the CD45RB isoform contains the CD45RB exon, whilst the CD45RO contains neither of the exons.

Morimoto et al in 1985 defined two subsets of T cells, CD45RO $CD4^+$ cells which mainly provide help for immunoglobulin synthesis by B cells and $CD45RA^+B^+$ $CD4^+$ cells which exert no helper functions, but induce $CD8^+$ T cells to become suppressive (Morimoto et al, 1986). $CD4^+$ T cells expressing CD45RO antigen are known to proliferate in response to soluble recall antigen, and are thus defined as memory cells, whilst $CD45RA^+$ $CD4^+$ cells do not proliferate to soluble recall antigen, and are thus naïve cells. Further study has redefined the initial two subsets into three depending on the degree of expression of each isoform of CD45 as assessed by FACS brightness: $CD45RA^{bright}$ B^{bright} -- naïve cells; $CD45RA^{dull}$ B^{bright} -- memory cells for IFN-g production; $CD45RA^{dull}$ B^{dull} -- memory cells for B cell help (Mason & Powrie, 1990).

All these experiments demonstrate cytokine production after in vitro polyclonal stimululation. Few experiments have been performed to assess the relative contributions of T cell subsets to IFN-g production during an immune response in vivo.

5.1.2 CELL SUBSETS INVOLVED IN IFN-g PRODUCTION IN VIVO.

The cells synthesizing specific cytokines has been examined during an in vivo immune response. Gessner et al (1989) examined the secretion of IFN-g by mouse spleen cells in response to infection with lymphocytic choriomeningitis virus using a cell spot ELISA. In vitro negative selection of T cell subsets demonstrated that T cells were the major producers of IFN-g, and that production was equally divided between $CD4^+$ and $CD8^+$ cells. IFN-g production during an in vivo immune response to a thymus-independent antigen has been shown by immunohistochemical staining of spleen cells to be equally divided between CD4 and CD8 cells, with a small contribution by NK cells (10%). The peak production of IFN-g was found days 6-7 after immunization (Van den Eertwegh et al, 1991).

If either T_{H1} or T_{H2} cells were to be activated in isolation, the predicted immune response would be of delayed type hypersensitivity or antibody dependent hypersensitivity respectively. This suggests that during the production of hypersensitivity the balance between the two types of helper cells is shifted, perhaps by excess or absence of IFN-g or IL-10, and this shift determines whether a mainly cellular- or a mainly antibody-dependent hypersensitivity is induced. In vivo evidence for the separation of helper T cells into T_H1 and T_H2 phenotypes has been identified in the murine response to Leishmania infection. C57BL/6 mice resolve infection and have high levels of IFN-g mRNA in their draining lymph node and spleen indicating the excessive activation of T_{H1} cells, compared with BALB/c mice which suffer fatal progressive disease and produce IL-4 mRNA in their lymph node as a result of excess activation of T_{H2} cells in the absence of T_{H1} cells (Heinzel et al, 1989). T cells from resistant BALB/c mice secrete IFN-g (detected as macrophage

activating factor), but susceptible mice secrete IL-3 and IL-4 which inhibit the leishmanicidal activity induced by IFN-g (Liew et al, 1989).

5.1.3 REGULATION OF CYTOKINE GENES.

The regulatory DNA sequences of the cytokines have been examined to determine the mechanism of the differential induction patterns. The regulatory gene sequences of the cytokines seem to have only limited homology except for a 10bp consensus sequence in the 5' flanking regions of some, including hGM-CSF, mGM-CSF, hIL-2, mIL-2, hIL-3, mIL-3, hIL-4, mIL-4, hIL-5, and hG-CSF (Stanley et al, 1985; Arai et al, 1990). Human IFN-g has a sequence which resembles the consensus sequence but it is in the reverse orientation. The factor(s) which transmit the activation signals from the secondary messengers, i.e. protein kinase C and calcium influx, to the nucleus are at present unknown, but there is a suggestion that the nuclear factor AP-1, which binds to phorbol ester-responsive elements (TREs) (Angel et al, 1987), may regulate both IL-2 and IFN-g genes as they both have the TRE sequence motif in their 5' flanking regions (Taniguchi, 1988).

The cytokine genes may also be regulated post-transcriptionally. Within the 3' untranslated region of many cytokines are particular conserved motifs of AU rich sequences, which have been shown to destabilizee mRNA species previously highly stable. The AT-rich sequence from human GM-CSF was inserted into the 3' coding region of the rabbit β-globin gene rendering this previously very stable gene unstable. The sequences were proposed to be recognition signals for an mRNA degradation pathway (Shaw & Kamen, 1986). They are currently thought to be used by cytokines and proto-oncogenes.

5.1.4 MEASURING mRNA IN ACTIVATED LYMPHOCYTE POPULATIONS.

Cells communicate with each other not only by cell-cell contact, but by the release of soluble mediators (cytokines) such as interferons, growth factors and interleukins. These factors are secreted from T cells which are bound to antigen presenting cells, and secretion occurs principally from the side of the T cell contacting the APC (Poo et al, 1988). The presence of these cytokines has typically been assessed by bioassay on sensitive cell lines or by immunoassay with antibodies to the cytokine. Both types of approach have several limitations. The free cytokine detected in supernatants is only representative of the difference between the amount of cytokine secreted and the amount of cytokine consumed by cells, bound to receptors or bound to natural antagonists. The detectable cytokine may at worst only represent the amount of cytokine that has leaked into the tissue fluid being assayed. The bioassays are typically time-consuming and commonly of debatable specificity due to the pleiotropy and redundancy of cytokines (Paul, 1989). The immunoassays are dependent on the availability of appropriate antibodies.

The measurement of cellular cytokine mRNA levels may represent more accurately the amount of cytokine production at any one time. This assumption has been tested for the measurement of IL-2. IL-2 secretion and IL-2 mRNA concentrations measured by northern blotting were found to correlate (Gauchat et al, 1986). mRNA and protein concentrations of both IFN-g and IL-2 produced by mitogen-activated $CD8^+$ T cell clones were strongly correlated, although IL-2 protein concentrations were sometimes low in the presence of high concentrations of mRNA (Fong & Mosmann, 1990). Correlations for other cytokines will depend on the stability of the mRNA, as very

short or long half-lives may upset the correlation. The assessment of cytokine mRNA levels may be made using hybridization methods with complementary probes such as cDNAs, cRNAs or oligonucleotides. This method of Northern blotting is certainly the simplest method but is regarded as being relatively insensitive, although advances in the generation of high specific activity labelled probes have improved sensitivity (Feinberg & Vogelstein, 1983). Northern blotting has become the standard method for some groups analysing cytokine mRNA (Gauchat et al, 1991). Ribonuclease protection analysis is a more sensitive method and has been used for cytokine analysis (Lowry et al, 1989) but requires relatively large amounts of mRNA. The polymerase chain reaction is the most sensitive method available for cytokine gene detection (Dallman et al, 1991) but its very sensitivity makes it more difficult to use as a quantitative measure. Quantitation has been attempted with a variety of internal checks to assess the semi-quantitative nature of the assay. The control genes used in the PCR techniques have included cellular actin (Dallman et al, 1991), cloned and cellular aldolase A (Chelly et al, 1988), and a synthetic cRNA containing primer sites for multiple cytokines each producing a similar sized product when specifically subjected to PCR (Wang et al, 1989). Measurement of product synthesized varied from specific hybridization to radioactive counting of ^{32}P-labelled primer incorporated in the product. The ability to quantitate the amount of mRNA is dependent on the efficiency of the cDNA synthesis, the efficiency of the PCR, and the amount of template in the reaction.

The sensitivity of the PCR technique has also allowed the analysis of mRNA within single cells. This technique, known as single-cell RNA phenotyping, is claimed to be able to detect less than 100 mRNA molecules (Rappolee et al, 1989). This degree of amplification was

achieved after 60 cycles of the PCR. The technique allows the *in vivo* or *in vitro* study of short-lived, low-copy number mRNA transcripts isolated from small numbers of cells.

5.1.5 OBJECTIVES.

The production of IFN-g during an *in vivo* immune response has not been studied extensively with respect to the T cell subsets producing it. Although it is known that $CD4^+$ and $CD8^+$ T cells synthesize IFN-g *in vitro*, the relative contributions during an *in vivo* response are ill defined.

A qualitative PCR was developed which would allow the detection of IFN-g mRNA transcripts within isolated cell populations. Initial development was made using mRNA from Concanavalin A-stimulated cells. Optimization of the PCR was performed with regard to Mg^{++} concentration and also the number of cycles required to allow detection of PCR product during its exponential increase phase. A new technique of cell separation was used to allow rapid isolation of the T cell populations to high purity without recourse to a fluorescence activated cell sorter (FACS). This new technique, known as magnetic activated cell sorting (MACS), allows isolation of cells much quicker than FACS (10^8 cells in 1½ hours by MACS, compared with 6 hours by FACS at 5,000 cells per hour) and without cellular activation by antibody cross-linking due to the small size of the superparamagnetic beads (<100nm) (Miltenyi *et al*, 1990). The beads are made of ferritin bound to dextran, and subsequently coupled to biotin. The beads do not affect cell viability. The technique has been used previously to deplete human bone marrow of T

cells prior to transplantation (Kogler et al, 1990). It gave recovery of cells between 50% and 70% depending on cell phenotype, and had less than 0.1% clonable T cells remaining.

An in vivo immune response to antigen by a primed sheep was studied at the lymph node level and the IFN-g mRNA levels in isolated cells assessed to discern the population of T cells responsible for its synthesis.

5.2 MATERIALS AND METHODS.

5.2.1 OPTIMIZATION OF THE PCR FOR SEMI-QUANTITATIVE ANALYSIS.

5.2.1.1 OPTIMIZATION OF THE PCR $MgCl_2$ CONCENTRATION.

The PCR of interferon-gamma, using the primers 343E and 474F was optimized with respect to magnesium concentration. PCR x 10 buffer contained 100mM Trizma.Cl pH8.4, 500mM KCl and 1mg/ml gelatin. All other factors were as in section 4.2.2.1. Different concentrations of magnesium chloride were included in each reaction tube (1,2,3,4,5 or 6 mM $MgCl_2$). Cycling times were:

95°C	0.8 minutes
50°C	1.0 minutes
72°C	2.5 minutes

35 cycles were performed. A 10µl aliquot of each reaction was run on a 2% agarose gel.

5.2.1.2 OPTIMIZING CYCLE LENGTH FOR THE PCR.

mRNA was extracted from lymphocytes stimulated with Concanavalin A for 24 hours. cDNA was synthesized as before (section 4.2.1) and used as template DNA. The PCR was performed using the reaction conditions optimized above in a volume of 150µl. From the end of the 18th cycle onwards and after every third subsequent cycle, a 10µl sample was removed from the reaction tube. These were electrophoresed on a 2% agarose gel and then transfered to Hybond N (Amersham) by Southern blotting overnight. Prehybridization was carried out for 2 hours at 65°C in Prehybridization solution 1 (Appendix 4) containing 20µg/ml of denatured salmon sperm DNA (Sigma). The previously cloned PCR product was labelled by random

primed synthesis to incorporate α-^{32}P-dCTP before hybridization at 65°C for 16 hours in hybridization solution 1 (Appendix 4). The blot was washed twice at 20°C in 2 x SSC/0.1% SDS, once in 1 x SSC/0.1% SDS at 65°C, and stringently washed twice with 0.5 x SSC/0.1% SDS at 65°C for 10 minutes. The blot was autoradiographed on Kodak S film at -70°C for 72 hours with an intensifying screen.

5.2.1.3 INDUCTION OF INTERFERON-GAMMA BY CONCANAVALIN A.

Efferent lymph fluid draining from a chronically-cannulated prefemoral lymph node was collected overnight into a bottle containing heparin (500IU), penicillin (5000IU) and streptomycin (5000µg). Cells were washed twice in ice-cold Hanks Buffered Salt Solution and counted. An aliquot of cells were lysed in 9ml of 4M GTC solution (Appendix 4). A further 10^8 cells were cultured in each flask at 5 x 10^6 cells/ml in Iscoves medium (Sigma) supplemented with BSA (500µg/ml), human transferrin (25µg/ml), palmitic, oleic and linoleic oils (all at 1µg/ml), fungizone (252µg/ml), penicillin (50U/ml) and streptomycin (50µg/ml). Concanavalin A (Sigma) was added to each flask at 10µg/ml. Cells were incubated at 37°C in 5% carbon dioxide. Cells and supernatant were harvested at intervals after stimulation by centrifugation at 400xg for five minutes. Supernatant was immediately frozen at -70°C until assayed for ovine interferon-gamma using the ELISA kit for bovine IFN-g from Australia (Commonwealth Laboratories, Victoria, Australia). Cells were resuspended and lysed in 9ml of 4M GTC solution. DNA was sheared by forcing the lysate through a 19G needle five times.

RNA was isolated by ultracentrifugation over 2ml of 5.7M caesium chloride (DEPC-treated) in an SW41 rotor at 28000 RPM for 16 hours at 20°C in a Beckman L70 ultracentrifuge. Careful removal of the supernatant was followed by resuspension of the RNA in 360µl of TES (Appendix 4) for 10 minutes. RNA was precipitated by addition of 40µl of 3M sodium acetate pH5.5 and 1ml of 100% ethanol. After 2 hours at -70°C the RNA was pelleted at 12000xg for 20 minutes and the pellet resuspended in 360µl of DEPC-treated distilled water. The RNA was precipitated again by the same method. RNA concentration and purity were measured using UV spectrophotometry at both 260nm and 280nm.

Interferon-gamma mRNA was analysed using the optimized semi-quantitative PCR described previously. Ten micrograms of total RNA was entered in a cDNA synthesis reaction using the Amersham cDNA Synthesis Kit. 10µCi of α-^{32}P-dCTP was included in the reaction to assess the synthesis of the cDNA. Fifty microlitre PCR reactions were performed on one tenth of the cDNA synthesized. Ten microlitre aliquots were removed from all the reactions after 20, 25 and 30 cycles and electrophoresed on a 2% agarose gel. The gel was treated as described for a Southern blot, transferred in 20 x SSC onto Hybond C (Amersham) and hybridized using α-^{32}P-dCTP-random-primed labelled pOVifn as probe. The probe was labelled to 6.5 x 10^8 cpm/µg. The membrane was prehybridized, hybridized and washed as in 5.2.1.2.

Media taken at the same intervals was analysed using the Bovine Interferon-gamma ELISA kit available from Commonwealth Serum Laboratories, Parkville, Victoria, Australia. This kit was developed using two high affinity monoclonal antibodies against bovine interferon-gamma (Rothel et al, 1990; Wood et al, 1990). The

ELISA takes the form of a sandwich immunoassay with a polystyrene plate precoated with one of the two monoclonal antibodies, and horseradish peroxidase-conjugated second monoclonal as the detector. Standard solutions containing bovine interferon-gamma are included in the kit. The kit is claimed to detect down to at least 50 pg/ml of bovine interferon-gamma. The kit is designed to be used as a test for Mycobacterium tuberculosis infection in cattle, when used in conjunction with a lymphocyte stimulation assay in response to M. tuberculosis and M. avium. The kit was assessed against ovine IFN-g in the original paper (Rothel et al, 1990) and has also been validated for use with ovine interferon-gamma by comparison with a standard anti-viral assay (G. Enterican, personal communication).

5.2.2 CELL ISOLATION USING MAGNETIC CELL SORTER (MACS).

Efferent lymphatic cells (2-3 x 10^8) were washed once in PBS/1% BSA/0.1% sodium azide/5mM EDTA (MACS buffer) and resuspended at 2-3 x 10^7/ml before incubation with anti-sheep CD4 (SBU-T4) or anti-sheep CD8 (SBU-T8) (Maddox et al, 1985) for 60 minutes, followed by washing once in MACS buffer and centrifugation at 1500RPM for 8 minutes. α-Amanitin (Sigma) was included in this staining mix at 10μg/ml to inhibit RNA polymerase II transcription of mRNA (Holmes & Higgins, 1990). Cells were then incubated with anti-mouse immunoglobulin biotin (Boehringer Mannheim) at 1/1000 for 60 minutes, washed once and then incubated with streptavidin (Boehringer Mannheim) at 1/1000 for 20 minutes. Cells were washed in MACS buffer again and then incubated with MACS biotin-microbeads for 5 minutes immediately prior to separation. All incubations were performed on ice. A MACS A2 separation column (3 x 10^7 cell capacity) was pre-cooled and equilibrated with ice-cold MACS buffer

prior to the cells being loaded. The non-magnetic cells were washed through with 15ml of MACS buffer, a 24G 1" needle determining the flow rate (approximately 0.6ml/hr). The magnetic cells were washed back up the column with MACS buffer to release non-magnetic cells trapped in the matrix, and then washed through with a further 10ml of buffer. The non-magnetic cells were collected as they left the column, while the magnetic cells were released by the removal of the column from the magnetic field. The magnetic cells were flushed through the column in 15ml of MACS buffer. 25µl aliquots of the pre-separation, non-magnetic, and magnetic fractions were stained with 50µl of 1/1000 anti-mouse Ig fluoroscein isothiocyanate (FITC) for 30 minutes. The quality of the selection was assessed on the FACScan as described previously (section 3.2.5), and by cell count.

5.2.3 ANALYSIS OF IFN-g mRNA DURING THE SECONDARY RESPONSE TO OVALBUMIN IN ISOLATED LYMPHOCYTE POPULATIONS.

On the day prior to surgery a 4 year old Finn sheep was injected with 0.5mg of ovalbumin in PBS intravenously to enhance a subsequent response to intradermal inoculation. The sheep had been injected with 1mg of ovalbumin in complete Freund's adjuvant nearly three years previously. The prefemoral lymph node of the sheep was cannulated by standard techniques (Hall & Morris, 1962). Lymphatic fluid was collected into a 250ml sterile bottle containing heparin and a penicillin/streptomycin mix. The fluid was discarded for the first six days to allow the effects of the surgery to settle down. On the seventh day after surgery the sheep was inoculated intradermally into the drainage area of the prefemoral lymph node with 250µg of ovalbumin in 1ml of PBS. Collections of lymphatic fluid were made each day. Cell counts and fluid volume produced

were measured continuously. Approximately 1 x 10^8 washed unfractionated cells were lysed in 9ml of 4M GTC. A sample of lymphatic supernatant was stored at -70°C for later analysis by the bovine IFN-g ELISA kit (Rothel et al, 1990).

Each day a sample of efferent lymphatic cells was stained as in section 5.2.2 for separation into $CD4^+$ and $CD8^+$ populations by MACS. The released magnetic cells were counted and assessed for purity by FACS. The cells were centrifuged and then lysed in 4M GTC and stored at -20°C until the RNA was isolated by ultracentrifugation over a 5.7M cesium chloride gradient as in section 5.2.1.3

Each RNA preparation was measured by UV spectroscopy at OD260nm. RNA from unseparated, $CD4^+$ and $CD8^+$ cells was converted to cDNA (section 5.2.1.3), and was then incorporated in the PCR described in section 5.2.1.3. 10µl of each of the PCR reactions was taken at 15, 20, 25 and 30 cycles. These were separated on a 2% agarose gel, transfered to Hybond N and hybridized with labelled pOVifn, as described in section 5.2.1.2. The membrane was autoradiographed on Kodak-AR film for 48 hours at -70°C.

5.3 RESULTS.

5.3.1 OPTIMIZATION OF THE PCR PROTOCOL.

Assessment of the role of magnesium in the optimization of the polymerase chain reaction was performed to enable a semi-quantitative type of PCR to be made later. Varying the magnesium concentration had a marked effect on the products formed during the PCR (Figure 34). At both 1mM and 2mM $MgCl_2$ the products visible on an ethidium bromide-stained gel were seen to be very largely the single 302bp band expected. At higher molarities up to six secondary bands of greater molecular size were visible. These bands were up to 1Kb in size, although the principal three bands were less than 564bp. Smearing of DNA of less than 302bp length was more apparent in reactions that contained lower concentrations of magnesium. A concentration of 2mM $MgCl_2$ was chosen as the optimum for all further polymerase chain reactions involving these particular primers as the larger bands seemed of more importance than the lower smearing because of their uniformity. This concentration is not unexpected because *Taq* polymerase requires a free magnesium concentration of approximately 1mM (Innis *et al*, 1988). The free Mg concentration is less than the total concentration because deoxynucleotides bind Mg ions in equimolar concentrations, and the total deoxynucleotide concentration in the PCR is 0.8mM. Hence the free Mg concentration in the optimized PCR is 1.2mM.

To assess the number of cycles required before reagents become limiting a PCR was performed in which product was sampled at intervals and assessed by both ethidium bromide staining and Southern hybridization using the pOVifn probe. A reaction was

prepared using the conditions described in the materials and methods section. Samples taken at 3 cycle intervals from the 18th cycle onwards were analysed by Southern hybridization probed with labelled pOVifn. This revealed that the PCR product first became visible at the 21st cycle (Figure 35). The PCR product also appeared at the 21st cycle on the ethidium bromide stained gel. This probably indicates that the probe was not labelled as efficiently as desired; it should detect much less than 10ng of DNA which is reckoned to be the limit for ethidium bromide staining. The blot did reveal additional bands present of smaller length than the 302bp product. These continued to accumulate until the last cycle point sampled (cycle 42).

Previous workers have determined that for a semi-quantitative assessment of mRNA levels in cells, the reaction samples should be taken whilst the PCR product is accumulating in an exponential manner before the nucleotides, primers or enzyme become limiting. The amount of amplified product is proportional to the abundance of the starting material (Chelly et al, 1988). From the gel and blot shown this would seem to be between the 24th cycle and the 30th cycle. This particular result, however, would only apply if the quantity of IFN-g mRNA in twelve-hour stimulated lymphocytes is typical of the amounts that are required to be amplified. A time course was performed to determine if the IFN-g mRNA at twelve hours was the peak level of IFN-g mRNA.

5.3.2 INDUCTION OF INTERFERON-GAMMA BY CONCANAVALIN A.

RNA was isolated from lymphocytes that had been stimulated with Concanavalin A for a variety of time periods up to 48 hours. The cDNA synthesis was assessed by α-^{32}P-dCTP incorporation and

Cherenkov counting of the acid-precipitatable material (Table 4). These figures indicate that the cDNA synthesis reactions incorporated between 0.07% and 0.29% of the radioactivity included in the reaction. The figure of 0.29% is high in comparison with the other figures, and cannot be explained by the general assumption that mitogen stimulation induces an overall increase in mRNA levels, as the incorporation of label in cDNA synthesised from RNA at 12, 36 and 48 hours are consistently at the 0.1% level. Although the incorporation figures for 2 and 24 hours are discrepant with respect to the other figures, the cDNA was included in the subsequent polymerase chain reaction.

A Southern blot of the time course at the three different sampling points revealed some variation in the detection of PCR product (Figure 36). The blot for product accumulated by 20 cycles showed a single band for each timepoint of the expected size. This first appeared faintly at 1 hour, but increased until 3 hours at which point it reached a plateau until the last sample at 48 hours. The band at 24 hours was greater than the other bands and this probably represents the greater amount of cDNA synthesised from the RNA available for use as PCR template.

The blot for product accumulated at 25 cycles showed a similar pattern but of greater intensity.

The blot for 30 cycles showed a different pattern. In each lane where previously one band was visible, two bands could now be seen. One band was of the same size as the previously seen PCR products, but the other was approximately 150bp larger. The second band was of the same intensity as the first throughout the timepoints. This suggests that the additional band was not present at all after 20 or

25 cycles, as a band would have been visible on both the stained gel and the blot. Study of the photograph of the gel showed that the loading wells appeared to have an unusual fluorescence pattern. It is possible that the wells have all torn at the top edge when the comb was removed. This would allow leakage of PCR product into a small crevice 1mm further back from the leading edge of the well. DNA would run along the bottom of the gel and this would correspond to a shift in size on a blot of 1-2mm. The presence of two bands demands that the amount of product present after 30 cycles must be interpreted with some care in this experiment.

The time-course of IFN-g protein accumulation was assessed with the bovine IFN-g ELISA kit (Figure 37). The concentration of IFN-g rises significantly from about 6 hours although IFN-g protein is detectable from 3 hours post-stimulation. The concentration increases considerably until 48 hours and has decreased a little by 72 hours.

In summary, IFN-g mRNA begins to accumulate 1 hour after stimulation with concanavalin A, and rises until 3 hours when it becomes maximal. It remains at this level until at least 24 hours, and begins to decrease by 36 hours. The IFN-g protein is detectable from 3 hours and accumulates linearly until 48 hours. The best time to sample the PCR seems to be at 25 cycles.

5.3.3 IN VIVO IMMUNE RESPONSE TO OVALBUMIN.

5.3.3.1 QUALITY OF CELL SEPARATION.

The MACS technique was found to give consistently good results using a biotin bridge method for coupling the biotin microbeads to the cell surface. $CD4^+$ cells typically comprised 50-65% of the efferent lymphatic output, and 3×10^7 cells could routinely be purified to 95% by overloading the A2 MACS column with $CD4^+$ cells (Figure 38). $CD8^+$ cells represented 10-20% of the efferent lymphatic output, and could be purified to 90% (Figure 39). Overloading of the column was more difficult due to the excessive number of cells required to achieve this ($>3 \times 10^8$) as the lymph production was usually 5×10^6/ml and 70-80ml per day i.e. $3.5\text{-}4 \times 10^8$ cells. Typically only $1.5\text{-}2 \times 10^7$ $CD8^+$ cells were purified on each occasion. As the column capacity was 3×10^7 cells, other cell phenotypes could be retained on it, and this explains the lower purity obtained for the $CD8^+$ cells.

5.3.3.2 IFN-g mRNA SYNTHESIS BY LYMPHOCYTES.

A DNA band of the correct size for the IFN-g PCR product was seen by 30 cycles of the PCR in all the reactions from cDNA of unseparated cells, including that using cDNA derived from day 0 (Figure 40). The intensity of the signal from each day was approximately equivalent and showed no signs of diminishing towards the end of the seven days. Although some variability in signal strength was noticeable, this was not considered significant because of the inherent problems associated with quantitation by PCR discusssed earlier. $CD4^+$ cells showed a variable level of PCR product with

each day. At day 0, PCR product only became visible after 30 cycles and even then at low level. By day 1 the level of PCR product was high and detectable at 25 cycles. This level was maintained over days 2 and 3, but was less by days 4 and 6. Day 7 registered as a high level of IFN-g mRNA, visible as PCR product by 25 cycles. $CD8^+$ cells, like the $CD4^+$ cells, did not produce IFN-g mRNA at day 0, but IFN-g mRNA was synthesized at high levels from day 1 to day 6 with little variation in levels detected. The level was much reduced on day 7.

5.3.3.3 IFN-g PRODUCTION MEASURED BY ELISA.

The concentration of IFN-g in each day's lymph fluid was assayed using the bovine ELISA kit. The output is show graphically in Figure 41. The concentration of IFN-g rose significantly to a peak at day 3, and then declined until it returned to normal by day 7. The IFN-g concentration detected for days 1 and 2 was not significantly elevated. The peak concentration (day 3) was two and a half times background.

5.4 DISCUSSION.

The analysis of mRNA by PCR techniques has been adapted to the analysis of cytokine mRNA levels in activated cell populations *in vitro* and *in vivo*. Methods for semi-quantitating the presence of cytokine transcripts have been described which involve the sequential assay of specific PCR product as the number of cycles increases (Dallman *et al*, 1991). The PCR technique is claimed to be up to one thousand times more sensitive than Northern blotting, capable of detecting only ten thousand molecules (Wang *et al*, 1989).

A time course for induction of IFN-g mRNA has previously been described in which lymphocyte stimulation was by phytohaemagglutinin and detection was undertaken by *in vitro* translation of recovered mRNA in *Xenopus laevis* oöcytes (Efrat *et al*, 1982). The synthesized IFN-g protein was then detected by an antiviral plaque assay. This method detected IFN-g mRNA initially at 6 hours post-stimulation, with rapid accumulation until a plateau was reached at 16 hours. The kinetics of synthesis of IFN-g mRNA *in vitro* have also been studied using Northern blotting (Efrat *et al*, 1984; Granelli-Piperno *et al*, 1986; Gauchat *et al*, 1988). Following phytohaemagglutinin stimulation of human PBLs, IFN-g mRNA concentrations peak at 24 hours and decline slightly by 48 hours. The results described in 5.3.1 indicate that IFN-g mRNA induced by Concanavalin A from sheep lymphocytes can reach relatively stable and high levels by 3 hours post-stimulation. This is likely to reflect the sensitivity of the PCR technique, although it is possible that ovine IFN-g mRNA is induced more rapidly.

Intracellular immunofluorescent staining of human mononuclear cells freshly isolated from blood has demonstrated that in vitro anti-CD3 stimulation induces IFN-g production with a biphasic peak in the number of producing cells (Andersson et al, 1988). At the 6 hour peak the IFN-g-producing cells were small resting lymphocytes, but by the 24 hour peak a mixture of small lymphocytes and blast transformed lymphocytes were stained. Studies by the same group have demonstrated a similar biphasic peak of production during stimulation by ionomycin and phorbol esters (Andersson et al, 1990). The single peak of IFN-g production at 24 hour described in 5.3.1 indicates that ovine IFN-g synthesis kinetics are similar to that described in other species, although the mRNA transcription appears to peak much earlier.

There is no doubt that ovine lymphocytes have the capability of rapidly synthesizing IFN-g protein which aids the commencement of an active immune response. The IFN-g response of lymphocytes to an in vivo primary antigenic stimulation has been studied at the protein level. Immunization of antigenically naïve sheep with inactivated maedi-visna virus to give a primary immune response (not an antiviral response) leads to no detectable synthesis of IFN-g in efferent lymph (P. Bird, personal communication). Emery et al, 1990, found a similar result with inoculation of ovalbumin into antigenically naïve sheep. These results are predictable as most IFN-g synthesis from $CD4^+$ or $CD8^+$ T cells is from the "memory" subset. The $CD4^+$ and $CD8^+$ T cells in rats and humans responsible for synthesis of IFN-g in response to Concanavalin A stimulation, or ionomycin and phorbol ester stimulation are thought to be of the memory phenotype, i.e. previously activated T cells (Mason & Powrie, 1990; Sanders et al, 1988; Andersson et al, 1990). These cells have been subdivided on the basis of their cell surface leucocyte common

antigen isoform. Memory type cells bear the CD45RA^{dull} B^{bright} phenotype and secrete IFN-g, or the CD45RA^{dull}B^{dull} phenotype and give B cell help. Naïve cells (CD45RA^{bright} B^{bright}) secrete little IFN-g in response to mitogen stimulation (Mason & Powrie, 1990).

In vivo synthesis of IFN-g during a secondary immune response has been studied in sheep inoculated with ovalbumin and a variety of adjuvants (Emery et al, 1990). In their experiment, IFN-g protein was detected, using the same bovine IFN-g ELISA kit as used here, from within 2 hours of antigen administration, and peak concentrations occurred 6-16 hours later. The peaks contained three to seven times the quantities of IFN-g measured prior to inoculation, and the IFN-g concentration remained at least double background up to 7 days post-inoculation. In the experiments described here the concentration of IFN-g rose significantly to a peak at day 3, and then declined until it returned to prestimulation concentrations by day 7. The IFN-g concentration detected for days 1 and 2 was not significantly elevated. The peak concentration (day 3) was two and a half times background. The major differences between the two experiments are that Emery's sheep were given their primary inoculation in adjuvant only one month prior to the secondary inoculation, whereas the sheep in this experiment received its primary dose of 1mg ovalbumin in Complete Freund's Adjuvant nearly three years prior to the secondary dose, but did receive 0.5mg of ovalbumin in PBS intravenously on the day prior to lymphatic vessel cannulation (one week before secondary antigenic stimulation).

Production of IFN-g has been attributed to T cells and natural killer cells (Sandvig et al, 1987; Andersson et al, 1988). The slower response of lymph node cells in the sheep described here may reflect the progressive loss of memory T cells from the lymph node, or the activation of memory T cells which have been quiescent for many months compared with activation of cells only recently committed to being memory T cells in Emery et al's experiment. A noticeable response would be delayed until the recruitment of sufficient memory cells from elsewhere in the sheep. Emery et al's sheep would have higher concentrations of anti-ovalbumin antibodies and also ovalbumin-specific B cells present in the lymph node. When the ovalbumin was injected into the sheep, it would be rapidly bound by both free antibodies and also B cell surface antibodies. It would thus be rapidly presented to specific T cells, hence stimulating earlier T cell activation and cytokine secretion.

This is the first reported analysis of IFN-g mRNA transcription in efferent lymph draining a lymph node during a secondary immune response. It represents a novel method of studying the contributions of different cellular populations to IFN-g production at a time when the antibodies required to stain ovine lymphocytes intracellularly for IFN-g are not available.

The efferent lymph sample on day 0 was taken prior to the sheep being stimulated with ovalbumin and at least seven days post surgery. The presence of IFN-g mRNA on day 0 in unfractionated cells can be seen to be due to the involvement of neither $CD4^+$ nor $CD8^+$ T cells. This observation leads to a conclusion that another cell type within efferent lymph actively contains IFN-g mRNA, even if these cells only synthesize or secrete IFN-g protein at low levels. Evidence from intracellular staining of human lymphocytes

has demonstrated that only half the cells stimulated by anti-CD3 antibodies to produce IFN-g protein were $CD3^+$, and $CD4^+$ or $CD8^+$, and that 25% were $CD16^+$ cells (presumptive natural killer, NK, cells) (Sandvig et al, 1987; Andersson et al, 1988). The remaining 25% of IFN-g producing cells were $IL\text{-}2R^+$, erythrocyte-rosetting cells but could not be ascribed to the B cell, T cell or monocyte lineages. Stimulation of human PBLs by ionomycin and phorbol ester indicated that 20% of lymphocytes produce IFN-g with a biphasic peak. Of the IFN-g-producing cells at the 6 hour peak, 96% were $CD3^+$, 44% $CD4^+$, 57% $CD8^+$, 10% $CD45R^+$ (stains B cells, NK cells and virgin T cells), and 85% $UCHL1^+$ (stains memory T cells). At the 24 hour peak, 80% were $CD3^+$, 44% $CD4^+$, 37% $CD8^+$, 21% $CD45R^+$, and 77% $UCHL1^+$. The identity of the 20% $CD3^-$ cells in these latter figures was not determined for technical reasons, but were likely to be NK cells. There are no significant numbers of NK cells in sheep efferent lymph. Cell staining demonstrates cells are either $CD4^+$ T cells (≈50%), $CD8^+$ T cells (≈20%), γ/δ TcR T cells(≈15%), or B lymphocytes(≈15%). As B cells are not thought to synthesize any lymphokines, the only remaining source of the IFN-g mRNA would appear to be the γ/δ T cells. Human T cells clones expressing the γ/δ T cell receptor have been found to potentially synthesize many cytokines. They synthesize less IL-2 and GM-CSF but more IFN-g than α/β T cell clones when stimulated by Concanavalin A. The production of IL-4, IL-5, and TNF-α was not significantly altered between α/β and γ/δ T cell clones (Morita et al, 1991). The cytokine synthesis profile of sheep γ/δ T cells is unknown at present, but would be a suitable subject for further study.

The activation requirements of γ/δ T cells are not fully understood, but they commonly exhibit reactivity to heat-shock proteins (Born et al, 1990), mycobacterial antigens (O'Brien et al, 1989; Holoshitz et al, 1989), and other microorganisms (Munk et al, 1990).

There is likely to be an active inflammatory response to the lymphatic cannulation surgery, which may involve T cell cytokine synthesis. One week post-surgery it is known that cellular morphology and lymphocyte surface molecules have returned to basal levels. It is possible that cells involved in the inflammatory response may still contain mRNA for cytokines including IFN-g. This could be due to a continuing immune response by $CD4^-$ $CD8^-$ T cells (i.e. the γ/δ T cells) or natural killer cells. Both of these cell types seem to be less specific in their activation requirements. Cytokine mRNA which was synthesized in the immediate post-surgery period may still be present in cells due to stabilization of mRNA by cellular proteins, possibly involving the 3' AU rich regions.

The mRNA may be present at high levels in a small population of cells which are poor at secreting IFN-g protein. As no IFN-g protein was detectable on day 0, the IFN-g mRNA detected in the unseparated cells may be explained in a number of ways: (i) it may be mRNA undergoing translation into protein; (ii) there may be a block in secretion of active protein; (iii) the mRNA detected may be regulated post-transcriptionally; (iv) consumption of IFN-g by cells may be so extensive that none is detectable using this method. Cytokines contain AU-rich 3' regions which destabilize the transcript and regulate their concentration post-transcriptionally (Shaw & Kamen, 1986). A higher number of cells expressing IFN-g mRNA and at a higher level, in the absence of detectable IFN-g protein, has previously been reported for rat $OX\text{-}22^{low}$ $CD4^+$ cells in

comparison with OX-22^{high} CD4^{+} cells following Concanavalin A-stimulation for 24 hours (McKnight et al, 1991). (OX-22 monoclonal antibody recognizes exon B of the CD45R gene). This was determined to be due to more rapid initial induction of IFN-g mRNA in the OX-22^{low} CD4^{+} cells, followed by active down-regulation.

In conclusion, this section of work has developed a technique for the sensitive assessment of IFN-g mRNA synthesis by PCR. This has been combined with a recently developed technique for relatively rapid and large-scale cell separation (MACS) to assess the contribution to IFN-g mRNA synthesis by CD4^{+} and CD8^{+} T cells during an in vivo immune response. Although these techniques could only be applied in one experimental sheep due to time considerations, the results gained suggest that this method of analysis should be capable of attributing IFN-g synthesis to individual cell groupings during responses to different immune stimuli. At the current stage of development the assay developed should be interpreted as primarily qualitative, although gross differences in IFN-g mRNA concentration can be appreciated. The use of control genes, such as actin, found at relatively constant concentration within the cDNA preparations can allow a semi-quantitative assessment of IFN-g mRNA concentration. This gene can also eliminate errors due to unequal amounts of template cDNA being used. This additional control could not be included in the experiment presented here due to time considerations.

The isolation of ovine genes for many other cytokines, e.g. IL-2, IL-4, IL-10, means the technique should be capable of assessing the role of many cytokines during in vivo immune responses. The sheep lymphatic vessel cannulation model may now be about to yield much

more specific information on the immune activity within and draining from lymph nodes. For each lymphokine the PCR procedure would need to be optimized as described for IFN-g.

The techniques may be used with a variety of cellular differentiation markers to assess the role of different cell subsets in IFN-g and other lymphokine synthesis. Currently the cytokine synthesis profiles of T cells expressing ovine MHC class II molecules DR and DQ are being assessed within the department. The possible future availability of markers for memory T cells, naïve T cells, natural killer cells, or suppressor cells may allow the use of these techniques in other situations.

The techniques described here are unlikely to be sufficient for the analysis of cytokine production by relatively infrequent cell types, such as γ/δ T cells. A modification of the mRNA isolation procedure would be required, such as that described by Rappolee et al (1989), which can isolate total RNA for PCR from 1-10^5 cells. Infrequent cell types may benefit from the use of MAC separation followed by additional purification by FAC sorter.

The potential for the technique is dependent on the further availability of ovine cell surface markers and ovine cytokine gene sequences.

Figure 34.

Submarine electrophoresis in 2% agarose of PCR products synthesized using primers 343E and 474F in the ovine IFN-g PCR. Varying magnesium concentrations were used to determine the optimum required. The 302bp PCR product of Ovifn is marked with >>.

Lane	Mg concentration
M	λ DNA markers
1	1mM
2	2mM
3	3mM
4	4mM
5	5mM
6	6mM
A	Anglian buffer (6.7mM)

Figure 34.

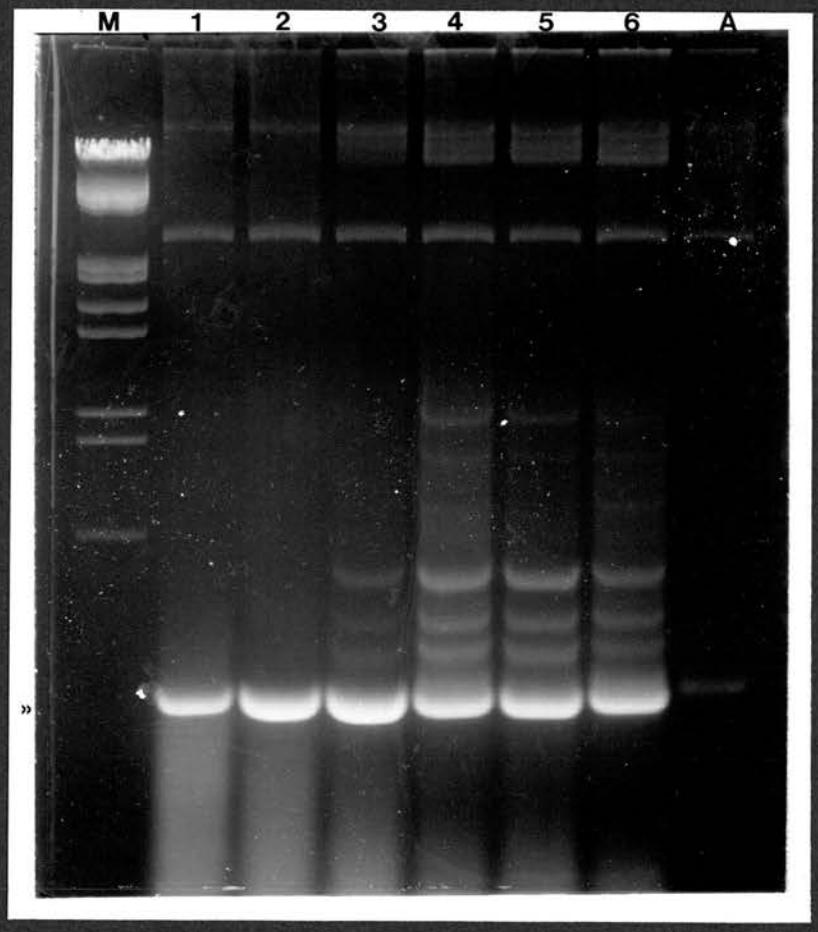


Figure 35.

A Southern hybridization of a polymerase chain reaction using primers 343E and 474F specific for ovine IFN-g sampled at 3 cycle intervals from cycle 18 (lane a) until cycle 42 (lane i). Lane n was a PCR containing no target DNA. Lane p was a PCR containing linearized pOvifn as target DNA. Membrane was hybridized with radio-labelled Ovifn. The specific product is marked «.

Figure 35.

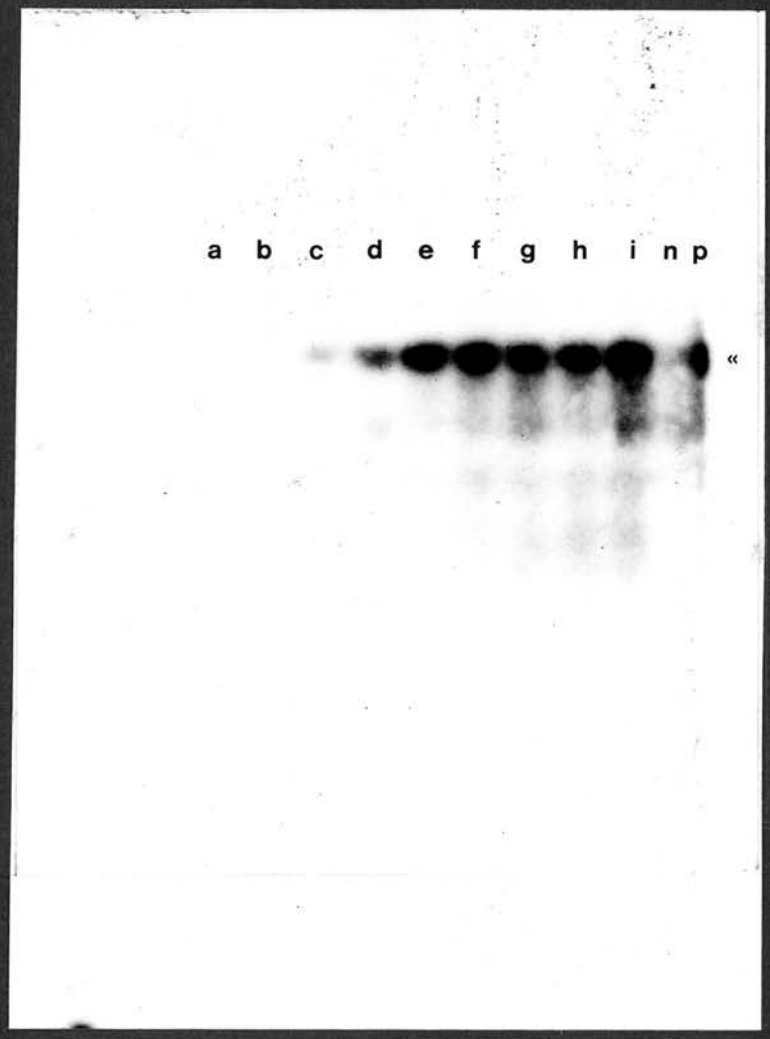


Figure 36.

Southern hybridization of polymerase chain reactions using cDNA derived from cells stimulated with concanavalin A for varying time periods. Aliquots removed from PCRs after 20 (A), 25 (B), and 30 (C) cycles. Hybridization with radio-labelled pOvifn.

Lane	Hours of con A stimulation
a	0
b	1
c	2
d	3
e	4
f	6
g	9
h	12
i	24
j	36
k	48
l	positive control

Figure 36.

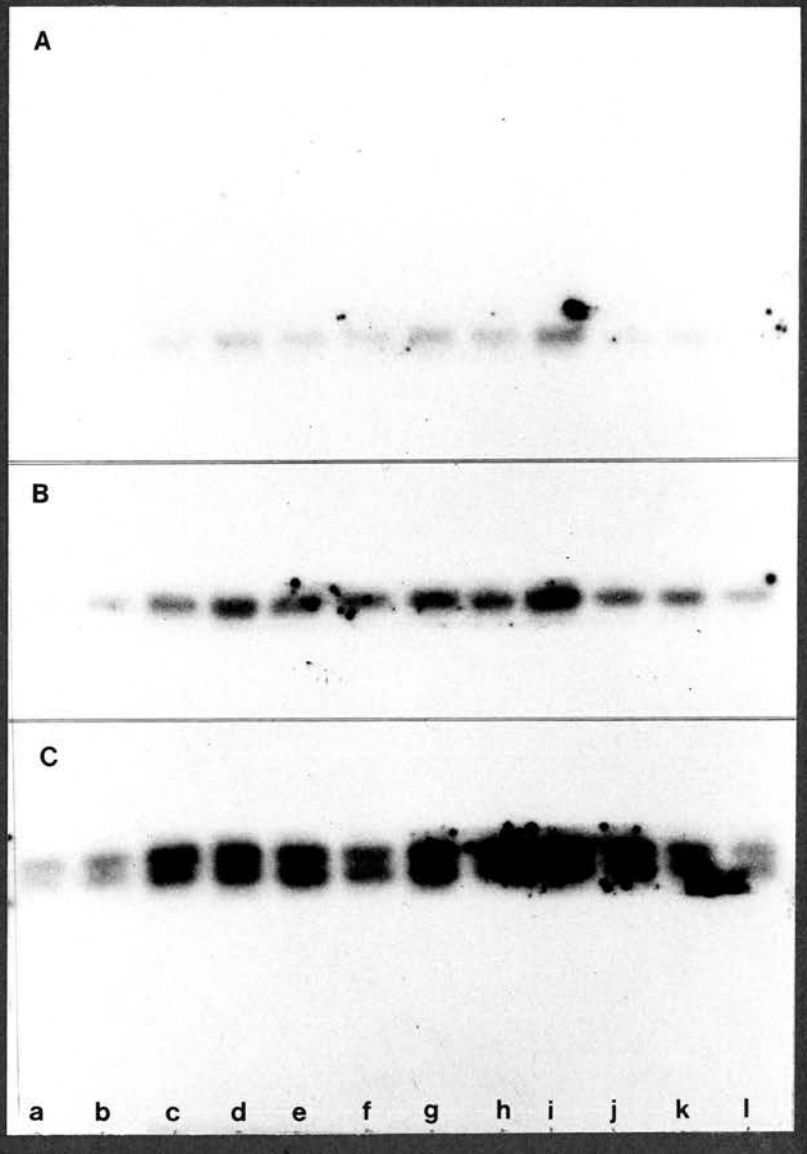


Figure 37.

Kinetics of ovine IFN-g appearance in supernatant from lymphocytes stimulated with concanavalin A for varying time periods. Quantitation of IFN-g was by ELISA and the results are given as the OD450nm.

Figure 37.

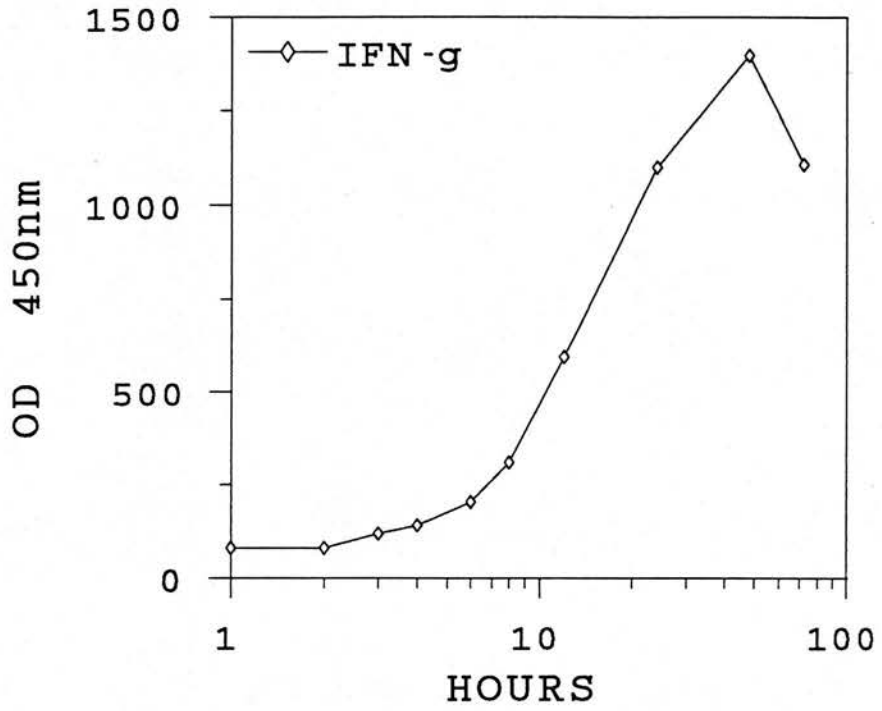
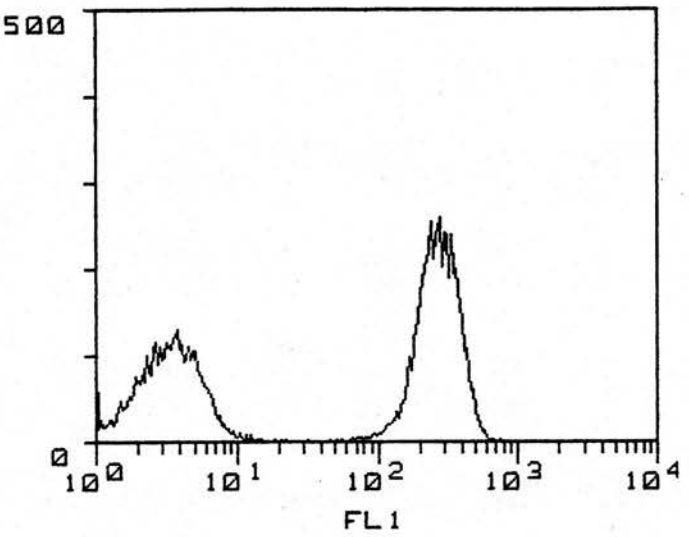


Figure 38.

A. FACS profile of efferent lymph cells stained with SBU-T4 prior to purification by MACS.

B. FACS profile of efferent lymph cells stained with SBU-T4 after purification by MACS.

Figure 38. A.



B.

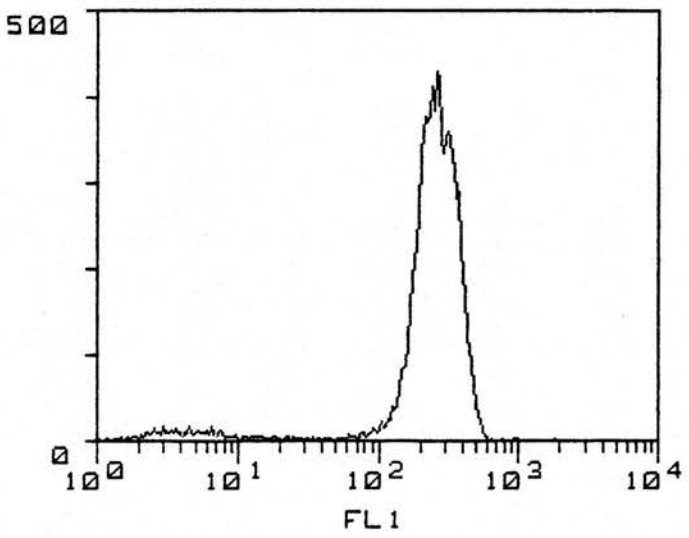
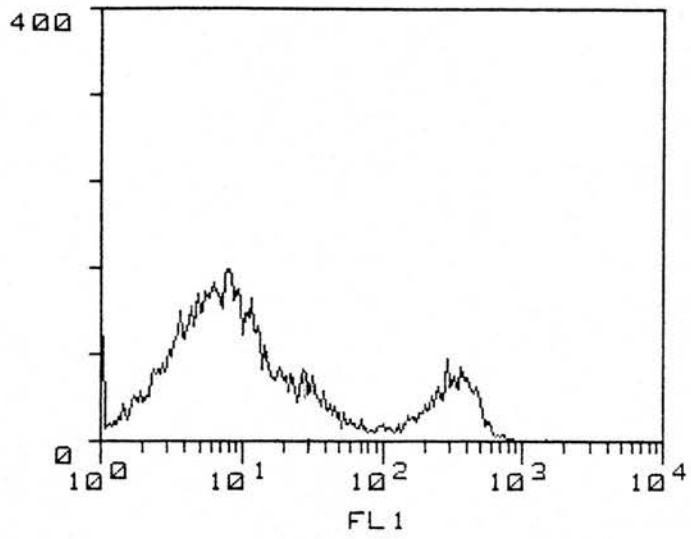


Figure 39.

A. FACS profile of efferent lymph cells stained with SBU-T8 prior to purification by MACS.

B. FACS profile of efferent lymph cells stained with SBU-T9 after purification by MACS.

Figure 39. A.



B.

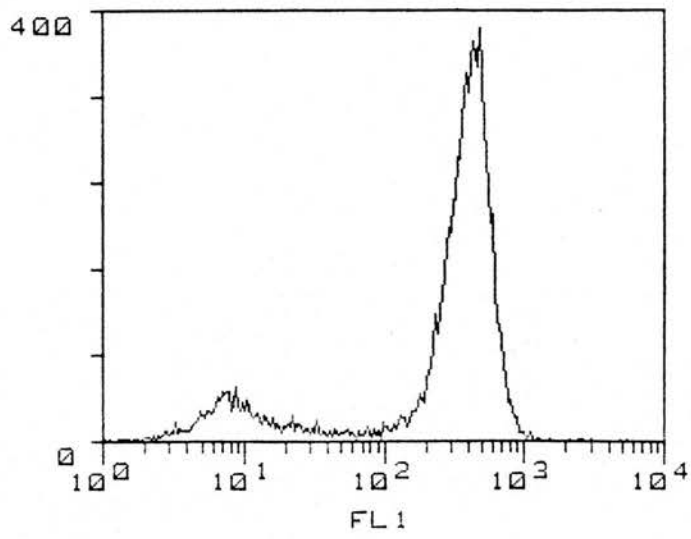


Figure 40.

Southern hybridization of PCR to determine ovine IFN-g mRNA appearance in prefemoral efferent lymph cells from a primed sheep on successive days before and after stimulation with ovalbumin. Cells were either unseparated (A), or separated by MACS purification into $CD8^+$ (B), or $CD4^+$ (C). 25 rounds of PCR were performed. Hybridization was with radio-labelled pOvifn.

Figure 40.

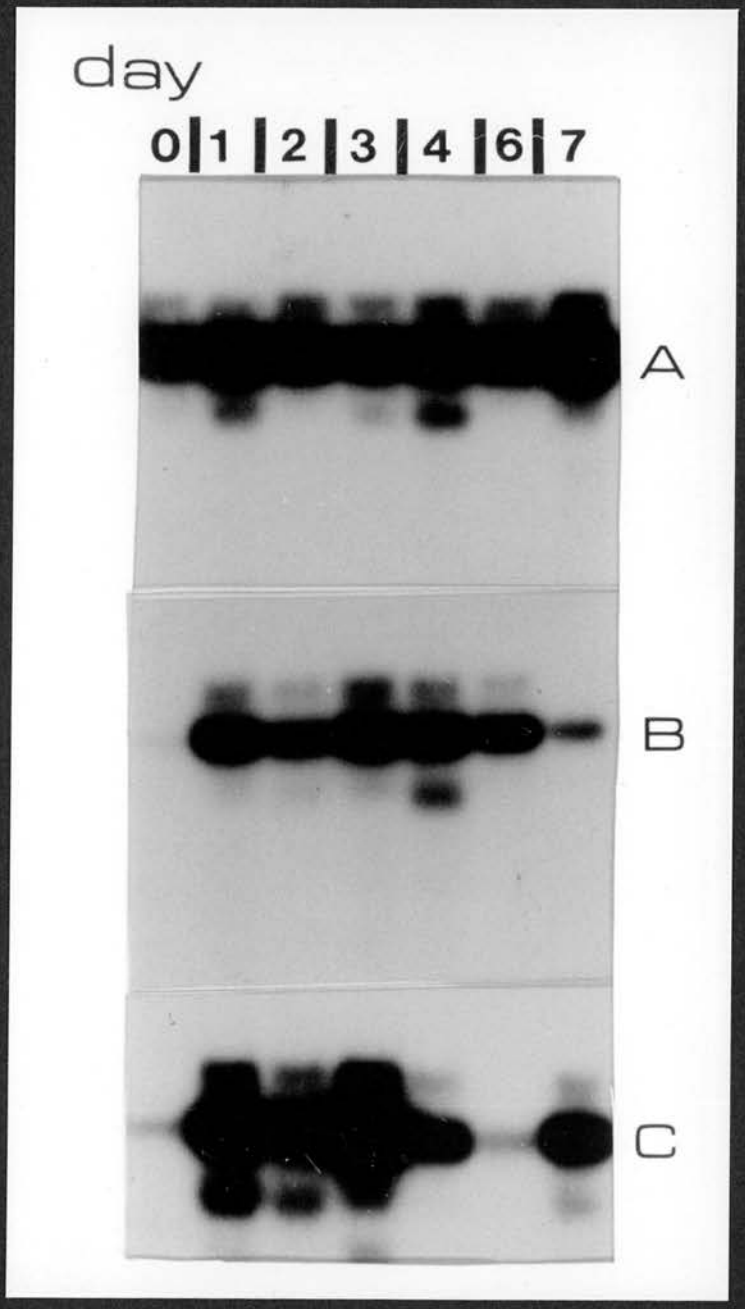


Figure 41.

Kinetics of ovine IFN-g appearance in prefemoral efferent lymph fluid from a primed sheep on successive days before and after stimulation with ovalbumin. Quantitation of IFN-g was by ELISA and the results are given as the OD450nm.

Figure 41.

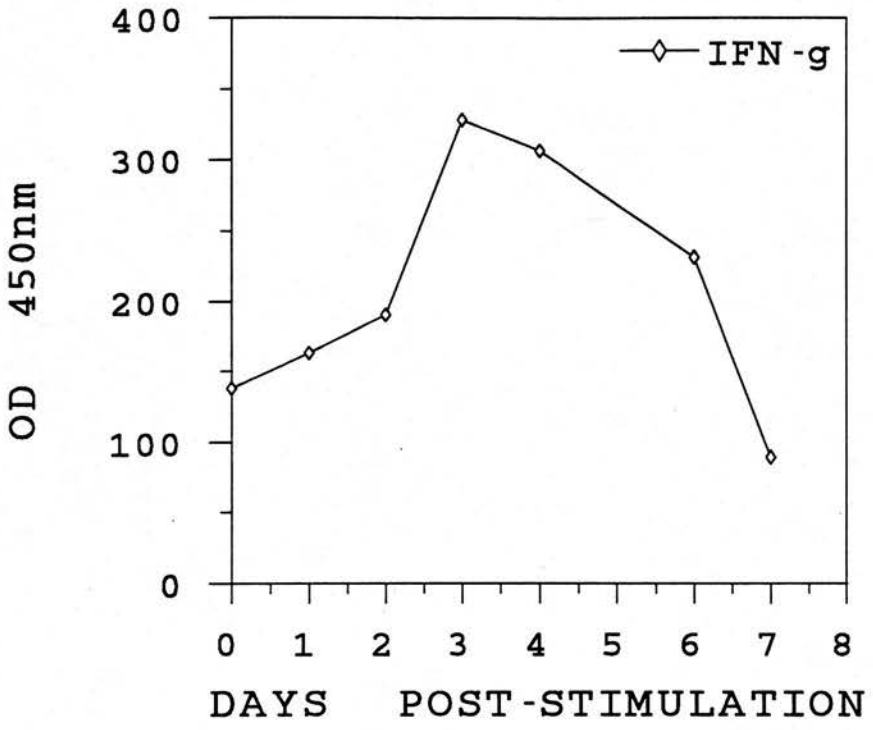

400
300
200
100
0
OD 450nm
IFN-g
0 1 2 3 4 5 6 7 8
DAYS POST-STIMULATION

Table 4.

Percentage incorporation of radioactive dCTP into single-stranded cDNA. mRNA is from concanavalin A-stimulated lymphocyte time course. Measurement is by TCA precipitation and Cherenkov counting. (CPM = counts per minute).

Table 4.

HOURS	CPM INCORPORATED	CPM TOTAL	% INCORPORATED
0	1727	1976153	0.09
1	2172	1595668	0.14
2	1268	1822310	0.07
3	1795	1401270	0.13
4	1793	1406156	0.13
6	1890	1492978	0.13
9	2585	1416475	0.18
12	1673	1597833	0.10
24	3880	1353828	0.29
36	1672	1603420	0.10
48	1507	1379617	0.11

APPENDIX 1.

WESTERN BLOTTING:

Running buffer:

Trizma	50mM
SDS	0.1%
Glycine	370mM

Transfer buffer:

Trizma	48mM
Glycine	39mM
Methanol	20%

Alkaline Phosphatase substrate:

Trizma	0.1M pH9.0
Nitro Blue Tetrazolium	180µg/ml
Bromo-chloro-indolyl-phosphate toluidine salt	90µg/ml
$MgCl_2$	3.5mM

APPENDIX 2.

TEA/DOC pH8.0:

Triethanolamine	15mM
Na deoxycholate	0.5%
pH with NaOH	

TEA/DOC/NaCl pH11.5:

Triethanolamine	15mM
Na deoxycholate	0.5%
NaCl	0.5M

Lysis buffer:

Trizma	20mM pH8.0
NaCl	140mM
Triton X100	0.5%
PMSF	0.2mM

APPENDIX 3.

MONOCLONAL ANTIBODIES.

MONOCLONAL	REACTIVITY	ISOTYPE
CC15	anti-T19	IgG2a
SBU-T4	anti-ovine CD4	IgG2a
SBU-T8	anti-ovine CD8	IgG2a
SW73.2	anti-ovine MHC class II	IgG2a (rat)
VPM5	anti-ovine IgM	IgG1
VPM16	anti-ovine MHC class II	IgG1
VPM19	anti-ovine MHC class I	IgG1
VPM36	anti-ovine MHC class II DQα	IgG1
VPM37	anti-ovine MHC class II DRβ	IgG1
VPM38	anti-ovine MHC class II DRα	IgG1
VPM41	anti-ovine MHC class II DQβ	IgG1

APPENDIX 4.

GTC buffer:

Guanidinium thiocyanate	4.0M
Sodium citrate, pH7.	25mM
Sarkosyl	0.5%
β-mercaptoethanol	0.1M
Sigma antifoam	1-2 drops

T4 kinase buffer:

Trizma	50mM pH7.6
$MgCl_2$	10mM
DTT	5mM
BSA	50µg/ml

1 x TAE gel running buffer:

Trizma	40mM
Acetic acid	20mM
EDTA	2mM pH8.4

1 x CIP dephosphorylation buffer:

$ZnCl_2$	1mM
$MgCl_2$	1mM
Trizma	10mM pH8.3

1x ligation buffer:

Trizma	50mM pH7.5
$MgCl_2$	10mM
ATP	500µM
DTT	500µM

2 x TSS:

PEG 4000	(40%)	4.5ml
LB		4.1ml
$MgCl_2$	1M	0.4ml
DMSO		1.0ml

LB Medium:

Tryptone	1%
Yeast extract	0.5%
NaCl	0.5%
NaOH 1N	0.1%

Ampicillin (Sigma) 150µg/ml
Kanamycin (sigma) 70 µg/ml
X-GAL (Northumbria Biologicals Limited, Cramlington, UK) 80µg/ml
IPTG (Northumbria Biologicals Limited, Cramlington, UK) 240µg/ml

TNE buffer:

Trizma	10mM	pH8.0
NaCl	100mM	
EDTA	1mM	

GTE buffer:

Glucose	50mM	
Trizma	25mM	
EDTA	10mM	pH8.0

TE buffer:

Trizma	10mM	pH7.6
EDTA	1mM	

1xYT:

Tryptone	0.8%
Yeast extract	0.5%
NaCl	0.25% pH7.5

Lambda DNA screening

Denaturation solution:

NaOH	0.2m
NaCl	1.5M

Neutralization solution:

Trizma	0.4M pH7.6
2 x SSC	

Equilibration solution:

2 x SSC

Prehybridization solution 1:

6xSSC	
5 x Denhardt's solution	
Sodium pyrophosphate	0.05%
Denatured salmon sperm DNA	100µg/ml
SDS	0.5%

Hybridization solution 1:

6 x SSC	
1 x Denhardt's solution	
Sodium pyrophosphate	0.05%
Yeast tRNA	100µg/ml

TNT:

Trizma	10mM pH8.0
NaCl	150mM
Tween 20	0.05%

SM medium:

NaCl	100mM
$MgSO_4$	15mM
Trizma	50mM pH7.5
gelatin	0.01%

TM medium:

Trizma	10mM pH7.6
$MgSO_4$	10mM

Lysogen extraction buffer:

Trizma	50mM pH7.5
EDTA	1mM
Dithiothreitol	5mM
PMSF	50µg/ml

TES:

Trizma	10mM pH8.0
EDTA	1mM
SDS	1%

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