MOLECULAR ANALYSIS OF OVINE CD1 EXPRESSION

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DEDICATION

This thesis is dedicated to the memory of my father,
John Rhind (1934-1995).

THE UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

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The CD1 family is a family of molecules with structural homology to MHC class I and low but significant sequence homology to both MHC class I and MHC class II. A key feature of the CD1 family is restricted tissue distribution. This distribution varies with isotype but includes cortical thymocytes, antigen presenting cells and intestinal epithelial cells. It has recently been shown that CD1 can present mycobacterial lipid antigens to T cells (Beckman et al. 1994, Sieling et al. 1995). Another major feature of CD1 is limited polymorphism which is in contrast to the extensive polymorphism associated with MHC molecules.

In humans, five CD1 genes exist (CD1A-E) which encode four CD1 molecules (CD1a-d). CD1 genes have also been described in mice, rats, rabbits and sheep. Studies in cattle and pigs have been at the protein level using anti-CD1 mAbs. Studies on ovine CD1 were initially carried out using mAbs to study expression in cells and tissues. More recently, four ovine CD1 cDNA clones (SCD1A25, SCD1B42, SCD1B52 and SCD1T10) have been isolated all of which have closest homology to human CD1B (Ferguson et al. 1996). SCD1B52 contains a precise deletion of exon 4 which encodes the $\alpha 3$ domain. Southern hybridization has indicated the existence of up to seven ovine CD1 genes.

The aim of the work carried out in this thesis was to further investigate the ovine CD1 family and clarify the existing information at the cellular and molecular level. Initial studies utilised existing anti-CD1 mAbs to clarify the pattern of tissue expression of ovine CD1. Two distinct clusters of mAbs were shown to exist - the majority recognise a molecule with tissue distribution similar to CD1b whilst three mAbs, SBU-T6, CC43 and CC118 demonstrate staining of tissue macrophages, the majority of B cells and monocytes in addition to thymocytes and dendritic cells. NH_2-terminal sequencing was subsequently used to establish the antigens recognised by the mAbs SBU-T6 and CC14. This technique demonstrated that the CC14 antigen was consistent with the predicted sequence of the SCD1B42 cDNA clone whereas the SBU-T6 antigen had closest homology to the predicted amino-acid sequence of the human CD1E gene. This is particularly noteworthy as no protein product of the CD1E gene has yet been described in any species.

Subsequent work attempted to isolate the gene encoding the molecule recognised by SBU-T6 using a transient expression system in which COS cells were transfected with a lymph node cDNA library contained within the vector pcDNA3. This was unsuccessful, however a sheep CD1D-like sequence was isolated from this library utilising primers based on the NH_2-terminal sequence of the SBU-T6 antigen. Expression of the SCD1D gene was investigated using *in situ* hybridization and RT-PCR. SCD1D transcripts were demonstrated in thymus, liver, intestine, lymph node and PBLs. A further experiment investigated the expression of the SCD1B52 gene (which contains a precise deletion of exon 4). These studies have extended the knowledge of the ovine CD1 family and establish it as one of the most complex described to date. This work has demonstrated that sheep clearly express multiple CD1 isotypes as in man and rabbits in addition to the multiple CD1B-like genes reported previously.

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Declaration

I declare that the composition of this thesis and the work presented herein are my own except where specifically stated.

Susan Rhind

CONTENTS

LIST OF FIGURES **Page**

LIST OF TABLES

ABBREVIATIONS

ATP	Adenosine triphosphate
APC	Antigen presenting cell
CD	Cluster of differentiation
cDNA	DNA complementary to mRNA
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetate
GSP	Gene specific primer
HBSS	Hanks' balanced salt solution
HRP	Horse radish peroxidase
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IPTG	Isopropylthio-beta-D-galactoside
kb	Kilobase
kD	KiloDalton
LAM	Lipoarabinomannan
LB	Luria broth
LC	Langerhans' cell
MHC	Major histocompatibility complex
MIIC	MHC class II compartment
mRNA	Messenger RNA
NCCI	Non-classical class I
NMS	Normal mouse serum

$OD_{(x)}$	Optical density at x nanometres
PAGE	Polyacrylamide gel electrophoresis
PBLs	Peripheral blood lymphocytes
PBMs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
TAE	Tris-acetate-EDTA
TCR	T cell receptor
TE	Tris-EDTA
X-gal	5-bromo-4-chloro-3-inolyl-beta-D-galactoside

1. INTRODUCTION

The CD1 family represents a group of genes and molecules with homology to both MHC class I and class II. Five CD1 genes exist in man (CD1A-E) for which four protein products have been described (CD1a-d). Sequence analysis of these genes has allowed division into group 1 (CD1A-C) and group 2 (CD1D) (Calabi et al. 1989c). No protein product of the CD1E gene has yet been described but on the basis of sequence, the gene has been placed in an intermediate position between the group 1 and group 2 families (Calabi et al. 1989c). CD1 genes have also been described in mice, rats and sheep whilst monoclonal antibodies (mAbs) have additionally identified CD1 molecules in cattle and pigs.

A key feature of CD1 expression is its presence on cells with integral functions in the immune response. Recent functional studies have shown that CD1b presents mycobacterial mycolic acid and lipoarabinomannan (LAM) to T cells (Beckman et al. 1994, Sieling et al. 1995) implying that CD1 molecules represent an additional arm of the immune response capable of presenting non-protein antigens such that the range of antigens which can be presented and subsequently recognised by T cells is increased.

There is marked interspecies variation in the CD1 family, notably rodents possess only members of the CD1d group. The CD1 family in sheep is particularly interesting as mAb studies have shown two distinct patterns of CD1 expression, one consistent with human CD1b, the other resembling human CD1c (Dutia and Hopkins, 1991; Hopkins and Dutia, 1991). More recent studies at the molecular level have identified four ovine CD1 clones all of which have closest homology to the CD1B (Ferguson et al. 1996). The work described in this thesis was undertaken to further analyse the nature of the ovine CD1 family using both immunochemical and molecular techniques.

The following introduction includes sections on cells and molecules involved in the immune response as a necessary prelude to the specific sections on the CD1 genes and molecules themselves. Section A deals with the MHC and cells of the immune system and is followed by Section B which more specifically deals with non-classical class I molecules and the CD1 family.

SECTION A

1A.1 The Major Histocompatibility Complex (MHC)

Inherent in the survival of an organism is the need to distinguish between self and non-self such that foreign antigens can be rapidly recognised and eliminated whilst maintaining tolerance to self antigens. That such a system of distinguishing between self and non-self existed was first shown in mammals by the phenomenon of skin graft rejection (Gorer, 1936). Subsequently, the highly polymorphic antigens responsible for this rejection were characterized and named major histocompatibility complex (MHC) antigens.

Three classes of MHC antigens exist in mouse and man - MHC class I, MHC class II and MHC class III. MHC class I and class II antigens present peptides to $CD8^+$ T cells and $CD4^+$ T cells respectively although there are exceptions to this general rule. MHC class III genes encode a variety of products including complement components but are not involved in antigen presentation and therefore will not be discussed further.

Early reports on the CD1 family identified the fact that these molecules structurally resembled MHC class I antigens although the CD1 genes were shown not to map to the MHC (Van de Rijn et al. 1983, Kahn-Perles et al. 1985, Calabi and Milstein, 1986). Since it is now generally accepted that CD1 molecules are related to both MHC class I and class II molecules, the MHC will be discussed in detail as important comparisons will subsequently be made between the two families in terms of both structure and function.

1A.1.1 MHC class I genes and molecules

MHC genes and antigens have been best characterized in man and in mouse. In man, the class I genes map to chromosome 6 and are termed HLA (Human Leucocyte Antigen) -A, HLA -B and HLA-C. In mice, the class I genes map to chromosome 17 and are termed H-2K, H-2D and H-2L. Many other class I genes also exist, however these genes do not demonstrate the characteristic genetic polymorphism and widespread tissue expression of classical class I molecules and as such are termed 'non-classical class I' genes (See Section 1B.1) .

Structure

MHC class I molecules consist of a 45kd α (heavy) chain which associates non-covalently with $\beta_2$microglobulin (β_2m). The heavy chain consists of three extracellular regions (α1, α2 and α3), a transmembrane region and a cytoplasmic tail.

X-ray crystallography has shown that the the α1 and α2 domains combine to form the antigen binding groove which consists of a β pleated sheet flanked by two α helices (Bjorkman et al. 1987a). This groove accommodates a short peptide (usually 8-9 amino-acids long) and this combination allows recognition of the peptide in an MHC restricted fashion. The allelic polymorphisms which are characteristic of the MHC occur mainly in residues in and around the groove allowing altered specificity of the MHC class I molecules (Bjorkman et al. 1987b). β_2m makes contact with all three of the extracellular domains of the α chain (Bjorkman et al. 1987a) hence the conformation of the α chain is dependant on this interaction with β2m (York and Rock, 1986).

MHC class I processing and presentation

The function of MHC class I molecules is to sample peptides from within the cell and present them to T cells such that foreign peptides can be identified and the appropriate immune response generated. Endogenous antigens (e.g. viral proteins) are degraded within the cytoplasm and processed into peptides capable of associating with MHC class I. Protein degradation is carried out by a particle called the proteasome which hydrolyses the protein to oligopeptides (Rechsteiner et al. 1993). Proteins are marked for degradation by ubiquitination (Hilt and Wolf, 1996) then the degraded peptides are transported into the rough endoplasmic reticulum via the TAP transporter. This peptide transporter is a heterodimer formed by polypeptides encoded by the *TAP1* and *TAP2* genes which are located within the MHC class II region. Once within the RER, these short peptides are then free to form associations with MHC class I and β_2-microglobulin. This stable complex can then be transported

via the Golgi to the cell surface. The molecular chaperone, calnexin, prevents the cell surface expression of class I molecules which are not fully assembled (Rajgopalan and Brenner, 1994).

Although this is the general dogma for class I presentation, exceptions to this do exist such that exogenous antigens can be presented by MHC class I antigens e.g. Pfeifer and colleagues (1993) demonstrate the existence of an alternative class I pathway for phagocytic processing of bacterial antigens.

The optimal length for peptide binding to MHC class I molecules is 8-9 amino acids as non polymorphic amino acids at the ends of the groove form hydrogen bonds with the amino and carboxy termini of the peptide thus restricting the length of peptide that can be bound (Bleek and Nathenson, 1993). Although this is optimal, longer peptides have also been shown to bind such that the carboxyl terminal residue is positioned outside the peptide binding site (Collins et al. 1994).

1A.1.2 MHC class II genes and molecules

MHC class II genes are again best characterized in man and mouse. In man, both α and β chains are encoded by genes within the HLA-D region (DP, DQ and DR). In the mouse, class II genes are in the H-2I region (IA and IE)

MHC class II molecules are found on antigen presenting cells i.e. B cells, macrophages and dendritic cells. In addition, following cytokine or inflammatory stimulation there is induction of MHC class II molecules on a variety of endothelial and epithelial cells. One example of this is the class II induction which occurs on keratinocytes in response to IFN-γ (Griffiths et al. 1989). Class II molecules consist of an α chain and a β chain and typically bind peptides of 12-15 amino acids in length.

The recently described MHC class II molecule HLA-DM is almost as closely related to class I molecules as class II molecules and has been shown to play a key role in antigen presentation (Morris et al. 1994, Fling et al. 1994). The functional role of HLA-DM is described below.

Structure

Each MHC class II molecule consists of a 33kd α chain and a 28kd β chain which associate non-covalently to form a heterodimer. The α and β chains each possess extracellular α1 and α2, and β1 and β2, domains respectively. The antigen binding groove is formed by the α1 and β1 domains with the class II β1 domain corresponding to the class I α2 domain (Brown et al. 1993). X-ray crystallography has been used to determine the three dimensional structure for MHC class II molecules which resembles MHC class I (Brown et al. 1993). As for MHC class I, the peptide binding groove is composed of a β pleated sheet flanked by two α helices. The antigen binding groove is open ended allowing binding of longer peptides - typically 15-24mers.

MHC class II processing and presentation

Exogenous antigens are internalized into an endosomal compartment in which association with class II molecules occurs. The compartment in which MHC class II/peptide complexes are assembled has been referred to as the MIIC (For MHC Class II Compartment - Amigorena et al. 1994). Class II α and β chains associate with the invariant chain (Ii) within the RER. A region of the invariant chain called CLIP associates with class II and inhibits peptide binding. The MHC class II molecule HLA-DM (which accumulates in the MIIC - Sanderson et al. 1994) is then responsible for facilitating the release of CLIP and allowing peptide to bind to the class II molecule (Sherman et al. 1995 and reviewed by Roche, 1995). Once free of invariant chain, the class II molecule can associate with exogenous antigen which has been internalized and degraded.

1A.2 Antigen Presenting Cells

The major 'professional' antigen presenting cells are dendritic cells, B cells, monocytes and macrophages which process and present antigen to T cells in association with MHC class II molecules. Certain endothelial and epithelial cells can

also express class II upon activation and are therefore be considered 'non-professional' APCs.

The dendritic cell is of particular interest as it expresses high levels of CD1 and is the most potent immunostimulatory cell known. The key role of dendritic cells in antigen presentation is well recognised. These cells have several features which contribute to their ability to stimulate the proliferation of resting T cells including high levels of MHC class II expression and the presence of cell surface adhesion and co-stimulatory molecules. Dendritic cells in the periphery exist in two stages of maturation - immature DCs which monitor non-lymphoid organs and process antigen prior to maturing into immunostimulatory cells which lose the ability to process antigen but acquire surface molecules to aid their function in initiating the T cell response (Sallusto and Lanzavecchia, 1994). Langerhans cells also express significant levels of CD1 and several reports have demonstrated that epidermal Langerhans cells mature into dendritic cells under the influence of the cytokines GM-CSF and IL-1 (Heufler et al. 1988, Koch et al. 1990).

1A.3. T Cell Ontogeny

Since MHC molecules function to present antigen to T cells, it is relevant to discuss mechanisms of T cell development and, in particular, the means whereby the T cell repertoire is generated.

T cells development begins in the embryo with haemopoietic stem cells seeding the thymus. Immature thymocytes reside in the thymic cortex where they undergo the processes of positive and negative selection and migrate to the medulla as mature thymocytes. The critical feature of thymocyte development and maturation is in educating the host to delete any cells which have the potential to generate autoreactive responses (negative selection) whilst maintaining the ability to recognise antigen in the context of self MHC molecules (positive selection).

1A.3.1 Positive and negative selection

The maturation of T lymphocytes depends on the interaction of the TCR on the immature thymocyte with MHC/peptide complex within the thymus. This process

results in the generation of CD4 T cells which are restricted by MHC class II and CD8 T cells which are restricted by MHC class I (minor exceptions to this general rule do exist). That peptide is involved in the selection process has been shown using TAP1 mutant mice. The empty class I molecules which appear on the cell surface in these mice can be loaded and stabilized with peptide (in the presence of β2m) such that the effects of different peptides on selection can be studied. Using this model with cultured foetal thymi, it was shown that only some peptides promoted positive selection (Ashton-Rickardt et al. 1994).

Two models to explain the process of positive and negative selection have been proposed. The first is the qualitative model which depends on conformational changes in the TCR or associated molecules modulating signal transduction. The second is the quantitative model where selection depends on alterations in the affinity or avidity of TCR binding. Current evidence suggests that the quantitative model is likely to be the most accurate. This conclusion comes from recent work by Alam and colleagues (1996) in which surface plasmon resonance was used to measure the kinetics of TCR interactions with ligands capable of inducing positive or negative selection. The results showed that the affinity of binding correlated directly to the outcome of selection i.e. lower affinity (within a 'window') resulted in positive selection and higher affinity in negative selection.

Studies in β2m deficient mice have shown that not all γδ T cells require β2m dependent proteins for positive selection (Correa et al. 1992). However selection of γδ T cells by non-classical class I molecules (H-2T region products) has been demonstrated (Dent et al. 1990, Wells et al. 1991).

1A.3.2 Extrathymic T cell development

In addition to thymic T cell development, a significant number of T cells develop extrathymically as proven by the capacity of both αβ and γδ T cells to develop in the absence of a thymus. The major organ involved in extrathymic T cell development is the intestine which supports the development of two T cell subsets which are unique to this area - $CD4^{+}CD8\alpha\alpha^{+}$ and $CD4^{-}CD8\alpha\alpha^{+}$ (Reviewed in Poussier and Julius,

1994). The developmental pathways and selection processes which govern the repertoire of these gut derived cells are still uncertain, however it is known that they do not develop through the $CD4^+CD8^+$ pathway as is the case for the thymus derived and selected cells. As a result of this 'unconventional' selection process, this cell population contains some autoreactive cells which are thought to have a role in the maintenance of epithelial integrity by destroying altered epithelial cells (Rocha et al. 1992). Experiments using β2m knockout mice have shown that in the absence of β2m, CD8+ γδ IEL developed normally in contrast to CD8+ αβ IEL which showed minimal development (Fujiura et al. 1996). Further, CD8αα and CD8αβ αβ IEL were dependant on MHC molecules for development whereas CD8αα γδ IEL were not.

1A.4 T Cell Subsets

As described in the previous section, T cells function to recognise antigen in the context of self-MHC molecules. The T cell interacts with MHC molecules via the T cell receptor which consists of either α and β subunits (αβ T cells) or γ and δ subunits (γδ T cells). The T cell receptor (TCR) can be considered as a 'multiprotein signalling machine' (reviewed in Janeway, 1992). The components of this 'machine' include the coreceptors CD4 and CD8 each of which associate with a tyrosine kinase ($p56^{lck}$) and CD45 (a transmembrane tyrosine phosphatase). These molecules combine to generate optimal signals following T cell antigen recognition. In addition to recognition by the TCR of MHC plus peptide, co-stimulatory signals are also required in most cases to initiate T cell responses. These co-stimulatory signals arise from the interaction of members of the CD28 family on T cells with members of the B7 family on the APC (Reviewed in June et al. 1994).

1A.4.1 Structure of the TCR

The basic structure of the αβ TCR was originally established by Saito and colleagues (1984) following analysis of cDNA sequences. The receptor is made up of an α and

β chain each with two extracellular Ig like domains, an amino terminal variable and carboxy terminal constant domain. X-ray crystallography of the β chain shows structural homology to immunoglobulins (Bentley et al. 1995). Functional TCR genes arise following rearrangement of V and J segments in α and γ chains and V, D and J segments in β and δ chains. As is the case for immunoglobulin genes, diversity in the receptor arises as a result of the combination of multiple germ line segments, combinatorial joining, junctional flexibility, N-region nucleotide addition and somatic mutation (Davis and Bjorkman, 1988). Although the γ and δ genes contain fewer germ line gene segments than α and β genes, the actual potential diversity of the γδ receptor is greater than the αβ receptor as a result of alternative joining of D gene segments.

The proposed site of antigen contact within this receptor is the V-(D)-J junctional region of the TCR which corresponds to the CDR3 region of Ig molecules with the CDR1 and CDR2 regions contacting the α helices of the MHC (Hedrick et al. 1988; Davis and Bjorkman, 1988).

A.4.2 γδ T cells

Several reports have demonstrated the ability of γδ Tcells to restrict non-classical class I and CD1 responses (see below). Due to the interest and speculation regarding the possible involvement of γδ T cells with the CD1 family, this subset will be discussed in more detail.

Man and mouse

Marked interspecies variations in the numbers, localization and repertoire of these cells exists. In general, γδ T cells have preferential localization in epithelial tissues. In mice, there are several populations of γδ T cells associated with epithelial sites. In the skin, there is a population of $Thy1^+$ dendritic epidermal cells (dEC) which are $CD4^-/CD8^-$ negative and express an essentially monomorphic γδ TCR (Allison and Havran, 1991). Intestinal intraepithelial γδ T cells differ in that they express CD8 and

as a result of considerable junctional diversity have a potentially large antigenic repertoire. Human epithelial tissues also possess γδ T cells however they are present in much lower numbers than in the mouse. The presence of γδ populations with invariant TCRs would be consistent with recognition of invariant antigens. Indeed both mouse and human γδ T cells reactive to heat-shock proteins (HSPs) have been demonstrated (O'Brien et al. 1989, Holoshitz et al. 1989, Born et al. 1990). The HSPs are among the most phylogenetically conserved proteins - antigens from a variety of bacteria (notably mycobacteria) and parasites have been identified as members of stress protein families. The mycobacterial stress proteins are among the immunodominant targets of both cell mediated and humoral response (Young et al. 1988).

Athough they serve vital roles in the cell in the absence of stress, induction of heat-shock/stress proteins occurs in response to a number of insults (Kauffman, 1990). Recognition by γδ T cells of these conserved antigens provides a mechanism for removal of stressed or damaged autologous cells (Young, 1990).

Ruminants

γδ T cells exist in much larger numbers in ruminants than in man and mouse. Greater than 50% of circulating PBMCs can be of the γδ phenotype in young ruminants although this percentage decreases with age (Hein and Mackay, 1991). The marked difference in numbers of γδ T cells between certain species has lead to the description of γδ 'high' (e.g. ruminants, chickens) and γδ 'low' species (e.g. man and mouse) and subsequent suggestion of possible functional redundancy within the γδ low species (Hein and Dudler, 1993). In addition, double negative ruminant γδ T cells express a surface molecule called T19 which is a 215kd cell surface molecule (Mackay et al. 1989, 1991). In cattle, the equivalent family is WC1 and has been characterised at the molecular level (Wijngaard et al. 1994). These molecules consist of extracellular repeating domains which exhibit strong homology to the macrophage scavenger receptor cysteine rich domain. Recent data has provided evidence for at

least 50 T19 genes in the sheep (Walker et al. 1994).The function of the T19/WC1 families are unknown at present although one possibility is that the molecule represents a functional homologue of the CD4/CD8 accessory molecules (Walker et al. 1994). Hanby-Flarida and colleagues (1996) utilise a mAb specific for WC1 to show that this receptor is involved in activation of γδ T cells by an undefined pathway.

Other significant features of ruminant γδ T cells are the lack of expression of CD2 and CD6 and the greater degree of receptor complexity which exists. In sheep, analysis of γ and δ chain constant regions has shown that although the δ chain is well conserved between species, the hinge region of the ovine γ chains differs from other TCR chains (Hein et al. 1990). In particular, they possess a longer extracellular C region domain than any other TCR chain and there are five cysteine residues in the extracellular C region. The consequences of this are purely speculative at present. However, it is tempting to speculate that there may be a link between these findings and the increased numbers of γδ T cells in this species.

γδ T cell function

The γδ T cell population remains a much studied yet poorly understood cell subset from a functional point of view. It is apparent that it recognises antigen in a manner which differs from the αβ T cell population as although some MHC class I or class II restricted clones have been identified (Kozbor et al. 1989; Flament et al. 1994), this would appear to be uncommon. Similarly, γδ T cell lines recognising non classical class I molecules (Bonneville et al. 1989, Vidovic et al. 1989) and CD1 (Brenner et al. 1986; Faure et al. 1990) have been described. γδ recognition of CD1 will be discussed in more detail in Section B.

Schild and colleagues (1994) use two γδ T cell hybridomas specific for MHC molecules to show that antigen recognition by these cells differs fundamentally from antigen recognition by αβ T cells. Neither of the two clones used required a functional antigen presenting pathway in the stimulator cells and alterations in

residues predicted to be important for αβ T cell recognition did not affect the specificity of binding.

Rock and colleagues (1994) analysed the lengths of the CDR3 regions of αβ and γδ TCRs and showed that γδ T cells are more similar to immunoglobulins than to αβ T cells. This implies a role for the γδ T cell as a mediator of cellular immunity without the need for APCs.

Interestingly, and in support of these data is the demonstration by Sciammas and colleagues (1994) that a γδ T cell clone (Tg14.4) recognises a transmembrane glycoprotein in a non MHC restricted manner. A soluble recombinant form of the antigen recognised by the clone was used to show that Tg14.4 was capable of recognising unprocessed glycoprotein in the absence of APCs. Consistent with this, Weintraub and colleagues (1994) showed that the γδ T cell G8, is stimulated by the non-classical class I molecule T22 in the absence of peptides. Further evidence for the redundancy of the TAP transporter system in γδ T cell responses was demonstrated by Moriwaki and colleagues (1993) who showed that the KN6 γδ T cell clone can be activated by both the TAP2-deficient cell line (RMA-S) and splenocytes from TAP1 mutant mice.

Further information on the role of γδ T cells has been obtained by utilising γδ TCR deficient mice ($TCR\delta^{-/-}$). Several studies utilizing these mice have indicated a role for γδ T cells in the immune response to intracellular bacteria and parasites (Tsuji et al. 1994, Mombaerts et al. 1993). Interestingly, a recent report has demonstrated impaired IgA responses in these mice suggesting a specialized role for γδ T cells in mucosal immunity (Fujihashi et al. 1996)

Recently it has been shown that γδ T cells can be stimulated by thymidine containing nucleotide conjugates (Constant et al. 1994) and also by phosphate modified sugars (Schoel et al. 1994). Tanaka and colleagues (1995) demonstrated stimulation by isopentenyl pyrophosphate. Morita and colleagues (1995) demonstrated that prenyl

pyrophosphate antigens are presented to γδ T cells through an extracellular pathway not dependent on classical or non-classical MHC molecules or on antigen uptake and processing. Thus, it is now becoming apparent that γδ T cells may represent a subset of T cells which are functionally distinct from αβ T cells and exist to increase the hosts ability to deal with foreign agents by increasing the range of substrates that can be recognised and subsequently induce an immune response.

1A.4.3 Other T cell subsets

Two further T cell subsets will be discussed due to their potential significance with regard to CD1 function. These are double negative (DN) T cells and $NK1^+$ T cells.

Double negative T cells

Double negative T cells represent a minor population of T cells which express neither the CD4 nor CD8 co-receptor. The majority of DN T cells are $\gamma\delta^+$ with a small percentage of $\alpha\beta^+$. The origin, differentiation and selection of TCR repertoires of DN T cells is controversial, however it is generally agreed that these cells are not progenitors of double positive immature thymocytes (Crispe et al. 1987) and that upon activation can acquire cytolytic or cytokine producing ability (Ballis and Rasmussen, 1990; Zlotnik et al. 1992). This subset of T cells has received particular attention as DN cells have been postulated as being potentially autoreactive due to an association with autoimmune disease e.g. SLE (Shivakumar et al. 1989). DN T cell lines have also been shown to recognise CD1a, CD1b and CD1c (Porcelli et al. 1989, 1992, Faure et al. 1990). The nature of this CD1 restriction is described in more detail in section B.1.3.

$NK1.1^+$ T cells

$NK1.1^+$ T cells are a subset of T cells so named because of expression of NK1.1 which is a member of the NKR-P1 family normally associated with NK cells. The $NK1.1^+$ T cells have a distinct phenotype characterized by an activation/ memory phenotype ($CD44^{high}$, $CD45RB^{low}$, $CD62L^{low}$), expression of the IL-2Rβ chain

(CD122) and are usually either $CD4^+$ or $CD4^-CD8^-$ (MacDonald, 1995). Notably, they also express a variety of NK cell markers including CD16, Ly-49A and Ly-49C. Other significant features of this subset include the expression of a lower TCR density and the use of restricted TCR repertoires. In the mouse, this is manifest by the use of Vα14Jα281 paired with Vβ8, Vβ7 or Vβ2 (Lantz and Bendelac, 1994; Ohteki and MacDonald, 1996). A human counterpart of this murine subset exists consisting of the invariant Vα24JαQ paired with Vβ11 (Dellabona et al. 1994) although there has been no demonstration to date of human $NK1.1^+$ T cells.

These cells have been shown to recognise an unconventional ligand as they develop normally in TAP deficient mice (Adachi et al. 1995). However, their requirement for β2-microglobulin implicates non-classical class I or CD1 molecules as potential candidates for their ligand (see Section 1B.3.5).

A key functional feature of this subset is the capacity to produce large amounts of the cytokine interleukin-4 (Yoshimoto and Paul, 1994) which suggests an involvement in Th1/Th2 lineage commitment. Further studies using $\beta 2m^{-/-}$ mice have shown that these mice produce minimal or no IL-4 whereas $\alpha\beta^{-/-}$, $CD8^{-/-}$, $CD4^{-/-}$ mice produced IL-4 (Yoshimoto et al, 1995b). In addition, by using goat anti-mouse IgD to stimulate IgE production, Yoshimoto and colleagues (1995a) showed that $CD4^+NK1.1^+$ T cells are essential for switching to IgE. Due to this potential for regulating the immune response and the dissimilarity of this cell population to either CD4 helper or CD8 cytotoxic T cells, it has been suggested that these cells may represent a third class of T cell - 'T regulator cells' (Vicari and Zlotnik, 1996). The role of CD1 as a ligand for this subset of T cells and resulting functional significance is discussed in Section 1B.3.5.

SECTION B

1B.1 Non-Classical Class I And MHC Related Molecules

In addition to the class I molecules described in Section A, there exists a number of genes which also map to the MHC but do not share the typical polymorphism and wide tissue distribution that is associated with classical class I molecules. These genes are termed non-classical class I (NCCI) genes and are of interest in that the CD1 genes, although not mapping to the MHC also share these features of limited polymorphism and restricted tissue distribution. In addition, many NCCI molecules possess truncated carboxy-termini which may result either in secretory isoforms or an unusual method of insertion into the plasma membrane (Stroynowski, 1995).

The demonstration of lack of polymorphism by NCCI molecules has inevitably led to much speculation about their role. Indeed, the lack of polymorphism which is considered characteristic of this group of molecules has been cited as evidence that these molecules are non-functional (Klein et al. 1991). However, the more popular, and currently more accepted view, is that the lack of polymorphism is a key to the distinct role of these molecules rather than an indicator of functional redundancy. NCCI molecules have been best characterized in man and mice although they have been described in many species (Shawar et al. 1994). A canine NCCI molecule (DLA-79) has recently been described by Burnett and Gerahty (1995).

1B.1.1 Human non-classical class I molecules

In man, the non-classical class I loci are HLA-E, HLA-F, HLA-G and the recently described MICA (HLA-X) and MICB (Bahram et al. 1994; Bahram and Spies. 1996). The best studied of these molecules is HLA-G.

HLA-G

HLA-G is present on villous trophoblasts (Kovats, 1990), in foetal eye tissue (Shukla et al. 1990) and foetal liver. HLA-G mRNA has also been demonstrated in PBLs and skin (Kirszenbaum et al. 1994; Ulbrecht et al. 1994) and more recently in normal and transformed mononuclear phagocytes (Yang et al. 1996).

Expression of HLA-G in the placental trophoblast has intriguing implications for immune regulation between mother and foetus. The trophoblast cells are interspersed

between maternal and embryonic cells throughout pregnancy and *in situ* hybridization has shown HLA-G transcripts to be prominent in first trimester placentas (Yelavarthi et al. 1991). Assuming some role for HLA-G as an antigen presenting molecule, it is generally considered that the presence of such a non-polymorphic member of the MHC family in such a 'critical' position allows a means whereby MHC restricted rejection of the foetal tissue would not occur. The fact that HLA-G has been shown to bind to CD8 is consistent with the ability of trophoblast cells to modulate T cell function (Sanders et al. 1991). The population of NK cells which are found in this area may also be functionally regulated by HLA-G. In support of this theory, HLA-G has been shown to protect transfected NK targets from lysis in a similar fashion to classical MHC class I molecules (Chumbley et al. 1994).

In addition to the membrane bound form, soluble HLA-G is also present and is generated by alternative splicing which results in termination of the open reading frame 21 amino acids after the α3 domain (Ishitani et al. 1992; Fuji et al. 1994). Other alternatively spliced forms of HLA-G have also been demonstrated which lack either the α1 domain or the α1 and α2 domain (Ishitani et al. 1992). Although traditionally considered as relatively non-polymorphic, a recent paper by Van der Ven and Ober (1994), demonstrates the presence of extensive nucleotide variability in the α2 domain of HLA-G in African Americans. However, as described above, alternatively spliced forms of HLA-G exist which do not contain these polymorphic regions so this paper is still consistent with a role for HLA-G as an immunologically 'neutral' molecule at the maternal/foetal interface.

Relevant to this is the work of Lee and colleagues (1995) which defines a unique peptide binding motif for HLA-G with a diversity significantly lower than classical class I molecules. In addition they demonstrate that the soluble form of HLA-G described above does not associate with TAP in the same manner as membrane bound HLA-G.

It is of interest that a primate, the cotton-top tamarin (*Sanguinus Oedipus*) has expressed class I genes which are most closely related to HLA-G and is highly susceptible to viral pathogens (Watkins et al. 1990).

The other human NCCI molecules i.e. HLA-E, HLA-F and MICA/ MICB have been less well studied. HLA-E has been shown to be highly transcribed in most tissues (Koller et al. 1988) however cell surface expression is variable (Shimizu et al. 1988, Srivastava et al. 1987). This is thought to be a result of defective binding of available endogenous peptides (Ulbrecht et al. 1992). A role for HLA-E is purely speculative at present, however it has been suggested that since HLA-E accumulates in the endoplasmic reticulum it may influence peptide binding by classical class I molecules or modulate peptide loading (Ulbrecht et al. 1992). HLA-F is the least well studied of the human NCCI molecules, however transcripts have been demonstrated in a number of tissues (Gerahty et al. 1990) including resting T cells and skin cells. More recently, transcripts have also been demonstrated in foetal liver (Houlihan et al. 1992). This implies that HLA-F may be involved in haematopoiesis or foetal development (Shawar et al. 1994).

The novel MHC class I genes MICA and MICB genes have recently been identified by Bahram and colleagues (1994) and Bahram and Spies (1996) and although sharing some features of NCCI genes including erratic tissue expression, conservation of certain amino-acids and divergence from known MHC class I genes, these genes exhibit polymorphism and evolutionary studies indicate that the MIC genes are a second lineage of evolutionarily conserved MHC class I genes (Bahram et al. 1994). The function of the molecules encoded by these genes is currently unknown.

1B.1.2 Murine non-classical class I molecules

The majority of the murine class I genes are located in the Q, T and M regions. Products of the H-2T and H-2Q loci have been shown, under some circumstances, to restrict T cell responses whereas one product of the H-2M locus, H-2M3 has been shown to present peptides with an N-formyl methionine.

H-2T

The best studied members of the T locus are the thymus leukaemia antigen (TL) and Qa-1 antigens.

The TL antigens were originally identified on leukaemic T cells (Old et al. 1963) and are also found on cortical thymocytes and Langerhans cells (Stroynowski, 1990). The TL antigen is predicted to fold into an antigen presenting structure and recently peptide has been shown to bind to TL (Sharma et al. 1996). Using transgenic mice which expressed the H-2T18^{d} on virtually all nucleated cells, Sharma and colleagues showed that TL antigens can bind peptides with blocked NH_2 termini implying that peptides have been selected from a novel degradative pathway. Another notable feature observed using this model was the presence of increased numbers of $CD4^{+}$ T cells displaying an altered T cell repertoire implying that TL has an influence on T cell differentiation. These studies suggest that TL may have adopted a functional niche in a similar fashion to H-2M3 whereby a distinct set of peptides can be bound and presented.

Qa-1 antigens are encoded by the T-23 gene. There have been several reports of H-2T region encoded antigens restricting T cell responses (Vidovic et al. 1989, Bonneville et al. 1989, Ito et al. 1990, Weitraub et al. 1994). In each of these cases, the responding T cells were γδ T cells and in the case of the G8 γδ line (Weintraub et al. 1994) the restriction by T22 was shown to be peptide independent.

Fragments of *M.bovis* heat shock protein 65 (HSP65) can stabilize Qa-1 surface expression implying that either heat shock induces chaperones which promote the expression of Qa-1 or that peptides derived from degraded HSPs could bind within the binding site and thus facilitate surface expression (Imani and Soloski, 1991).

Analysis of the putative peptide binding groove of Qa-1 shows replacement of threonine at positions 143 and 147 with serine which has the effect of opening up the peptide binding groove at the carboxyl end (Connoly et al. 1993). The same threonine residues are found in some other NCCI molecules (human and primate). Although the peptide binding groove of Qa-1 is similar to most other class I molecules, there is a requirement by some Qa-1 for the product of the Qa-1

determinant modifier (Qdm) gene (Aldrich et al. 1988). The Qdm peptide has recently been identified (Aldrich et al. 1994) and it is possible that despite the apparent capability to bind a range of peptides, preferential chaperoning of certain peptides exists. A further notable feature of Qa-1 molecules is their ability to form heterodimers with other class I molecules (Wolf and Cook, 1995). It is postulated that the formation of such heterodimers may allow cell surface expression of class I molecules which are unstable (either due to weak β2m association or lack of peptide) such that they are capable of interaction with exogenous peptide or effector molecules on other cells (Wolf and Cook, 1995; Soloski et al. 1995).

H-2Q

H-2Q region genes encode both cell surface and secreted molecules (Robinson, 1987). The best studied Q region molecules are the Qa-2 antigens which are encoded by the Q7 and Q9 genes. Qa-2 antigens exist in both soluble and membrane bound forms as a result of premature stop codons in exon 5 (corresponding to the TM domain of classical class I molecules). This results in both the production of soluble Qa-2 and Qa-2 antigens that are attached to cell surfaces via glycosylphosphatidylinositol (GPI) lipid anchor. GPI-anchored Qa-2 can undergo post-translational cleavage also resulting in the generation of a soluble form of the molecule (Tabaczewski et al. 1994). The potential role of such GPI anchored Qa-2 molecules is speculative however it is known that GPI linked class I proteins can move across domain boundaries defined on the surface of cells (Edidin and Stroynowski, 1991) and it is also proposed that GPI-anchoring targets Qa-2 molecules to specialized endocytic vesicles (caviolae) which function in internalization of membrane attached proteins. Qa-2 has also been shown to be defective in CD8 binding, a feature which has been attributed to differences in the α3 region (Teitell et al. 1993).

Analysis of peptides which are capable of binding to Qa-2 has revealed conflicting results. Rotzschke and colleagues (1993) described the Qa-2 molecule as one with a high stringency due to binding of a limited range of peptides. In contrast, however,

Joyce and colleagues (1994) utilizing different techniques, demonstrated that indeed Qa-2 molecules can bind 'a large array' of different peptides. This challenges the popular view that the limited polymorphism of non-classical class I molecules has been maintained such that these molecules can bind and present relatively invariant antigens.

H-2M3

H-2M3 (initially known as Hmt) is a product of the H-2M locus. This NCCI molecule has been shown to present N-formylated peptides to CTLs - a feature which was originally demonstrated by examination of the maternally transmitted antigen (Mta) which consists of H-2M3, β2m and Mtf peptides, the latter being the product of mitochondrial Mtf genes (ND1) (Shawer et al. 1990). Subsequently, these findings were extended to show that N-formylated peptides of pathogens can also be presented by H-2M3. Since bacterial protein synthesis is initiated with an N-formyl methionine (as is mitochondrial protein synthesis), this provides a means whereby H-2M3 can present bacterial peptides to CTLs as has been shown for *Listeria monocytogenes* (Kurlander et al. 1992, Pamer et al. 1992) thus exploiting an inherent difference between microbial and mammalian antigens. More recently it has been shown that in addition to N-formylated peptides, H-2M3 can also present a minor histocompatibility antigen encoded by the *COI* mitochondrial gene (Morse et al. 1996).

The rat also expresses an M3 homologue - RT1.M3 (Wang et al. 1995). X-ray crystallography of this molecule bound to a nonamer of rat ND1 reveals that although the overall structure of M3 is similar to classical class I molecules, a novel method of peptide binding exists whereby the N-formylated peptides are positioned such that the peptide is moved one residue along the groove and the N-terminal pocket is closed (Wang et al. 1995).

H-2M3 thus demonstrates a function which provides an explanation for limited polymorphism indicating that the H-2M locus has specifically evolved and been retained to recognise invariant 'non-self' antigens.

1B.1.3 The neonatal receptor for Ig - FcRn

The neonatal receptor for IgG, FcRn, is responsible for unidirectional transport of immunoglobulin from the intestinal lumen, across enterocytes and into the blood stream. This occurs due to binding of IgG at the acidic pH of the gut (pH 6.0) followed by receptor mediated endocytosis and subsequent release of the IgG at the higher pH of the blood (pH 7.4). FcRn was initially isolated and purified from neonatal rat intestinal epithelium (Jakoi et al. 1985). This was followed by cloning and sequencing of the molecule by Simister and Mostov (1989).

Sequence and structural analysis revealed that FcRn was homologous to MHC class I molecules albeit that it is more divergent from classical class I molecules than the non-classical class I molecules whilst being more closely related than CD1 molecules. Subsequent studies using mouse intestinal cDNA libraries revealed the existence of a similar molecule in this species (Ahouse et al. 1993). FcRn has also been shown to be functionally expressed on the surface of adult rat hepatocytes (Blumberg et al. 1995) where it is thought to function in the transport of IgG either from the blood to bile or by providing a mechanism for the transcytosis of IgG from the apical to the basolateral surface of the hepatocyte. This expression of FcRn in the liver and other extraintestinal tissues (lung, spleen and endothelial cells) together with pharmacokinetic studies of IgG_1/Fc fragments in $\beta 2m^{-/-}$ mice (Ghetie et al. 1996) implies that FcRn may also be involved in the maintenance of serum IgG levels.

Story and colleagues (1994) isolated the human homologue of this receptor (hFcRn) utilising an $\alpha 2$ domain probe based on the structure of the rat FcRn. The implication therefore is that hFcRn is involved in IgG transport across the placental barrier.

Recent analysis of the crystal structure of the rat FcRn (Burmeister et al. 1994) has intriguing implications for the study of CD1. Previously, the relationship between these two molecules was solely as MHC related molecules. However, a key amino acid residue, (Pro 162), responsible for closure of the peptide binding groove in FcRn such that it cannot bind peptide is shared with all CD1 molecules. In addition, sequence identity between CD1 and FcRn is highest in the $\alpha 2$ domain implying possible structural similarity within this region.

The genomic structure of a mouse FcRn gene has recently been described (Kandil et al. 1995) which reveals an exon-intron organisation similar to that of MHC class I genes. Two notable differences however are that the 5'UT region of the FcRn gene is split into two exons and that the majority of the cytoplasmic region and the entire 3'UT are encoded by a single exon. This is also a feature of the CD1 genes (also MICA and H2-T18) although Kandil and colleagues (1995) suggest that as these genes do not form a phylogenetic cluster, this similarity was probably acquired independently.

1B.1.4 Mechanisms of antigen presentation by NCCI molecules

As discussed in the individual sections, there is variability in the dependence of the NCCI molecules on a functional TAP transporter system to generate an antigenic epitope. Several groups have demonstrated that the murine TL antigen is expressed independently of TAP function (Moriwaki et al. 1993; Holcombe et al. 1995; Rodgers et al. 1995) and, in addition, that TL is thermostable at physiological temperatures in the absence of peptide implying that binding of specific peptides is not required for transport and stable expression at the cell membrane (Holcombe et al. 1995). As discussed earlier, there is also recent data to show that TL binds peptides with blocked NH_2 termini (Sharma et al. 1996). However, despite these marked variations when compared to classical MHC class I function, TL antigens can nevertheless mediate a transplantation rejection response and TL specific CTLs can be elicited (Morita et al. 1994; Sharma et al. 1996).

In contrast however, the Qa-2 antigen has been shown to be dependent on the TAP system (Tabaczewski et al. 1994; Stroynowski,1995) and this fact taken in conjunction with its wider tissue distribution and potential to bind a diverse array of peptides (Joyce et al. 1994) indicates a closer similarity to classical class I molecules. Different again, is the M3 molecule which, as described above binds a limited array of N-formylated peptides. Thus, although generally considered as a group, the NCCI molecules exhibit characteristics ranging from 'class I-like' in terms of processing and peptide specificity e.g. Qa-1, to H-2M3 which although resembling class I in terms of processing and presentation is highly restricted in terms of peptides which

will bind to the TL antigens which appear to possess an alternative antigen processing pathway possibly selecting peptides from a novel degradative pathway (Holcombe et al. 1995, Sharma et al. 1996).

Hence when considering possible relevant similarities between the NCCI molecules and the CD1 family, it may be more prudent to consider the NCCI molecules not as a relatively homogeneous family but as a family consisting of many functionally divergent members, some of which may have relevant similarities or may provide clues to the function of the CD1 family.

1B.2 The CD1 Family

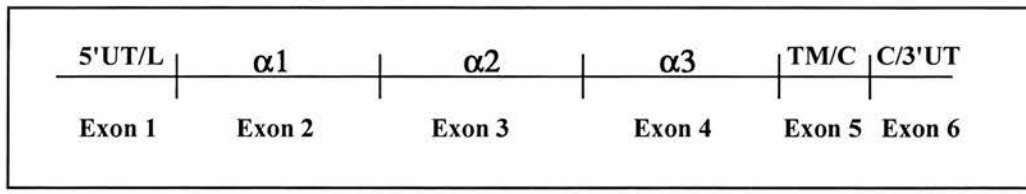
The CD1 family is a family of molecules with structural homology to MHC class I and low but significant sequence homology to both MHC class I and MHC class II. Members of the CD1 family have been demonstrated in a number of species and it is clear that marked interspecies variation exists in terms of both the numbers of CD1 genes present and the isotypes of CD1 which these genes encode.

CD1 genes and molecules will now be discussed in more detail both in general terms and with interspecies comparisons.

1B.2.1 CD1 genes

CD1 genes have been identified in man, mice, rats, rabbits and sheep. All the CD1 genes possess separate exons which encode the six functional domains of the molecule. The exon arrangement is shown below and consists of 5' untranslated and leader; external α1, α2 and α3 domains; transmembrane and proximal cytoplasmic tail and the remainder of the cytoplasmic tail and 3' untranslated region (Martin et al. 1987; Calabi et al. 1989b, 1989c). The highest sequence homology exists within the α3 domain - in this domain, there is at least 75% identity between all CD1 sequences. The exon structure of the CD1 genes strongly resembles that of MHC class I and is one of the the most significant similarities between the two families. However, the genes encoding the cytoplasmic/ 3' untranslated domains of CD1 and

MHC class I differ in that three exons encode this region in MHC class I compared to only one exon in CD1 (Calabi et al. 1991).



A key feature of the CD1 genes is a lack of significant polymorphism. This is in marked contrast to the extensive polymorphism which is associated with MHC class I genes. In humans, sequencing of CD1 clones from different individuals has revealed only single amino acid changes (Martin et al. 1987; Aruffo and Seed, 1989) and a similar situation has been reported in the mouse (Bradbury et al. 1988). In the rat, only three allelic variations were found within the 12 rat strains tested (Ichimaya et al. 1994).

Relationship of CD1 to the MHC

The pattern of exon arrangement illustrated above is similar to MHC class I genes with the exception of additional exons encoding the longer cytoplasmic tails of MHC class I molecules. Although the α3 regions of both CD1and MHC class I molecules are highly conserved, there is no significant homology at the nucleotide level between the two domains (Martin et al. 1986). Comparisons between the CD1 α3 domain and MHC class I and class II molecules have shown that the CD1 α3 domain is related to both MHC class I α chains and MHC class II β chains (Calabi and Milstein, 1986; Martin et al. 1986). By analysing consensus sequences for CD1 a-d, Balk and colleagues (1989) showed that the CD1 α3 region is more homologous to the MHC class II β2 domain than the MHC class I α3 domain. Therefore it is apparent that the CD1 genes should be considered as being equally related to both class I and class II MHC genes.

Interspecies comparison of CD1 genes

Interspecies comparisons of the CD1 genes are interesting as this provides clues to the evolution of the CD1 family. An interesting feature of the CD1 genes is that homology is greater between the same isotype in different species than between different isotypes in the same species (Calabi et al. 1991). This implies that the different CD1 isotypes evolved structural features relative to conserved functions prior to the radiation of mammalian species (Porcelli, 1995).

1B.2.1.1 Humans

In humans, five CD1 genes have been described (CD1A-E) which encode four protein products (CD1a-d). No product of the CD1E gene has yet been described. CD1 genes in other species are generally classified according to their homology to the human genes.

Sequence comparisons of the CD1 genes in the α1 and α2 domains allow division into two main groups with CD1 A-C forming one group and CD1D forming the second. The CD1E gene is in an intermediate position (Calabi et al. 1989c). Comparison of sequences at the 3' end however reveals an alternative grouping dividing the CD1 molecules into 3 distinct types (Porcelli, 1995). CD1e has a cytoplasmic tail significantly longer than the other CD1 isotypes (Calabi et al. 1991). CD1b,-c and -d possess a 7 amino acid cytoplasmic tail containing a YXXZ motif (where Z is a hydrophobic residue) which is associated with sequences which target to an endosomal compartment in other proteins e.g. CD3 chains (Letourneur and Klausner, 1992). It has recently been shown that CD1b does indeed localize to an endocytic compartment and that this localization is dependent upon the tyrosine-based motif in the cytoplasmic tail (Sugita et al. 1996).

CD1a in contrast, has a shorter tail (3 amino acids following the membrane anchor sequence) and contains two cytoplasmic cysteine residues which may be involved in disulphide-linked interactions with other proteins (Porcelli, 1995). In addition, the arrangement of the cysteine residues generates a similar motif to that of CD8α which

interacts with $p56^{lck}$ (Turner et al.1990). It is interesting to speculate that this may indicate a functional difference between CD1a and the remaining CD1 isotypes.

1B.2.1.2 Rodents

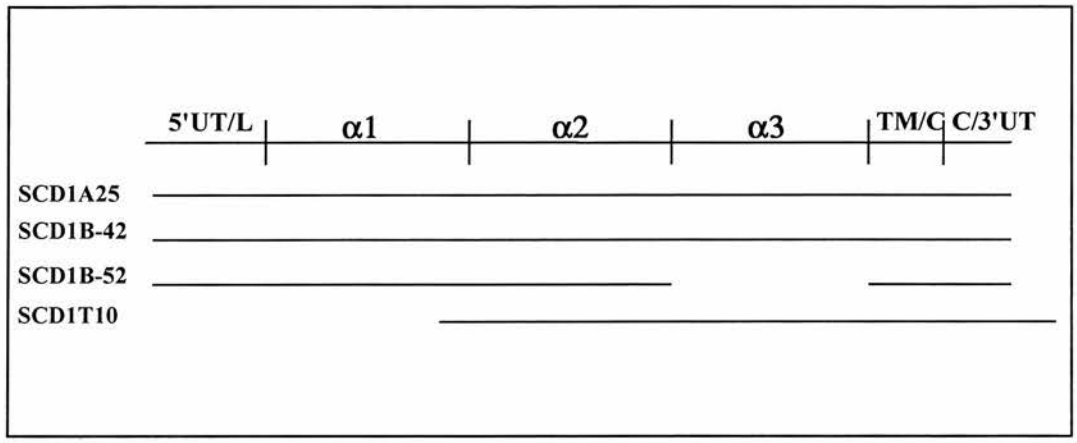
In the mouse, two CD1 genes exist - CD1D.1 (mCD1.1) and CD1D.2 (mCD1.2) which show greater than 95% identity in their coding regions. The presence of 2 such closely related genes may imply evolutionary pressure to maintain this locus (Bix and Locksley, 1995). These genes have closest homology to human CD1D. Recent studies in the rat have revealed a similar situation i.e. the existence of members of the CD1D group only (Ichimiya et al. 1994).

1B.2.1.3 Rabbits

The CD1 family in rabbits is interesting as this species has members of both groups of CD1 genes i.e. CD1B-like and CD1D-like. Probing genomic libraries from cotton-tail and domestic rabbit with a human CD1 probe yielded a clone from the domestic rabbit which was CD1B-like and a clone from the cotton-tail rabbit which was CD1D-like (Calabi et al. 1989b). Southern blot analysis of domestic rabbit DNA has identified the presence of up to eight genes in this species, only one of which is a member of the CD1D group based on hybridization with a probe from the cotton-tail rabbit (Calabi et al. 1989b).

1B.2.1.4 Ruminants

In ruminants, the majority of studies on the CD1 family have been at the protein level (Section 1B.2.2.3). Recent molecular studies in the sheep have however shown the presence of up to seven CD1 genes in this species (Ferguson et al. 1996). Four genes have been cloned and sequenced and all four clones possess closest homology to human CD1B (Ferguson et al. 1996). The nature of these clones is illustrated below.



Identification and cloning of ovine CD1

The sheep CD1 sequence SCD1A25 was identified by screening a sheep foetal thymocyte library with a human CD1C probe (α3 domain and flanking intron sequence). A sheep CD1 probe was subsequently generated by PCR amplification of the α3, transmembrane and cytoplasmic domains. This probe was used to screen both the foetal thymocyte library and a lamb thymocyte library and resulted in the identification of the clones SCD1B-42, SCD1B-52 and SCD1T10. Further molecular techniques were used to establish full length sequence for SCD1A25 and SCD1B-42. SCD1T10 is a truncated CD1 sequence which begins 26 bp upstream of the start of the α2 domain. The 3'UT region of SCD1T10 is notably longer than the other sheep genes i.e. 642 bp and lacks a consensus polyadenylation signal and a polyA tail. The SCD1B-52 clone contains a precise deletion of the α3 domain. The nucleotide identity between equivalent exons in SCD1B-42 and SCD1B-52 is >93%. Of particular note also is the fact that the two sequences are 96% identical in the 3'UT region. These two observations combine to suggest that SCD1B-42 and SCD1B-52 are alleles.

The information presented in the above section implies that the CD1 genes originated prior to the radiation of mammalian species since members of the same families of CD1 genes are found in both man and rabbit. Further, Calabi and colleagues (1989b)

conclude that the slow rate of evolution of these genes (as indicated by the interspecies gene similarities) indicates that the absence of members of the CD1a-c family in the mouse is due to a deletion occurring after the mouse and rabbit/human separation.

1B.2.2 CD1 molecules and tissue distribution of CD1

Initial studies on the CD1 family utilizing CD1 specific mAbs demonstrated the unusual tissue distribution of the CD1 molecules on cortical thymocytes (McMichael et al. 1979, Rienhertz et al. 1980). The anti-CD1 mAbs were subsequently used to study biochemical properties of the CD1 molecules. Immunoprecipitation demonstrated that the CD1 α chain was associated with β2m implying structural relationship to MHC class I molecules (Cotner et al. 1981). mAb studies on the CD1 molecules will be discussed in more detail in the following species specific sections.

CD1 molecules consist of a 43-49kd α chain non-covalently associated with β2-microglobulin (β2m). As with MHC class I molecules, the extracellular α1, α2 and α3 domains are each 90 amino acids long and contain variable numbers of N-linked glycosylation sites (depending on isotype). As a result, the α chain varies in size according to isotype (see table below).

ISOTYPES (MAN)	RAT	MOUSE	RABBIT	SHEEP/ CATTLE
CD1a (49kd)				
CD1b (46kd)			+	+
CD1c (43kd)				?
CD1d (49kd)	+ (55kd)	+(50kd)	+	

Althought the exon arrangement and association with β2m suggests that CD1 molecules are similar to MHC class I molecules, analysis of the nucleotide and amino-acid sequences demonstrate that the two families are only distantly related (Martin et al. 1986, Calabi et al. 1989c). The domain with greatest homology between the two proteins is the α3 domain however a considerable degree of

divergence exists even in this domain. It appears particularly significant that identity between all CD1 sequences in the α3 domains is much higher than between MHC class I sequences (Calabi et al. 1991). In CD1, 55.6% of the 90 amino-acid residues in the α3 domain are completely conserved which is in contrast to only 26.75% of residues in MHC class I (Hughes, 1991). This suggests that there has been strong evolutionary pressure to maintain the conserved nature of this domain implying an important conserved function.

Structural analysis of the α1 and α2 domains suggests that CD1 does not possess a structural homologue of either the MHC class I or class II peptide binding groove (Calabi et al. 1989c). In addition, CD1 contains more hydrophobic regions in the α1 and α2 domains than the MHC molecules. This is particularly pronounced in CD1b and CD1c) and may be of significance when considering the potential of lipid antigens to bind to CD1 and is discussed in more detail in Section 1B.3.2.

1B.2.2.1 Expression of CD1 molecules in man

CD1a-c

CD1 was the first differentiation antigen to be recognised by a mAb when in 1979 McMichael and colleagues described the recognition of the majority of human thymocytes but not peripheral cells by the monoclonal antibody NA1/34. This antibody was subsequently shown to recognise the CD1a molecule (Initially named HTA1 or T6). The mAbs 4A76 and M241 were subsequently shown to recognise CD1b and CD1c respectively.

Initially, mAbs recognising these thymocyte antigens were considered to be homologues of murine TL antigens (see Section 1B.1.2). Subsequently however they were shown to have a lower molecular weight (Van de Rijn et al. 1983) and not to map to the MHC (Calabi and Milstein, 1986).

CD1a is expressed on cortical thymocytes and dendritic cells. In addition, this molecule is also present on epidermal Langerhans' cells whereas CD1b is only expressed on cortical thymocytes and dendritic cells.

CD1c in addition to being expressed on cortical thymocytes, dendritic cells and 10% of Langerhans' cells is also expressed on a subset of B lymphocytes (Small et al. 1987).

CD1d

The gene encoding the fourth CD1 molecule, CD1d, was isolated from a human thymocyte library before the CD1d molecule itself was detected by mAbs (Balk et al. 1989). Subsequently, Bilsland and Milstein (1991) raised a specific antisera to the CD1D gene product utilizing a CD1d fusion protein to immunize mice. This monoclonal antibody (NOR3.2) defined a 49kd molecule expressed on a cortical thymocyte cell line. Blumberg and colleagues (1991) demonstrated CD1d expression on human B cells and intestinal epithelial cells utilizing an anti-murine CD1 mAb, 1H1.

A later study by Canchis and colleagues (1993) revealed a wider distribution of CD1d including many non-lymphoid tissues where expression was demonstrated on epithelial cells, vascular smooth muscle cells and parenchymal cells.

Thus, consistent with the genetic analysis revealing the CD1D gene to be the most divergent member of the CD1 family, CD1d expression is similarly divergent when compared to the other CD1 isotypes.

1B.2.2.2 CD1 molecules in rodents

The murine CD1 molecules, CD1d.1 and CD1d.2 (mCD1.1 and mCD1.2) are expressed mainly on gastro-intestinal epithelium epithelium and hepatocytes. Within the thymus, CD1 transcription is very low illustrated by the fact that it is not detectable by Northern hybridization (Bleicher et al. 1990, Bradbury et al. 1988). However, its presence is detectable by the sensitive S1 nuclease protection assay and by ribonuclease mapping (Bradbury et al. 1990).

Murine CD1 is expressed at relatively high levels within the intestine, however there have been differing reports on the localization of murine CD1d within this tissue. Bleicher and colleagues (1990) described expression on all epithelial cells within the gut (by immunofluorescence) using the mAbs 3C11 and 1H1. In contrast, *in situ*

hybridization experiments to detect mRNA in this region (Lacasse et al. 1992), showed that only Paneth cells at the base of the crypts contain CD1 mRNA. Further controversy over the exact location of mCD1 expression in the gut has recently arisen. Sydora and colleagues (1996) were unable to detect mCD1 expression on intestinal epithelial cells by flow cytometry (utilizing the mAb 3C11) however low levels of expression were detected on intestinal intraepithelial lymphocytes.

In the rat, immunochemical techniques demonstrated CD1 on intestinal epithelial cells and hepatocytes using the mAbs 1H1 and 3C11 (Burke et al. 1994). Using molecular techniques however, rat CD1D shows several differences in expression when compared to murine CD1D (Ichimaya et al. 1994). These include a quantitative difference in the thymus in that CD1 mRNA can be detected by Northern hybridization and that CD1 mRNA expression by intestinal epithelial cells is lower than in other organs and is not detectable by Northern hybridization, only by the more sensitive RT-PCR. In addition, high levels of expression were also detected in non-lymphoid organs - liver, heart, kidney and lung.

1B.2.2.3 CD1 molecules in ruminants

The CD1 family in sheep and cattle has been characterized at the protein level using a number of mAbs. In sheep, the mAbs recognise a 46kd molecule in association with β2m which is most closely related to human CD1b in terms of expression (Bujdoso et al. 1989; Hopkins and Dutia, 1991; Dutia and Hopkins, 1991). One mAb, SBU-T6 (Mackay et al. 1985) recognises a molecule with a wider tissue distribution - in addition to staining thymocytes and dendritic cells, expression has also been shown on B cells and Langerhans' cells. As such, this mAb has been considered as a CD1c or pan-CD1 mAb (Dutia and Hopkins, 1991). In cattle, similar results have been published with the majority of mAbs showing a CD1b like reactivity with the exception of SBU-T6 (MacHugh et al. 1988; Parsons and MacHugh, 1991; Howard et al. 1993b). The mAbs which show CD1b-like reactivity have been clustered as BoCD1w2 and SBU-T6 as BoCD1w1 (Howard and Naessens, 1993). More recently, two additional mAbs (CC43 and CC118) have been described which recognise an α

chain of 43kd in cattle. In addition to staining of thymocytes and dendritic cells, these mAbs also stain B cells and monocytes. However staining of cells in *Bos taurus* but not *Bos indicus* indicates recognition of a polymorphic antigen (Howard et al. 1993a). These two mAbs have been clustered as BoCD1w3 (Howard and Naessens, 1993).

CD1 antigens have also been described in goats (Davis and Ellis, 1991) and pigs (Pescovitz et al. 1984, Denham et al. 1994). As in sheep and cattle, both these species also possess an isotype of CD1 which exists on B cells as identified by SBU-T6 in goats and 76-7-4 in pigs.

1B.2.3 Modulation of CD1 expression

CD1 expression has been shown to be modulated under the influence of cytokines and also in certain neoplastic diseases. In general, CD1 expression appears to be upregulated in inflammatory disease and in vitro under the influence of certain inflammatory cytokines.

1B.2.3.1 CD1 modulation by cytokines

Although CD1 expression is not constitutive on human monocytes, expression of CD1a-c has been induced by stimulation with the cytokines GM-CSF and IL-4 (Porcelli et al.1992) and CD1c expression has been induced by GM-CSF alone (Kasinrerk et al. 1993). Cytokines which induce MHC class I and class II expression e.g. IFNγ, do not however induce CD1 expression (Kasinrerk et al. 1993). This GM-CSF/IL-4 induced CD1 expression can be suppressed by IL-10 (Thomssen et al. 1995). Taken together, this evidence for CD1 modulation both by pro-inflammatory and anti-inflammatory cytokines again points to the CD1 molecule having an important role within the immune system. The fact that some cytokines have differential effects on CD1 and MHC class II expression (e.g. IFNγ, IL-10) shows that CD1 expression is not regulated in parallel with MHC class II molecules

implying an alternative role for CD1 other than classical antigen presentation. This also indicates that transcription factors for CD1 differ from those for MHC class II.

1B.2.3.2 CD1 modulation in disease

The expression of CD1a on Langerhans' cells within the gingival epithelium has been shown to increase under the influence of IL-1 (Walsh et al. 1986) and IL-6 and TNFα (Ishii et al. 1990). Bruynzeel-Koomen and colleagues, (1988), report an association between the expression of CD1 antigen and Fc receptor for IgE on epidermal LC from patients with atopic dermatitis. In addition to modulation of CD1 expression as a result of elaboration of inflammatory cytokines, there are also some interesting reports of CD1 modulation on neoplastic cells. In particular, a study of CD1 expression on leukaemic B and T cells (Salamone et al. 1990) describes CD1 expression on neoplastic cells of both the myeloid and lymphoid lineage in acute and chronic leukaemias. The detection of CD1a on leukaemic cells of T lineage was restricted to cells with an immature phenotype while in contrast, CD1a was preferentially expressed on the surface of cells of mature B phenotype.

Other notable features include the fact that some patients showed a great variability in the percent of CD1 positive cells when samples were analysed on different days (occurring without concomitant variation in other cell surface antigens), and that there is a positive correlation between expression of CD1a and CD25 observed in acute non-lymphoblastic leukaemia suggesting a link between CD1 expression and activation events in these cells. The finding that CD1 expression on B cell neoplasms appears mainly on cells of mature B phenotype, whereas CD1a on T cell neoplasms is restricted to cells with an immature phenotype mirrors the normal physiological pattern of CD1 expression on these cell types.

1B.3. CD1 Function

The function of the CD1 family of molecules has been the subject of much debate over recent years. The existence of these molecules on cell types with central roles in antigen presentation (i.e. dendritic cells, B cells, monocytes) together with their

degree of homology to both class I and class II major histocompatibility antigens is consistent with a role in antigen presentation.

The other major cell type expressing CD1, which is not in the above category of antigen presenting cells, is the cortical thymocyte. High levels of CD1 are present in a region where MHC class I appears to be downregulated, hence a role for CD1 molecules in T cell ontogeny is possible e.g. as a surrogate class I molecule. The role of CD1 in T cell ontogeny is discussed in more detail in Section 1B.3.5.1.

Finally, there is some evidence for the involvement of CD1 molecules in a cellular activation pathway. It has been shown in one T cell model that addition of CD1 mAbs induced an increase in intracellular Ca^{2+} and IL-2 production (Theodorou et al. 1990). In addition to antigen presentation, MHC class II molecules have been shown to have a signalling role in both B cells and T cells (Odum et al. 1991, reviewed in Wade et al. 1993) hence a combined antigen presentation and signalling role could also exist for CD1.

1B.3.1 CD1 as a restriction element for T cells

The major focus of attention by researchers interested in functional aspects of the CD1 family has been the capacity for CD1 to act as a restriction element for certain populations of T cells.

Recognition of CD1 by T cells was first shown by Porcelli and colleagues (1989) who demonstrated specific recognition of CD1a and CD1c by DN αβ (BK6) and γδ (IDP2) T cell lines respectively. Subsequently, recognition of CD1c was shown by the DN γδ T cell clone J2B7 (Faure et al. 1990) and recognition of CD1b by the DN αβ T cell line, DN1 (Porcelli et al. 1992). A human intestinal intraepithelial lymphocyte (IEL) T cell line exhibiting CD1c specific cytotoxicity has also been described (Balk et al. 1991).

More recently, NK1.1+ T cells have also been shown to exhibit CD1 restricted responses (Bendelac et al. 1995a, c) - the significance of this reactivity is discussed in Section 1B.3.5.

As discussed in section B.2.1, although the domain structure of the CD1 molecules resembles that of MHC class I molecules, there are also similarities with MHC class II molecules. In view of this, a recent report of CD1 restricted $CD4^+$ T cells is particularly interesting (Cardell et al. 1995). Non-classical class I and CD1 restriction was demonstrated in peripheral $CD4^+$ T cells of MHC class II deficient mice which showed some similarities to $NK1^+$ T cells (i.e. memory phenotype, high cytokine production) although unlike $NK1^+$ cells they possess a diverse TCR repertoire. These data suggests that CD4 may be able to serve as a co-receptor for CD1 recognition in a similar fashion to CD8 (which has been shown to associate with CD1a by immunoprecipitation [Snow et al. 1985, Ledbetter et al. 1985]). Whilst all of the above experiments demonstrated the potential for CD1 recognition by T cells, there is no indication of the precise nature of the ligand being recognised. Subsequent research has begun to address this question with the result that two major groups of antigens have been shown to be capable of being presented by CD1 molecules.

1B.3.2 Presentation of antigen by CD1

Peptide presentation by CD1

A peptide binding motif for murine CD1 has recently been demonstrated (Castano et al. 1995). Screening random peptide phage display libraries with *Drosophila* cells expressing recombinant soluble CD1-β2m complexes revealed a well defined core motif. Features of both class I and class II associated peptides are demonstrated in this analysis. The 'class-II like' properties are the long (20-22mer) peptides (MHC class I typically binds 8-10mers), hydrophobic amino acids at certain positions and an affinity of interaction similar to MHC class II associated peptides. The major 'class I-like' property is the existence of three highly restricted anchors, which, if mutated, markedly reduce peptide binding (Castano et al. 1995).

Lipid/ Glycolipid

Recognition of a microbial antigen by T cells in a CD1 restricted manner was first shown in 1992 by Porcelli and colleagues. In these studies, the cell line DN1

(generated by repeated stimulation of αβ double negative T cells with $CD1^+$ monocytes and soluble extract of *M. tuberculosis*) proliferated in a CD1b restricted manner in response to mycobacterial antigen. These results have recently been extended by Beckman and colleagues (1994) to demonstrate the specific nature of the antigen that is involved in this response. Purification of the mycobacterial antigen which is responsible for the proliferative response of line DN1 revealed it to be a mycolic acid - a mycobacterial cell wall constituent. Thus it was shown that the immune system has devised a method to allow the presentation of non-protein antigens to T cells.

More recently, it has been demonstrated that mycobacterial lipoarabinomannan (LAM) can be presented by CD1b. Sieling and colleagues (1995) isolated two mycobacteria reactive double negative αβ T cell clones, LDN4 and BDN2, from the skin of a leprosy patient and normal PBMCs respectively. Biochemical methods were used to show that the fraction responsible for proliferation of these T cell clones was LAM and that CD1b was the restriction element involved in the recognition. In addition, it was shown that LAM could induce the production of IFNγ and small amounts of IL-4 by these CD1 restricted T cells.

Summary of CD1 and antigen presentation

Considering the available sequence data within the putative antigen binding region of the CD1 molecule which implies significant structural divergence from classical MHC molecules within this region (Calabi et al. 1989c) and the suggestion that the proline residue in position 162 may result in closure of the peptide binding groove (as in FcRn - see Section 1B.1.3), it would seem more likely that if antigen presentation is a function of the CD1 family, then it must be 'unconventional'. Analysis of nucleotide sequence in the α1 and α2 domains reveals a lack of positive selection of non-synonymous nucleotide substitutions which are characteristic of polymorphic MHC genes (Hughes, 1991). This therefore suggests a lack of selective evolutionary pressure for amino acid variability within this region which would not

be consistent with binding of diverse arrays of peptides as is the case for classical MHC molecules.

Hence the reports of lipid presentation are easier to reconcile with the sequence information than the report of the murine CD1 peptide binding motif identified above. Relevant to this debate, Cheroutre and colleagues (1995) suggest that peptides binding to mCD1 were only defined because the starting material was a peptide display library or alternatively, that CD1 can bind a variety of hydrophobic molecules including both peptides and non-peptides. An additional caveat to the study using peptide display libraries is the fact that certain peptides have been shown to bind to proteins that under normal physiological circumstances do not bind peptide (Devlin et al.1990). Despite these reservations however the possibility remains that members of the CD1d group of CD1 molecules do bind and present peptide under physiological conditions. If this is true then the implication is that the functions of the CD1a-c group and the CD1d group are distinct.

1B.3.3 CD1d/ mCD1 - functional considerations

That the CD1d group of molecules may have a different function from the other CD1 molecules is suggested by its markedly different patterns of expression. Whilst this may simply reflect a molecule that is performing the same function but in different tissues, the presence of this molecule in rodents that lack the other CD1 isotypes suggests that it has been retained to perform some necessary function.

Of particular interest is the expression of CD1d on intestinal epithelial cells which may indicate a role in mucosal immunity. Intestinal intraepithelial lymphocytes represent a somewhat unique population of T cells which predominantly express CD8, have a restricted T cell repertoire and are capable of spontaneous cytotoxicity (Taunk et al. 1992). Panja and colleagues (1993) demonstrate direct evidence of CD1d involvement in T cell proliferation by showing that an anti-CD1d mAb blocks the response in a proliferation assay with intestinal cells as stimulators. Thus it appears that CD1d is involved in T cell - epithelial interactions within this region and it is plausible that there may be a role for CD1 involving sampling/ uptake of

lumenal peptides with a resultant involvement in the induction of the immune response or induction of systemic oral tolerance (Cheroutre et al. 1995).

Balk and colleagues (1994) show that CD1d is expressed on the surface of human intestinal epithelial cells (IECs) in a β2m independent manner and with no N-linked carbohydrate such that the molecule was reduced to 37kd. The conformational alterations which would be likely to result from these changes would make it seem unlikely that antigen could be presented by this molecule, at least in the conventional sense. It is noteworthy however that there is some debate as to requirement of mCD1.1 for β2m as Brutkiewicz and colleagues (1995) demonstrate the requirement of β2m for mCD1.1 expression in their system.

1B.3.4 Antigen processing pathways for CD1

As described in Section A.1, MHC class I and class II molecules have distinct processing pathways which generate the complex of MHC molecule and peptide at the cell surface. NCCI molecules exhibit varying dependence on a functional TAP transporter system to generate an antigenic epitope (Section 1B.1.4)

CD1 molecules have been shown to be expressed on cells of patients with a TAP deficiency (Hanau et al.1994) implying that CD1 does not utilize the MHC class I system for loading antigen. In addition, presentation of mycobacterial antigen to the double negative, CD1b restricted T cell line DN1 (Porcelli et al.1992) was strongly inhibited by chloroquine suggesting that CD1 antigen presentation may have more similarities to MHC class II presentation than to classical MHC class I presentation. Consistent with this, Seiling and colleagues (1995) demonstrated that the presentation of mycobacterial LAM also requires uptake of the antigen into an endosomal compartment. It has recently been demonstrated by Sugita and colleagues (1996) that CD1b localizes to endosomal compartments as a result of a tyrosine motif in the cytoplasmic tail. This motif (YXXZ - where X is any amino-acid and Z is a hydrophobic amino-acid) is also found in CD1c and CD1d thus it is likely that these molecules also localize to endosomal compartments where presumed loading of antigen occurs.

1B.3.5 CD1 and NK1.1$^+$ T cells

As described in Section 1A.4.3, members of the NK1.1$^+$ T cell subset have recently been shown to recognise CD1 molecules. Bendelac and colleagues (1995a, 1995c) demonstrate a role for the CD1 ligand both in positive selection of thymocytes and in stimulation of mature T cells.

1B.3.5.1 CD1 and NK1.1$^+$ T cell ontogeny

The strong expression of CD1 by cortical thymocytes but not by medullary thymocytes would be consistent with CD1 having a role in T cell ontogeny particularly as the relationship between CD1 and MHC Class I expression in the thymus is a reciprocal one i.e. only low levels of MHC I are seen in the cortex with higher levels in the medulla.

It is therefore interesting to speculate that CD1 may perform some role in the process of thymic selection - fulfilling the role of MHC class I in a region where the latter is only present at low levels (Gambon et al. 1988). Several reports have described a covalent association of CD8 with CD1 in the thymus (Snow et al. 1985, Ledbetter et al. 1985, Blue et al.1989). The significance of this finding is not clear although it has been suggested that a switch from CD8/CD1 to CD8/Class I association may represent a maturation stage in T cell ontogeny (Blue et al. 1989).

As described in Section 1A.4.3, Vα14+ cells in mice and the corresponding Vα24 subset in man are a class of T cells which mainly express an invariant TCR i.e. Vα14Jα281 in mice and Vα24JαQ in humans (Lantz and Bendelac, 1994). Using mAbs directed against Vα24 and Vβ11, Dellabona and colleagues (1994) show that Vα24-JαQVβ11 receptors are present within the DN cell subset in all individuals. This striking conservation both within and between species of a restricted repertoire would be consistent with constraints imposed by a non-polymorphic ligand such as the non-classical class I molecules or CD1.

Adachi and colleagues (1995) investigate the receptor-ligand relationship further by showing that the ligand for Vα14Jα281 T cells is present in class II $^{-/-}$ mice and TAP$^{-/-}$ mice but is lost in β2m $^{-/-}$ mice. Evidence is also provided that this β2m

associated molecule is not encoded within the MHC and that cells are positively selected by bone marrow derived haematopoietic cells (thymic epithelial cells are not required), a feature also previously described by Bix and colleagues (1993).

Bendelac and colleagues (1995a) extended this research by showing that the NK1.1 subset of thymocytes in mice was CD1 specific. T cell hybridomas derived from $NK1.1^+$ T cells were stimulated by thymocytes from various mouse strains - this stimulation was abrogated in $\beta 2m^{-/-}$ mice and in the presence of the anti-mouse CD1.1 mAb 3C11. Recognition of the ligand was shown to depend on TCR expression and the ligand had a restricted tissue distribution similar to CD1. Further experiments utilizing recombinant vaccinia virus expressing the mCD1.1 gene to infect mouse fibroblast cell lines resulted in cells capable of stimulating the hybridomas - again this stimulation was blocked with 3C11. Following on from this work, Bendelac (1995c) showed that immature $CD4^+CD8^+$ thymocytes were the likely source of CD1 for positive selection of developing thymocytes by excluding cells of myeloid origin (i.e. dendritic cells and macrophages), B cells and mature (medullary) T cells. These results suggest that the the main cell type selecting the $V\alpha 14\text{-}J\alpha 281^+$ CD1-specific thymocytes are CD1 expressing $CD4^+CD8^+$ cortical thymocytes.

Thus for the first time there is evidence suggesting that CD1 participates in the positive selection of specific T cell subsets providing an explanation for CD1 expression on cortical thymocytes.

1B.3.5.2 CD1 and activation of NK1.1+ T cells

The studies described above in which Bendelac and colleagues (1995a) demonstrate CD1 recognition by $NK1.1^+$ T cells also show that the CD1 ligand is capable of inducing activation of mature T cells. As discussed in Section 1A.4.3, it is known that this subset of T cells has a role in IL-4 production and thus in Th2 lineage commitment and IgE production. It has been postulated that stimuli which elicit IgE production e.g. helminth infection and exposure to allergens occur at sites where CD1 is constitutively expressed (i.e. intestinal epithelium and skin) thus allowing

IL-4 production by $NK1.1^+$ T cells in response to the stimuli (Yoshimoto et al. 1995b).

It has also been shown that there is an associated expression of the Fc receptor for Ig with CD1 on Langerhans' cells from atopic dermatitis patients (Bruynzeel-Koomen et al. 1988).

SJL mice (which are known to produce limited amounts of IgE in response to a variety of stimulants) have recently been shown to have a marked deficiency of $CD4^+$ $NK1.1^+$ T cells (Yoshimoto et al. 1995a). This further supports a key role for $NK1.1^+$ T cells in Th1/ Th2 lineage commitment as a result of their capacity for IL-4 production, indeed it has been postulated that expression of CD1d by intestinal epithelial cells may contribute to the propensity for type 2 responses following oral immunization (Fearon and Locksley, 1996).

In summary then, it is apparent that this family of molecules can be compared to both MHC class I and class II molecules but current evidence points to a unique function distinct from that of classical MHC molecules. Within the family, the division between the CD1a-c isotypes and the CD1d isotypes would now appear to be one of both sequence and function - possibly implying different evolutionary requirements by different species for the CD1 molecules and the functions they perform.

1B.4 Thesis Objectives

As discussed in preceding sections, the CD1 molecules can be divided into two groups, CD1a-c and CD1d. The predicted amino acid sequence of the CD1E gene places the putative CD1e molecule in an intermediate position between the two groups. It is clear that the mouse is not the most useful model for research into the CD1 family as mice only possess members of the CD1d group and whilst much research has been performed using this model, it is obvious that research into the CD1 family using mice can only address questions relevant to group 2 genes and molecules.

The sheep is a useful model for the study of CD1 for the following reasons. Ready access to high CD1 expressing tissues from abattoir specimens and blood and lymph

compartments render this a system highly amenable to research. A number of mAbs are available which recognise ovine CD1. Previous work using these mAbs has indicated the presence of at least 2 isotypes of CD1 - an isotype with CD1b like distribution (recognised by the majority of mAbs) whilst SBU-T6 (and CC43 and CC118 in cattle) recognise a molecule with a wider distribution possibly more similar to to CD1c. Recent molecular analysis of the ovine CD1 family has shown that up to seven ovine CD1 genes may exist. Four of these genes, which are all CD1B-like, have been cloned (Ferguson et al. 1996). Although four ovine CD1 genes have been identified, these all encode CD1b-like molecules. Immunochemical research suggests that there are at least two CD1 isotypes on the basis of tissue reactivity of anti-CD1 mAbs.

The aims of this thesis were therefore to extend the understanding and knowledge of the ovine CD1 family. Initial work was centred on characterizing previously available and new mAbs in more detail. This involved both immunochemical and biochemical analysis of the ovine CD1 antigens. Subsequent work utilized the information gained at the protein level to enable further investigation of the ovine CD1 genes. The combination of these two approaches has resulted in the identification of a novel CD1 NH_2-terminal sequence with closest homology to the predicted sequence of the human CD1E gene and the cloning of a sheep CD1D-like gene.

CHAPTER 2

MATERIALS AND METHODS

The companies supplying the reagents used in this work are referred to in the text. All company addresses are listed in Appendix III.

2.A IMMUNOCHEMISTRY

2A.1 Monoclonal Antibodies

The monoclonal antibodies (mAbs) used in this research are listed in Tables 1 and 2 with appropriate references. Table 1 lists the anti-CD1 mAbs used and Table 2 lists other mAbs used as controls and in double staining FACS experiments.

2A.2 Immunostaining

Tissue preparation

Unless otherwise specified, tissue was obtained from lambs at the local abbatoir. Tissue was cut into small blocks, mounted in OCT embedding medium (Tissue Tek) and snap frozen in liquid nitrogen. 8-10μm cryostat sections were cut onto Vectabond (Vector Labs.) treated slides, air dried overnight, fixed for 10 minutes in ice cold acetone then stained or stored at -70^{0}C.

Cytospins

Cytospins for immunostaining were prepared by spinning 100μl of cell suspension (5×10^5 ml^{-1}) at 1000 rpm for 5 minutes using a Cytospin-2 (Shandon). Cytospins were then fixed in ice cold acetone for 10 minutes before staining or storage at -70^{0}C.

Staining

Staining was carried out using an indirect immunoperoxidase technique with a commercially available kit (Vectastain Elite ABC kit, Vector Labs.). Slides were rehydrated for 10 minutes in blocking buffer (0.08% Tween 20, 2% normal sheep serum, 0.1% bovine serum albumin in PBS). Incubation with primary antibody was carried out for one hour at room temperature. Slides were washed in blocking buffer

TABLE 2.1

Anti-CD1 mAbs used in immunochemistry with isotype and original reference listed.

TABLE 2.2

mAbs used as controls and in double staining flow cytometry experiments. Isotype and original references are listed.

TABLE 2.1

MONOCLONAL	ISOTYPE	REFERENCE
SBU-T6	IgG_1	Mackay et al. 1985
VPM5	IgM	Bujdoso et al. 1989
TH97A	IgG_{2a}	Davis et al. 1986
CC13	IgG_3	Machugh et al. 1988
CC14	IgG_1	"
CC43	IgG_1	Howard et al. 1993a
CC118	IgG_1	"
CC20	IgG_{2a}	Howard et al. 1993b
CC40	IgG_1	"
CC90	IgG_1	"
CC122	IgG_1	"

TABLE 2.2

MONOCLONAL	SPECIFICITY	ISOTYPE	REFERENCE
VPM53	Anti-campylobacter	IgG_1	McOrist et al. 1987
VPM30	ov B cell	IgM	Naessens and Howard, 1991
VPM65	ov CD14	IgG_1	Gupta et al. 1996
VPM46	ov MHC II	IgG_{2a}	Dutia et al. 1990
SBU-T8	ov CD8	IgG_{2a}	Maddox et al. 1985
SBU-T4	ov CD4	IgG_{2a}	Maddox et al. 1985
DU2104	pan B cell	IgM	Mackay et al. 1992
VPM19	ov MHC I	IgG_1	Hopkins and Dutia. 1990

then incubated with biotinylated secondary antibody for 30 minutes. Slides were washed again in blocking buffer then incubated in HRP-streptavidin complex for 30 minutes. Prior to visualization of the bound antibody-HRP complex, endogenous peroxidase was blocked by incubating sections for 20 minutes at 37^0C in 10mM β-D glucose, 5 units ml^{-1} glucose oxidase (Glucose oxidase type II-S, Sigma), 1mM sodium azide, 0.1M PBS. Visualization of bound antibody-HRP complex was carried out in either a solution of filtered 3,3' -diaminobenzamine (DAB, Sigma) (10mg DAB, 20mls PBS, 40µl H_2O_2) or using a DAB substrate kit (Vector Labs.) according to manufacturers instructions. In cases where this kit was used, a black stain was obtained by adding nickel solution to the substrate prior to use.

Following colour development (5-7 minutes), sections were washed in tap water, lightly counterstained in haematoxylin, dehydrated through graded alcohols and mounted in DePeX mounting medium (BDH).

2A.3 Flow Cytometry

Cell Preparation

Single cell suspensions of thymocytes, splenocytes and mesenteric lymph node were prepared by teasing out fresh tissue into RPMI medium. The resulting cell suspension was washed twice then overlaid onto Lymphoprep (Nycomed) and spun at 800 x g for 20 minutes at room temperature. Cells were resuspended in FACS buffer (PBS, 0.5% BSA, 0.05% sodium azide, pH 7.2), washed, counted and resuspended to give $4x10^6$ cells ml^{-1}.

Blood was collected by jugular venipuncture into heparinized containers. Lysis of RBCs was performed by adding 10 mls of blood to 35 mls of prewarmed lysis buffer (0.16M NH_4Cl, 0.017M Tris pH7). White cells were then centrifuged at 200 x g and lymphocytes purified by harvesting over Lymphoprep as above. Cells were then washed twice in RPMI and resuspended in FACS buffer.

Afferent and efferent lymph was obtained from chronic cannulation of the prefemoral efferent and pseudoafferent lymphatics (Hall, 1967; Hopkins et al. 1986). The lymph

was collected into sterile plastic bottles bottles containing 400u of heparin. Cells were washed twice in FACS buffer then resuspended to give $4x10^6$ cells ml^{-1} .

Single Staining Flow-Cytometry

$4x10^5$ cells were incubated for 30 minutes at 4^0C with 25µl of mAb (either saturated supernatant or ascites titrated to give optimal dilution). Cells were washed twice in FACS buffer then incubated for 30 minutes at 4^0C with 25µl of FITC conjugated anti-mouse Ig (The Binding Site). Cells were washed twice in FACS buffer then analysed on a FACScan (Becton Dickinson).

Double Staining

For double staining using mAbs with different isotypes, $4x10^5$ cells were incubated with 25µl of each mAb (one of which was biotinylated) for 30 minutes at 4°C. Cells were washed twice in FACS buffer then incubated with 25µl of isotype specific anti-mouse conjugated FITC and 25µl of streptavidin-phycoethrin (SA-PE, Amersham International) for 30 minutes at 4^0C. Cells were then washed and analysed as above.

For double staining using antibodies of the same isotype, cells were first incubated with the mAb to be labelled with FITC. Cells were washed as above then, following incubation with FITC-antiglobulin conjugate and washing, free sites of the FITC conjugate were blocked by incubating with the same primary antibody for 30 minutes at 4^0C. Cells were then incubated with the second biotinylated antibody prior to labelling with SA-PE as above.

The mAbs and conjugates used in flow cytometry experiments were titrated to establish optimal working concentrations. Isotype specific negative controls were used in single staining experiments and similarly for the mAb to be labelled with FITC in double staining experiments. Biotinylated normal mouse serum was used as a control for the biotinylated mAb in double staining experiments

2A.4 Purification of Ascitic Fluid

Immunoglobulin (Ig) from ascitic fluid was purified by caprylic acid precipitation as follows. 1ml of ascitic fluid was acidified by addition of 25µl glacial acetic acid (BDH) followed by the precipitation of unwanted protein by the addition of 50µl caprylic acid (octanoic acid). This was incubated on a roller at 20^0C for 30 minutes then spun at high speed in a microcentrifuge for 5 minutes. The mixture was then incubated on ice for 30 minutes after which time solidified caprylic acid was removed from the surface and the supernatant which contained the Ig dialysed extensively into 0.1M $NaHCO_3$, pH 8.4 for 24 hours.

2A.5 Biotinylation of Monoclonal Antibodies

Purified immunoglobulin from ascites was adjusted to a concentration of 1mg ml^{-1} in 0.1M $NaHCO_3$, pH 8.4. Biotinylation was then carried out by incubating the Ig for 5 hours with biotin (biotin-amido caproate N-hydroxysuccinimide ester) in DMSO (10 mg ml^{-1}) at a biotin to protein ratio of 75µg : 1mg. The biotinylated Ig was then extensively dialysed against 0.1M PBS, 0.01% sodium azide, pH7.2 for 24 hours at 4^0C before a final 2 hour dialysis against PBS azide containing 20% glycerol. Biotinylated mAbs were then stored at -20^0 C.

2A.6 Immunoprecipitation

2A.6.1 Iodination of cells

Iodination of thymocytes was carried out using an Iodogen chemical labelling method. A 1mg ml^{-1} solution of Iodogen (1,3,4,6-tetrachloro-3α,6α-di-phenylglycouril) in dichloromethane was made and eppendorf tubes were prepared by drying 100µl of the Iodogen solution onto the tube under a stream of nitrogen in a fume hood. $2x10^7$ thymocytes were suspended in 0.1ml of PBS and transferred to an iodogen treated tube.

1mCi of sodium iodide (I^{125}, Amersham International) was added and incubated at 20^0C for 15 minutes whilst being shaken every 2 minutes. Iodinated cells were then washed twice in 20mls of PBS.

Labelled cells were then lysed in 1ml TNT lysis buffer (Appendix I) containing 0.2mM phenylmethylsulfonylchloride, for 30 minutes on ice. Nuclear material was then removed by centrifugation at 13,000 x g for 10 minutes. The supernatant was removed and stored at -70^{0}C.

2A.6.2 Precipitation of Antigen

Standard ELISA plates

ELISA plates (Immulon 2, Nunclon, Denmark) were coated with 75µl goat anti-mouse immunoglobulin (Sigma) at a concentration of 10µg ml^{-1} in borate buffered saline pH 8.2 (Appendix I) and incubated at 4^{0}C for 12 hours. Plates were then washed five times in PBS/0.01% Tween 20 prior to addition of 75µl of mAb (either neat saturated supernatant or ascites at a dilution of 1:1000 in PBS) and incubation at 20^{0}C for 1 hour. Plates were then washed as above and incubated overnight at 4^{0}C with 75µl of I^{125}-labelled lysate. After washing, bound antigen was eluted by boiling for 3 minutes in 75µl of SDS-PAGE sample buffer (see Section 2.7).

Streptavidin coated ELISA plates

Enhancement of the signal obtained from immunoprecipitates was achieved by using streptavidin-coated microtiter plates (Boehringer Mannheim). The streptavidin coated plates were incubated with a 1:2000 dilution of anti-mouse Ig-biotin overnight at 4^{0}C. Plates were washed in PBS/0.1% BSA then incubated in specific mouse mAb for 4 hours at 4^{0}C. Plates were washed as above then incubated with I^{125} labelled lysate overnight at 4^{0}C. Bound antigen was eluted as previously described.

2A.6.3 Autoradiography

Following elution, labelled antigen was fractionated by polyacrylamide gel electrophoresis (PAGE) as described in section 2A.7. Gels were stained, destained and dried as described in section 2A.8. Dried gels were then placed in a film cassette and exposed to X-ray film (X-Omat TR, Kodak) at -70^{0}C.

2A.7 Polyacrylamide Gel Electrophoresis (PAGE)

Proteins were fractionated by SDS PAGE. Resolving gels were either single concentration 12% or 5-20% linear gradient polyacrylamide gels and consisted of 12% acrylamide, 0.32% bisacrylamide in 375mM Tris-HCl pH8.7, 0.1% SDS, 0.05% w/v ammonium persulphate (APS), 0.05% v/v TEMED. The stacking gel consisted of 3% acrylamide, 0.1% bisacrylamide in 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.05% w/v APS, 0.1% TEMED. Gels were run in Tris-glycine buffer containing SDS (255mM glycine, 0.1% SDS, 50mM Tris HCl).

Samples were boiled for three minutes in sample buffer (2% SDS, 20% glycerol, 0.125M Tris-HCl pH 6.8, 0.004% bromophenol blue) prior to electrophoresis through vertical slab gels using Bio-Rad Mini Protean II gel equipment. Gels were run at a constant 200 Volts for 30 minutes or until the dye front reached the bottom of the gel. Pharmacia low molecular weight markers (range 14.4kd-94kd) were run on each gel.

2A.8 Staining of SDS-PAGE Gels

Gels for autoradiography were stained in 0.025% Coomassie brilliant blue-R in 20% v/v methanol, 5% v/v acetic acid for 30 minutes. Gels were then destained in several changes of 20% v/v methanol, 5% v/v acetic acid and dried onto wet 3mm filter for 3 hours at 80^0C on a vacuum drier (Biorad Labs. Gel drier, model 583).

2A.9 Two-Dimensional Electrophoresis

First Dimension

First dimension gels were cast in capillary tubes within a casting tube (Biorad). A gel solution consisting of 8M urea (Schwarz/Mann Biotech), 3.5% acrylamide, 0.2% bisacrylamide, 10% w/v Nonidet-P40 (NP40), 5% ampholines (pharmalyte pH3-8, Pharmacia) was made in milliQ water. Once polymerized, first dimension gels were connected to sample chambers and positioned in the apparatus (Mini-protean II 2-D cell, Biorad). Samples were dissolved in sample buffer (8M urea, 5% ampholine, 2% NP40), loaded into the chamber and overlayed with 8M urea, 1% ampholine.

First dimension gels were run with an upper chamber (anode) buffer of 0.1M NaOH and lower chamber (cathode) buffer of 0.1M H_3PO_4. Following completion of the first dimension, gels were extruded from the capillary tubes using equilibration buffer (0.125M Tris pH 6.8, 10% glycerol, 2.3% SDS) and used directly in the second dimension or stored at -70^{0}C.

Second Dimension

Vertical slab gels were prepared for the second dimension as described in section 2A.7. First dimension gels were placed on the surface of the stacking gel with the top end (anode) of the gel at the left side. Excess equilibration buffer was removed with blotting paper and the gel run in Tris-glycine buffer as described above. 2D gels were then either blotted (Section 2A.10) or dried and subjected to autoradiography (Section 2A.6.3).

2A.10 Western Blotting

Following SDS-PAGE separation, the proteins were transferred to nitro-cellulose membrane (Hybond C, 0.45µm, Amersham International) using a semi-dry electroblotter (Trans-Blot SD, Bio-Rad). Transfer was performed in blotting buffer (25mM Tris, 20% v/v methanol) for 1 hour at a constant current of 120mA. Following blotting, non-specific sites on the membrane were blocked in a solution of 5% v/v fat free dried milk in PBS for 1 hour at 20^{0}C before incubation overnight in primary antibody diluted in PBS. Blots were then washed 5 times over 30 minutes in wash buffer (PBS, 0.1% Tween 20) at 20^{0}C. Detection of bound antibody was carried out by incubating blots for 1 hour at 20^{0}C in a 1:1000 dilution of anti-mouse Ig-biotin (Sigma) followed by incubation for 30 minutes in a 1:2000 dilution of streptavidin- alkaline-phosphatase conjugate (Boehringer Mannheim).

Blots were washed twice in 0.1M Tris-HCl pH 9.5 then developed in a solution of 0.1M Tris HCl pH 9.5, 4mM $MgCl_2$, 0.2mg ml^{-1} nitro-blue tetrazolium (NBT, Sigma), 100mg ml^{-1} 5-bromo-4 chloro-3 indolyl phosphate (BCIP). The reaction was stopped by washing in tap water.

2A.11 Protein Detection on Blots

Proteins were directly visualized on blots using 'Protogold Colloidal Gold Sol' (BioCell). Proteins were blotted onto nitrocellulose as described above and the membrane blocked in PBS/0.3% supplied detergent for 30 minutes at 37^{0}C. The membrane was washed 3 times over 15 minutes in PBS/0.3% detergent, 3 times over 3 minutes in distilled water then incubated in 'Protogold' solution for 3 hours with continuous shaking. In order to enhance the stain, the membrane was washed 5 times over 10 minutes in distilled water and incubated in equal quantities of initiator and enhancer for 20 minutes, washed thoroughly and dried.

2A.12 Enhanced Chemiluminescence (ECL)

ECL was used to provide increased sensitivity in development of membranes which were probed with the mAb SBU-T6. Blotting, blocking of non-specific sites and washing were as in section 2A.10 with the exception of washes being carried out in a solution of PBS, 0.5% Tween 20. Blots were then incubated in a 1:1000 dilution of anti-mouse Ig-biotin (Sigma) for 1 hour at 20^{0}C, washed as above then incubated in a 1:2000 dilution of biotinylated HRP streptavidin complex (Amersham) for 1 hour at 20^{0}C. Following extensive washing over a 45 minute period, detection of bound HRP complex was carried out using ECL western blotting detection reagents (Amersham) as follows. Washed membranes were incubated in the detection reagents for exactly 1 minute, excess reagent was drained off then covered on both sides with cling-film. Membranes were then place protein side up in a film cassette and a sheet of X-ray film placed (X-Omat TR, Kodak) on the surface for 15-60 seconds. Further exposures were made if necessary based on the results of the short exposures.

2A.13 Affinity Purification of Antigen

2A.13.1 Preparation of thymus lysate

Thymus tissue from 4 lambs was homogenized in 1 litre of ice cold HBSS for 1 minute. Cells were spun at 600 x g for 15 minutes at 4^{0}C and resuspended in TNT lysis buffer (Appendix I) containing 0.2mM PMSF. Cells were then lysed on ice for 30 minutes and nuclei removed by spinning at 600 x g for 20 minutes at 4^{0}C. The

supernatant was retained and sodium azide added to 0.05%. The lysate was centrifuged to remove high molecular weight debris at 20,000 x g for 2 hours at 4^0C and filtered through Millipore prefilters before being used directly on an Affigel column or storage at -70^0C.

2A.13.2 Monoclonal antibody affinity chromatography

Ascites was purified by caprylic acid precipitation as described in Section 2A.4. Purified Ig was dialysed into 0.1M phosphate buffer pH7.2 containing 0.05% azide overnight at 4^0C. Dialysis was continued for a further 4 hours in fresh buffer. Dialysed protein was then linked to washed Affigel 10 (Biorad) at 30mg protein ml^{-1} Affigel suspension by incubating overnight at 4^0C. Ig linked Affigel was centrifuged (200 x g), the supernatant removed and the Affigel blocked by incubating on a rotor in 1M ethanolamine pH8 for 1 hour at 20^0C. Affigel was centrifuged and washed in PBS. 0.1% azide before being poured into a column. Non-specifically bound material was removed from the column by washing in 15mM triethanolamine (TEA) 0.5% sodium deoxycholate (DOC) pH8 for 30 minutes followed by a 30 minute wash in 15mM TEA, 0.5% DOC, 0.5M NaCl, pH11.3.

The column was then washed with 10mls PBS, 0.1% azide followed by 10mls TNT buffer before running the lysate (prepared as described in Section 2A.12.1) through the column at a very slow rate over 48 hours. Following absorption of antigen, the column was washed in TNT buffer followed by TEA/DOC pH8 for 30 minutes each before elution of antigen from the column with TEA/DOC/NaCl pH11.3. Eluted fractions were immediately neutralized by the addition of 0.3ml 0.5M TEA pH7.9 ml^{-1} fraction. Following elution, the column pH was immediately returned to pH7.3 by washing with PBS. Eluted fractions were tested by ELISA (see Section 2A.13) and the peak fractions pooled and stored at 4^0C.

2A.14 ELISA

Eluted antigen was diluted 1:10 in PBS/azide then 75µl was added to each well of an ELISA plate and incubated overnight at 4^0C. Plates were then washed three times in

PBS/0.1% Tween 20 and non-specific sites blocked by incubating in 2% BSA in PBS for 30 minutes at 20°C. Plates were washed as above, 75µl mAb was added and the plates were incubated for 1 hour at 20°C. After a further wash, 75µl of a 1:1000 dilution of anti-mouse Ig-HRP conjugate (SAPU) was added and incubation continued for 30 minutes in the dark at 20°C. Plates were washed again then incubated in the dark with HPX substrate (0.8% O-phenylenediamine, 0.1M Na_2HPO_4, 0.05M citric acid, 0.0016% v/v H_2O_2) for 15-20 minutes. The reaction was stopped by adding 50µl 2M sulphuric acid. The optical density of the reaction was measured at 492nm on a Dynatech MR 3000 micro ELISA plate reader.

2A.15 Deglycosylation of Antigen

Purified antigen was deglycosylated using N-glycosidase F (Boehringer Mannheim). Antigen was boiled for 3 minutes in 0.75% SDS then incubated overnight at 37°C in 0.01M EDTA, 1% Triton-X 100 containing 0.1unit N-glycosidase-F.

2A.16 Isolation of Antigen for NH_2-Terminal Sequencing

Precipitation of antigen

Antigen eluted from Affigel columns was precipitated by the addition of 4 volumes of ethanol (BDH) overnight at -20°C in Corex tubes. Precipitated antigen was recovered by centrifugation at 13000 x g for 20 minutes. The pellet was air dried and resuspended in SDS sample buffer.

Gels and blotting

The gel system was as described in section 2A.7 with the following modifications. Molecular biology grade reagents were used and all buffers were made fresh prior to each isolation. The stacking gel contained 375mM Tris-HCl pH 8.7 and the gel was prerun in 0.125M Tris-HCl pH 6.8 containing 50µM glutathione. A dye was used to monitor the progress of the gel. Once the dye had reached the interface between stacking and resolving gel, the buffer was replaced with normal running buffer

(50mM Tris, 255mM glycine, 0.1% SDS) containing 0.1mM thioglycolate and run as normal.

The gel was then blotted onto Pro-blott membrane (Applied Biosystems) using a wet blotting system. The gel was soaked for 5 minutes in blotting buffer (10mM CAPSpH11 [3 cyclohexylamino-1-propane sulfonic acid], 10% methanol) and the membrane was wetted in methanol for 3 minutes prior to equilibration in blotting buffer. Blotting was then carried out at 50V for 2 hours. After blotting, the membrane was rinsed in deionised water, saturated in 100% methanol for 5 seconds and then stained in 0.1% Coomasie-blue R-250 in 40% methanol/1% acetic acid for 1 minute. The membrane was destained in 50% methanol, rinsed extensively in deionised water, dried and bands of interest excised.

2B MOLECULAR BIOLOGY

All DNA sequencing was carried out by Mr. I. Bennet on a LI-COR DNA sequencer (model 4000L).

2B.1 RNA Isolation

The following steps were taken to minimise RNAase contamination. Where appropriate, solutions were treated with diethyl pyrocarbonate (DEPC). 0.2% DEPC was added to solutions which were well mixed, stored for at least 1 hour at 4^0C then autoclaved. Sterile plasticware was used and gloves worn at all times.

2B.1.1 Isolation of total RNA

Total RNA was isolated from cells using RNAzol B (Biogenesis). For thymocyte RNA, fresh tissue was placed on ice and cell suspensions made in RPMI. Cells were washed twice, the medium removed then resuspended in RNAzol at 1ml per $5x10^7$ cells. For PBLs, cells were isolated by ammonium chloride lysis of RBC (section 2A.3), density centrifugation followed by washing twice as above. Cells in tissue culture flasks were washed once in PBS then lysed directly by addition of RNAzol B to the flask. Following lysis, chloroform was added to 10%, samples vigorously shaken and stored on ice for 5 minutes. Samples were centrifuged at 13000 x g for 15 minutes, the upper aqueous phase was transferred to a fresh tube and an equal

volume of ice-cold isopropanol added. Precipitation was carried out for 1 hour on ice then samples were centrifuged at 13000 x g for 15 minutes. RNA pellets were washed with 75% ethanol, air dried then resuspended in 1mM EDTA pH7. If not for immediate use, RNA was stored in aliquots at -70^{0}C.

2B.1.2 Isolation of mRNA

PolyA+ RNA was isolated either directly using the Invitrogen Fasttrack mRNA kit (Invitrogen) or by selecting polyA RNA from total RNA using streptavidin paramagnetic particles (Promega) according to manufacturers instructions.

2B.1.3 RNA analysis

Quantitation of RNA was determined by spectrophotometry with an OD_{260} of 1.0 being equivalent to 40µg ml^{-1} of RNA. Absorbence readings at A280 were also measured to check sample purity - pure RNA preparations have A260/A280 ratios of 2.0.

Gel analysis was also used to demonstrate the intact nature of the RNA. Clear 28S and 18S bands were required to indicate that the RNA had not undergone degradation. Approximately 5µg of total RNA was run on a denaturing agarose gel as follows. 1.5g of agarose was added to 82mls of phosphate buffer (20mM sodium phosphate pH7). The agarose was melted then cooled to 60^{0}C. 18ml of formaldehyde was then added in a fume hood immediately prior to pouring the gel. RNA samples were denatured in 3 volumes of denaturing load buffer (60% v/v deionised formamide, 7.2% formaldehyde, 20mM phosphate buffer, 0.1M EDTA) for 15 minutes at 52^{0}C. 1 volume of dye mix (30% Ficoll, 0.05% bromophenol Blue, 100mM phosphate buffer, 0.25µg µl^{-1} EtBr) was then added and samples run for 90 minutes at 80v in 20mM phosphate buffer. The buffer was changed every 15 minutes.

2.B.2 cDNA Synthesis

2B.2.1 Standard

cDNA synthesis was carried out using a first strand cDNA synthesis kit (Amersham International). Random hexanucleotide primers or oligo-dT primers were used to initiate the cDNA synthesis from total or polyA$^+$ RNA according to the manufacturers instructions.

Alternatively, synthesis was carried out using Superscript II RNase H$^-$ Reverse Transcriptase (RT) (GibcoBRL) as follows. 5µg total RNA plus 150ng random primers (Amersham International) were made to 12µl in SDW and heated to 70^{0}C for 10 minutes then chilled on ice. Buffer (250mM Tris-HCl pH8.3, 375mM KCl, 15mM $MgCl_2$) dithiothreitol (GibcoBRL) and dNTP mix were then added and the mixture incubated at 25^{0}C for 10 minutes. The mixture was heated to 42^{0}C for 2 minutes prior to addition of 200 units RT and incubation at 42^{0}C for 50 minutes. The reaction was then inactivated by heating at 70^{0}C for 15 minutes.

2B.2.2 Adaptor ligated cDNA

cDNA synthesis was also carried out to allow 'RACE' (**R**apid **A**mplification of **c**DNA **E**nds) PCR reactions to be performed. For this technique, cDNA synthesis was carried out using the Marathon cDNA amplification Kit (Clontech) according to the manufacturers instructions. Briefly, 1µg of polyA$^+$ RNA was used as a substrate for first and second strand cDNA synthesis. Adaptors were then ligated onto the ends of the cDNA population allowing the subsequent use of adaptor primers in PCR reactions (see section 2B.3)

2B.3 Polymerase Chain Reaction

PCR reactions were carried out in sterile 0.5ml eppendorf tubes with final reaction volume of 50µl or 100µl. The basic components of the PCR reaction were as follows. 1x PCR reaction buffer (50mM KCl, 10mM TrisCl pH8.3, 1.5mM $MgCl_2$), 100µM each dNTP (Pharmacia), DNA template, 25-50 pmoles of each primer. These constituents were mixed then briefly spun and overlaid with 50µl mineral oil

(Sigma). Reaction tubes were then heated to 94^{0}C for 3 minutes and cooled to 80^{0}C prior to the addition of 1 unit of Taq (*Thermus aquaticus* DNA) polymerase enzyme (GibcoBRL) per reaction. Cycling was carried out using a Hybaid Omnigene thermal cycler. Cycling parameters varied according to substrate and primers and as such are described in the relevant results chapters. Oligonucleotide primers were purchased from Cruachem Ltd. or Perkin-Elmer. Sequences of all the primers used in this work are listed in Appendix II.

2B.4 Analysis of PCR Products

Following PCR, 10% of the reaction was analysed on a 1% w/v agarose gel (Type I: low EEO, Sigma). Agarose was melted in TAE buffer (Appendix I) containing 1μg ml^{-1} ethidium bromide. Samples were mixed with 1/5 volume of 5x DNA sample buffer, loaded and the gel run at 70-80V for 60-90 minutes. EcoRI/HindIII digested lambda DNA markers (NBL) were run on each gel.

2B.5 Purification of DNA

DNA from PCR reactions which was subsequently to be cloned was purified by excising the relevant band from the gel and subjecting it to one of two purification methods. Small fragments of less than 500bp were purified using Spinbind DNA extraction units (FMC Bioproducts) according to the manufacturers instructions. Larger PCR products and plasmids were purified using the Geneclean II kit (Bio 101 Inc) according to the manufacturers instructions.

2B.6 Cloning of PCR Products

PCR products which had been purified as described above were cloned using the TA cloning kit (Invitrogen). Briefly, ligations were set up as 1:1 to 1:3 molar ratio of vector : PCR insert. 1-2 μl of the ligation reaction was then used to transform INVαF cells and the transformed cells were plated onto LB/ampicillin/X-gal plates (section 2B.6.1) and incubated overnight at 37^{0}C. Transformed colonies were identified by

blue/white selection and positive clones were picked and grown overnight in 10ml LB containing 50μg/ml ampicillin.

2B.7 Bacterial Cultures

2B.7.1 Growth media

All bacterial strains were cultured in Luria Bertani (LB) broth (Appendix I) with the exception of MC1061/p3 which were cultured in TYM medium (Appendix I). The medium was supplemented with ampicillin (50μg/ml) since the vectors pCRII, pBluescript and pCDNA3 all contain the ampicillin resistance gene. LB/agar plates or TYM/agar plates were made with 1.5% (w/v) bacteriological agar (Oxoid) supplemented with 50μg/ml ampicillin and stored at 4^0C. When plating INVαF' cells containing the vector pCRII from the TA Cloning kit (see section 2B.6), each 10cm plate was supplemented with 50μgml^{-1} 5-bromo-4-chloro- 3-indolyl-β-D-galactoside (X-gal, Melford laboratories) to allow blue-white selection.

2B.7.2 Competent cells

The *E. coli* strains used in this work were INVαF' (TA cloning kit), XL1-blue and DH5α (GibcoBRL). In addition, MC1061/p3 were made supercompetent according to the method of Simmons (1993). Single colonies were picked into 5ml TYM and grown for 4 hours then diluted to 100ml and grown to midlog OD_{600}= 0.5. Cells were then diluted to 500ml and again grown to midlog OD_{600}= 0.5. Cultures were rapidly chilled, pelleted at 2800 x g for 15 minutes then resuspended in 30mM CH_3COOK, 50mM $MnCl_2$, 100mM KCl, 10mM $CaCl_2$, 15% glycerol (v/v). Cells were pelleted again at 1500 x g for 10 minutes then gently resuspended in 10mM 3-[N-Morpholino] propanesulfonic acid (pH 7), 75mM $CaCl_2$, 10mM KCl, 15%glycerol before being aliquoted into prechilled eppendorf tubes and snap frozen in liquid nitrogen. Competent cells were then stored at -70^0C.

2B.8 Preparation of Plasmid DNA

Plasmid DNA for sequencing was isolated using Qiaprep-spin columns (Qiagen Ltd) according to the manufacturers instructions. Preparation of plasmid DNA for analysis of insert sizes and screening of multiple colonies was performed as follows. 6ml overnight cultures were pelleted at 400 x g for 10 minutes and resuspended in 1M glucose, 1M Tris pH 8, 0.5M EDTA pH8. Cells were then lysed in 10M NaOH, 20% SDS for 10 minutes at 20^{0}C. Precipitation of bacterial DNA was carried out by addition of 3M CH_3COOK, incubation on ice for 10 minutes and high speed centrifugation for 3 minutes. The supernatant was then phenol/chloroform extracted, precipitated with isopropanol, washed in ethanol and resuspended in 50 µl TE buffer (Appendix I).

2B.9 Restriction Endonuclease Digestion of DNA Samples

Restriction endonuclease digests of plasmid DNA to allow analysis of inserts was carried out as follows. The reaction was typically carried out in a 20µl volume containing 2µl plasmid DNA, 10 units of enzyme and the appropriate enzyme buffer. Digests were carried out at 37^{0}C for 2-4 hours for the majority of enzymes used. Digestion using the enzyme BstXI was carried out at 45^{0}C. Following digestion, inserts were analysed by running 10µl of the reaction on an agarose gel (as in section 2B.4) alongside uncut plasmid.

2B.10 *In Situ* Hybridization (ISH)

2B.10.1 Preparation of slides and tissues

Slides

Glass slides for ISH were prepared as follows. Slides were washed overnight at 60^{0}C in 10% Decon, rinsed in running tap water for 4 hours then placed in distilled water for 2 hours. Slides were then baked overnight at 150^{0}C prior to treatment with 3-aminopropyltriethoxysilane (TESPA) as follows. Baked slides were incubated for 5 seconds at 20^{0}C in 2% TESPA in acetone, washed twice in acetone then finally rinsed in water before baking dry at 42^{0}C.

Tissues

Tissues were excised immediately post-mortem and fixed in 4% paraformaldehyde. Paraffin sections were then cut onto TESPA treated slides. Sections were stored at 4^0C for 1-2 weeks before use.

2B.10.2 Riboprobe synthesis

Inserts for riboprobe synthesis were contained within the vector pCRII thus allowing the use of the T7 and Sp6 promoters to prime the synthesis reaction. Two restriction digests of the plasmid were set up and the cut plasmids then purified using Geneclean II. The synthesis reaction was set up in a 20µl reaction containing 2µl digoxigenin (DIG) labelling mix (Boehringer Mannheim), 3 µl cut plasmid DNA, 10mM DTT, 60U RNasin (Pharmacia), 60U RNA polymerase in 1x appropriate buffer. The reaction was allowed to proceed for 3 hours at 37^0C then terminated by adding 80µl DEPC-water. 20µg glycogen was then added and the probe was ethanol precipitated for 1 hour at -20^0C. RNA was pelleted at high speed for 15 minutes, washed twice in 75% ethanol resuspended in DEPC-water and stored at -70^0C.

2B.10.3 Hybridization

Sections were dewaxed in xylene for 10 minutes then rehydrated through graded alcohols. Slides were then washed in DEPC-PBS for 5 minutes then transferred to 0.2N HCl for 15 minutes followed by incubation in 0.3% Triton-X for 15 minutes. Slides were rinsed then incubated in proteinase K solution for 5-20 minutes. After a brief rinse, slides were fixed in 4% paraformaldehyde, rinsed then prehybridized for 1-2 hours in 2xSSPE (Appendix I)/ 50% formamide. Riboprobes were diluted in hybridization buffer (1mM Tris, 1x Denhardts, 2x SSPE, 50% formamide, 0.5% SDS, 0.5% dextran sulphate, 0.25mg/ml salmon sperm DNA), heated to 85^0C for 4 minutes then cooled on ice. 30µl was then added to each slide, a coverslip applied and hybridization carried out overnight at 42^0C.

Following hybridization, coverslips were removed in 4x SSPE then slides washed in 2x SSPE for 30 minutes at 20^0C, 0.1x SSPE for 10 minutes at 20^0C, 0.1x SSPE for

30 minutes at 50^{0}C, 0.1x SSPE for 20 minutes at 20^{0}C. Slides were washed in DIG buffer 1 (Appendix I) for 5 minutes then DIG antisera (10% normal rat serum, 1:2000 anti-digoxigenin-Fab fragments (Boehringer Mannheim) in DIG buffer 1) applied for 2 hours at 20^{0}C. Slides were washed in DIG buffer 1 2x 15 minutes then DIG buffer 3 (Appendix I) 1x 5 minutes before applying substrate for colour development (NBT, X-phosphate, levamisole). Colour development was allowed to proceed for up to 2 hours then slides were rinsed in PBS, water and mounted in crystalmount (Biogenesis).

2B.11 Generation of cDNA Library

2B.11.1 cDNA synthesis

mRNA was prepared as described in Section 2B.1. cDNA synthesis was carried out using a cDNA synthesis kit (Pharmacia) according to manufacturer's instructions using 5µg mRNA as a substrate for first and second strand cDNA synthesis. Double stranded cDNA was purified on a S-300 column and BstXI adapters (Invitrogen) were ligated overnight at 12^{0}C followed by a second S-300 purification. Following these proceedures, 100µl of purified, adaptor ligated library was obtained. This was stored at -20^{0}C whilst the results of test ligations were obtained (Section 2B.11.3)

2B.11.2 Preparation of vector

INVαF' cells were transformed with the vector pCDNA3 and resultant colonies grown overnight in LB/ ampicillin as previously described. Plasmid DNA was purified using Qiagen mini plasmid kit columns (Qiagen Ltd) then digested using the enzyme BstXI overnight at 45^{0}C. Digested plasmid was ethanol precipitated, resuspended in phosphatase buffer and treated with phosphatase (Shrimp alkaline phosphatase, Gibco BRL) for 1 hour at 37^{0}C. Phosphatase was inactivated by heating the reaction for 15 minutes at 65^{0}C. Digested, phosphatased plasmid was purified using Geneclean II and adjusted to a concentration of 50µgml^{-1}.

2B.11.3 Library ligation and purification

Test ligations using the double stranded adaptor ligated DNA described above were set up using 0.1μg of vector and varying quantities of column effluent. A random selection of colonies were grown up in overnight culture and analysed to establish the size range of library inserts. Based on colony numbers obtained following transformation of competent cells with a percentage of the ligation reaction, a large scale ligation was set up as follows. 1μg of prepared vector was combined with 50μl column effluent in a total volume of 70μl ligation buffer (66mM Tris-HCl pH7.6, 1mM spermidine, 10mM $MgCl_2$, 15mM DTT, 0.2mg/ml DNase-free BSA). 100 units of T4 ligase was added and the reaction allowed to proceed overnight at 12^0C. A total of one third of the ligation reaction was used to transform supercompetent DH5α cells which were plated onto 10cm LB/ampicillin/agar plates and incubated overnight at 37^0C. Colonies were removed by the addition of prewarmed LB and scraping with an L-shaped glass spreader. Cells were then pelleted and plasmid DNA extracted using the Qiagen Maxi -plasmid kit (Qiagen Ltd).

2B.12 Library Expression and Screening

2B.12.1 Transfection of COS cells

COS 7 cells were used for the transfection and were cultured in DME (Appendix II), 10% FCS, 200mM L-Glu, 100U ml^{-1} penicillin-streptomycin. Cells were plated out the night before use at 2 x 10^6 cells per 80cm^2 flask. The following day, flasks were washed in 2 x 25 ml DMEM and 5ml transfection mix consisting of 2mg DEAE-dextran, 0.1mM chloroquine, 20μg purified DNA in 5ml DMEM was added. After incubation for 3 hours at 37^0C, the transfection mix was poured off and flasks washed in 25ml PBS. 5ml 10% DMSO in PBS was added and left on the cells for 2 minutes at 37^0C. Flasks were washed twice in 25 ml PBS and incubated overnight in 30ml culture medium. The following day, cells were trypsinised and transferred to new flasks, grown for 1 more day and harvested for Dynal bead selection (see below).

2B.12.2 Magnetic separation of transfected cells

Cells were harvested from flasks by incubating in 0.02% EDTA pH7.2 (prewarmed) at 37^{0}C for 30 minutes. Cells were pelleted at 400 x g for 5 minutes, resuspended in 1ml of mAb tissue culture supernatant and incubated on ice for 1 hour. Cells were pelleted at 400 x g (4^{0}C), washed twice in cold PBS/0.1% BSA and resuspended in 1ml PBS. Washed cells were incubated with washed Dynal beads (Dynabeads M-450, Dynal) at 2x10^7 beads per ml of sample for 30 minutes at 4^{0}C. Positive cells were selected against a magnet (Dynal MPC), washed four times in cold PBS and transferred to an eppendorf tube.

2B.12.3 Preparation of plasmid DNA

Plasmid DNA from positively selected cells was prepared as follows. Excess PBS was removed from the beads/cell mixture using the magnet. 400µl 0.6% SDS, 10mM EDTA was added, the solution vortexed and incubated at 20^{0}C for 30 minutes. 100µl 5M NaCl was added, the solution vortexed and incubated at 4^{0}C overnight. Following centrifugation at 13000g for 5 minutes, the supernatant was removed and phenol chloroform extracted twice. 0.02µg tRNA was added as a carrier and the DNA ethanol precipitated, resuspended in 100µl TE and ethanol precipitated a second time. The pellet was finally resuspended in 20µl TE and stored at -20^{0}C.

2B.12.4 Preparation of spheroplasts

500µl MC1063/p3 *E. Coli* were transformed with 5µl of HIRT DNA by incubating on ice for 30 minutes followed by a 5 minute 'heat-shock' at 37^{0}C. Transformed bacteria were grown for 1 hour in LB then plated onto LB/agar plates containing 50µg/ml ampicillin and incubated overnight at 37^{0}C. The following day, a solution of LB/ampicillin (50µg ml^{-1}) was used to create a slurry of bacterial clones using an L-shaped glass spreader. The bacteria were grown in liquid culture (LB/amp) from OD_{600} = 0.1to OD_{600}=0.7. At this stage chloramphenicol was added to 150µg/ml and cultures continued for a further 16 hours. 100ml of this overnight culture was

centrifuged at 3000 x g for 10 minutes at 4^0C and the pellet resuspended in 5ml 20% sucrose/ 50mM Tris-HCl pH8 at 4^0C. 1ml of 5mg ml^{-1} lysozyme in 0.25M Tris-HCl pH8 was added and incubated on ice for 5 minutes then 2ml of 2.5M EDTA added followed by a further 5 minute incubation on ice. 2ml of 50mM Tris-HCl pH8 was added and incubated at 37^0C for 5 minutes. The solution was placed on ice, conversion to spheroplasts checked under light microscopy and 20ml prewarmed DME/ 10% sucrose/ 10mM $MgCl_2$ added dropwise with frequent inversion then incubated at 20^0C for 20 minutes.

2B.12.5 Transfection of COS cells with spheroplasts

COS cells were plated out the night before at $1x10^6$ cells per 3ml DME/10% FCS/ 200mM L-Glu, 100U ml^{-1} penicillin-streptomycin in 6cm diameter tissue culture petri plates (Falcon 3004, Becton Dickinson, Plymouth, U.K.). Media was removed the following day and 3ml of spheroplast suspension added to each dish. Dishes were taped up with 'Parafilm' (American National Can, Greenwich) and spun at 500 x g for 10 minutes with no brake setting. Fluid was aspirated and 1.5ml of a 50% (w/v) solution of polyethylene glycol (PEG) at room temperature was added slowly to the centre of each dish. After 1 minute, PEG was washed off with 3 x 3ml of DME and cells were incubated for 4 hours at 37^0C in DME/ 10% FCS/ 15$\mu g ml^{-1}$ gentamycin sulphate. Debris was washed off and cells incubated for 48hrs in DME/ 10% FCS/ 200mM L-Glu/ 15μg ml^{-1} gentamycin sulphate prior to harvesting by incubating in prewarmed EDTA pH 7.2 for 15 minutes at 37^0C and Dynabead selection as described in section 2B.12.2.

2B.13 Transfection of C127 Cells

2B.13.1 Transfection

C127 cells were grown in DME, 10% FCS, 200mM L-Glu, 100U ml^{-1} penicillin-streptomycin (DME 10). Cells were seeded into 6 well plates at 5×10^5 cells per well and transfected the next day (70% confluent). Transfection was carried out using DOTAP Transfection Reagent (N-[1-(2,3-dioleoyloxy) propyl] -N,N,N-trimethyl-

ammoniummethylsulphate - Boehringer Mannheim) as follows. For each transfection, 5μg of DNA and 60μl of DOTAP were each made to 100μl in PBS, mixed together and incubated at 20°C for 10 minutes. 3ml of medium was then added to the DOTAP/ DNA mixture and the cells cultured in this transfection mixture overnight. The following day, this was removed and replaced with medium containing the selecting agent (see below). Cells were grown for a further 3 days then harvested and seeded at 3 different concentrations in 6 well plates (10^3, 10^4, 10^5 cells per well).

2B.13.2 Selection of transfected cells

Since the transfecting DNA was contained within the vector pCDNA3 which contains the ampicillin resistance gene, transfected cells were selected using the antibiotic geneticin disulphate (G418, Gibco-BRL). Cells were maintained at all times in DME 10 plus G418 at a final concentration of 0.5mg ml^{-1}. Medium was changed every 3 days and transfected cells were identified as individual clones appearing after approximately one week of culture. Once individual clones were identified, they were isolated and transferred to 24 well plates then cultured as normal.

2B.13.3 Analysis of transfected cells

Once cells had grown to confluence in T175 flasks, they were removed for FACS analysis or preparation of cytospins. Medium was removed, cells rinsed in versene then incubated in EDTA pH7.2 at 37°C until cells detached. Cells were then washed in FACS buffer and analysed as described in section 2A.3. For preparation of RNA, cells were washed in cold PBS then an appropriate amount of RNAzol added directly to the flask.

2B.14 Southern Blotting

2B.14.1 Blotting

Agarose gels were run as described in section 2B.4. Nucleic acids were blotted onto Hybond N^+ (Amersham International) using overnight capillary transfer in a buffer of 0.4N NaOH. Following blotting, the membrane was rinsed in 2xSSC (Appendix I) then used for hybridization.

2B.14.2 Generation of radiolabelled probes

DNA oligonucleotides to be used as probes were labelled with [γ^{32}P]ATP (Amersham International). The labelling reaction consisted of 10pmol DNA, 3ml [γ^{32}P]ATP, 50mM Tris-HCl pH7.6, 10mM MgCl2, 10mM 2-mercaptoethanol, 0.6u T4 polynucleotide kinase (United States Biochemical Corporation). The mixture was incubated at 37^0C for 30 minutes then 65^0C for 5 minutes.

2B.14.3 Hybridization

Membranes were prehybridised at 50^0C for four hours in 0.5% dried milk, 0.02M $NaPO_4$ pH7, 2.85N SSC, 1mM EDTA, 0.1mgml^{-1} yeast RNA, 1% SDS. The labelled oligonucleotide(s) were added and hybridization carried out overnight at 50^0C.

Membranes were washed in 1xSSC twice for 15 minutes each at 22^0C then in 0.1xSSC for 15 minutes at 52^0C. This wash was repeated until background signal was reduced. Membranes were dried and exposed to X-ray film (X-Omat TR, Kodak) for 1-4 hours.

CHAPTER 3

EXPRESSION OF OVINE CD1

3.1 Introduction

In humans, five CD1 genes exist for which four protein products have been described (CD1a, CD1b, CD1c and CD1d). The CD1a-c isotypes have a broadly similar distribution being present on cortical thymocytes, dendritic cells, Langerhans' cells (CD1a and CD1c) and a subpopulation of B cells (CD1c). In contrast, CD1d has a much wider distribution with expression on epithelial cells, vascular smooth muscle and parenchymal cells. In particular, expression on intestinal epithelial cells and hepatocytes is a prominent feature of the CD1d isotype (Canchis et al. 1993).

In the sheep, previous studies have shown that the majority of mAbs form a distinct cluster consistent with recognition of the ovine homologue of human CD1b (Dutia and Hopkins, 1991). The mAbs which have been shown to constitute this cluster are CC13, CC14, CC20, CC40, CC90, CC132, CC122, TH97A and VPM5 (Hopkins and Dutia, 1991; Hopkins et al. 1993). Transfection of COS cells with cDNA clones encoding human CD1 molecules has shown that CC20 recognises a homologue of human CD1b (Howard et al. 1993b). The mAb, SBU-T6, has been shown to have a wider tissue distribution (Mackay et al. 1985; Dutia and Hopkins, 1991). As such, this mAb has previously been considered to be specific for CD1c or reactive to all CD1 isoforms (Dutia and Hopkins, 1991).

In cattle, two additional mAbs, CC43 and CC118 have recently been described (Howard et al. 1993a). These recognise a molecule in cattle with a much wider tissue distribution than previously described for CD1 mAbs in this species. In addition, these mAbs immunoprecipitate a 43kd α chain compared to the 46kd α chain recognised by the CD1b specific mAbs and SBU-T6.

In cattle, the mAbs which show CD1b-like reactivity have been clustered as BoCD1w2, SBU-T6 as BoCD1w1 and CC43 and CC118 as BoCD1w3 (Howard and Naessens, 1993).

In order to clarify the situation in the sheep with the previously studied mAbs and characterize the mAbs CC43 and CC118, immunohistology was carried out on a variety of tissues together with FACS analysis of blood, lymph (afferent and efferent), and thymocytes. Although in most cases staining was carried out using all the mAbs, CC20 was taken as representative of the CD1b mAbs due to availability and the fact that it has been characterized by molecular means (Howard et al. 1993b). Following studies on normal tissues, immunohistology was also carried out on sections of gut from sheep infected with *Mycobacterium paratuberculosis*. Briefly, infection with this organism can result in chronic disease of intestinal and lymphoid tissues termed paratuberculosis or Johne's disease. The disease can be classified as 'tuberculoid' or 'lepromatous' according to the major lesions present. The former is characterized by the infiltration of many lymphoid cells plus scattered focal aggregates of macrophages with low mycobacterial load; the latter by heavy infiltration of macrophages packed with mycobacteria. The reason this work was carried out was to investigate whether infection with this bacterium would result in any detectable upregulation of CD1 expression. This was considered a possibility because of the recent evidence that CD1 is capable of presenting mycobacterial mycolic acids and lipoarabinomannan to T cells (Beckman et al. 1994, Sieling et al. 1995).

3.2Results

3.2.1 Immunohistology

All the mAbs used demonstrated the same pattern of staining within the thymus i.e. strong staining of cortical thymocytes and isolated dendritic cells (DCs) within the medulla. This staining is characteristic of CD1 a-c isotypes and is shown in Figure 3.1.

Within the skin, all the mAbs stain DCs in the dermis (Figure 3.2). In addition, the mAb SBU-T6 also stains Langerhans' cells (LCs) within the epidermis (Figure 3.2a and 3.2b). As shown in Figure 3.2c, CC118 stains DCs but not LCs. CC43 exhibited the same staining pattern as CC118.

In peripheral lymphoid organs, the mAbs SBU-T6, CC43 and CC118 demonstrate an identical pattern of reactivity. In the spleen, there is staining of mantle zone B cells in secondary follicles and also cells in the marginal zone and scattered red pulp constituents (Figure 3.3). Within lymph nodes, there is a marked staining of cells within the follicular mantle (Figure 3.4a) in addition to paracortical dendritic cells (Figure 3.4b). The CD1b mAbs fail to stain B cells, however they do stain subcapsular and paracortical dendritic cells. The staining characteristic of all the CD1b mAbs is shown in Figure 3.4c which demonstrates staining of lymph node DCs by CC20.

In the liver, SBU-T6, CC43 and CC118 also demonstrate an identical pattern of reactivity and strongly stain Kupffer cells within the parenchyma (Figure 3.4d).

Within the intestine, the mAbs SBU-T6, CC43 and CC118 again form a cluster with consistent staining of lamina propria macrophages (Figure 3.5a and 3.5b). No staining of intestinal intraepithelial lymphocytes is observed with these mAbs. In addition and consistent with the pattern of staining seen in spleen and lymph node, these 3 mAbs also stain B cells in the dome region of small intestinal Peyers patches and colonic lymphoid nodules (Figure 3.5c and 3.5d). Only very weak staining is evident within the centre of the lymphoid tissue (F). No reactivity was observed using the CD1b specific mAbs on intestinal tissue from 6-12 month old animals. However, studies using tissue from late foetal lambs revealed a consistent pattern of reactivity of these mAbs with crypt epithelial cells. Figure 3.6a and 3.6b demonstrate this staining which is in contrast to that seen with SBU-T6 which fails to stain the epithelial cells but stains lamina propria macrophages as described above (Figure 3.6c). Additional studies using foetal tissue demonstrated the presence of a network of CD1 positive dendritic cells within the ileal peyers patches of the intestine recognised by SBU-T6, CC43 and CC118. No positive lymphocytes were observed in this region (Figure 3.7a and 3.7b). The significance of these results is discussed below.

Within the central nervous system, staining of microglial cells was observed with SBU-T6 and CC118. Figure 3.8 shows staining of these cells in the cerebellar

medulla with CC118 (3.8a) and SBU-T6 (3.8b). No staining was seen in this tissue using the CD1b mAbs.

Immunostaining of cytosmears of afferent lymph and mammary macrophages was also carried out. The results are shown in Figure 3.9. Figure 3.9a shows afferent lymph DCs staining with SBU-T6, Figure 3.9b with CC20. Figure 3.9c shows mammary macrophages stained with SBU-T6 and developed using an alkaline phosphatase system (this section was obtained from Dr. P. Berthon).

CD1 expression in disease

Sections from *M.paratuberculosis* infected sheep were also stained with a range of mAbs. Sections from lesions characterized as either tuberculoid or lepromatous were examined. Figure 3.10 shows the results of staining sections from a case of lepromatous paratuberculosis with group 1 and group 2 mAbs. Figure 3.10a and 3.10b show staining of lymphoid cell aggregates by the mAbs CC118 (a) and SBU-T6 (b). It is evident from Figure 3.10a that, as in lymph node and spleen germinal centres, there is regulation of CD1 expression within the aggregate of lymphoid cells as demonstrated by the crescent shaped region in the stained area in Figure 3.10a. Figure 3.10c shows staining of isolated clusters of cells by the mAb CC20 in lamina propria lymphoid tissue. Figure 3.10d is a higher magnification (x400) which demonstrates clearly that the positive cells are arranged in isolated clusters.

Figure 3.11 shows the results of staining sections from a case of tuberculoid paratuberculosis with group 1 and group 2 mAbs. Figure 3.11a and 3.11b show intense staining of lamina propria macrophages by the mAbs CC118 (a) and SBU-T6 (b). Figures 3.11c and 3.11d show intracytoplasmic staining of intestinal epithelial cells by the mAb CC20.

The results of immunohistology are summarized in Table 3.1 with the CD1b type mAbs being assigned to group 1 and SBU-T6, CC43 and CC118 assigned to group 2. Group 2 is further subdivided into group 2a (SBU-T6) and group 2b (CC43 and CC118).

FIGURE 3.1

Immunoperoxidase stained sections of frozen thymus showing cortex (**C**) and medulla (**M**). Mabs (a) CC118, (b) SBU-T6, (c) CC20 and (d) CC40 all demonstrate strong staining of cortical thymocytes and isolated dendritic cells within the thymic medulla (**DC**).

Magnification a, b, c - x400.

Magnification d - x100.

FIGURE 3.1

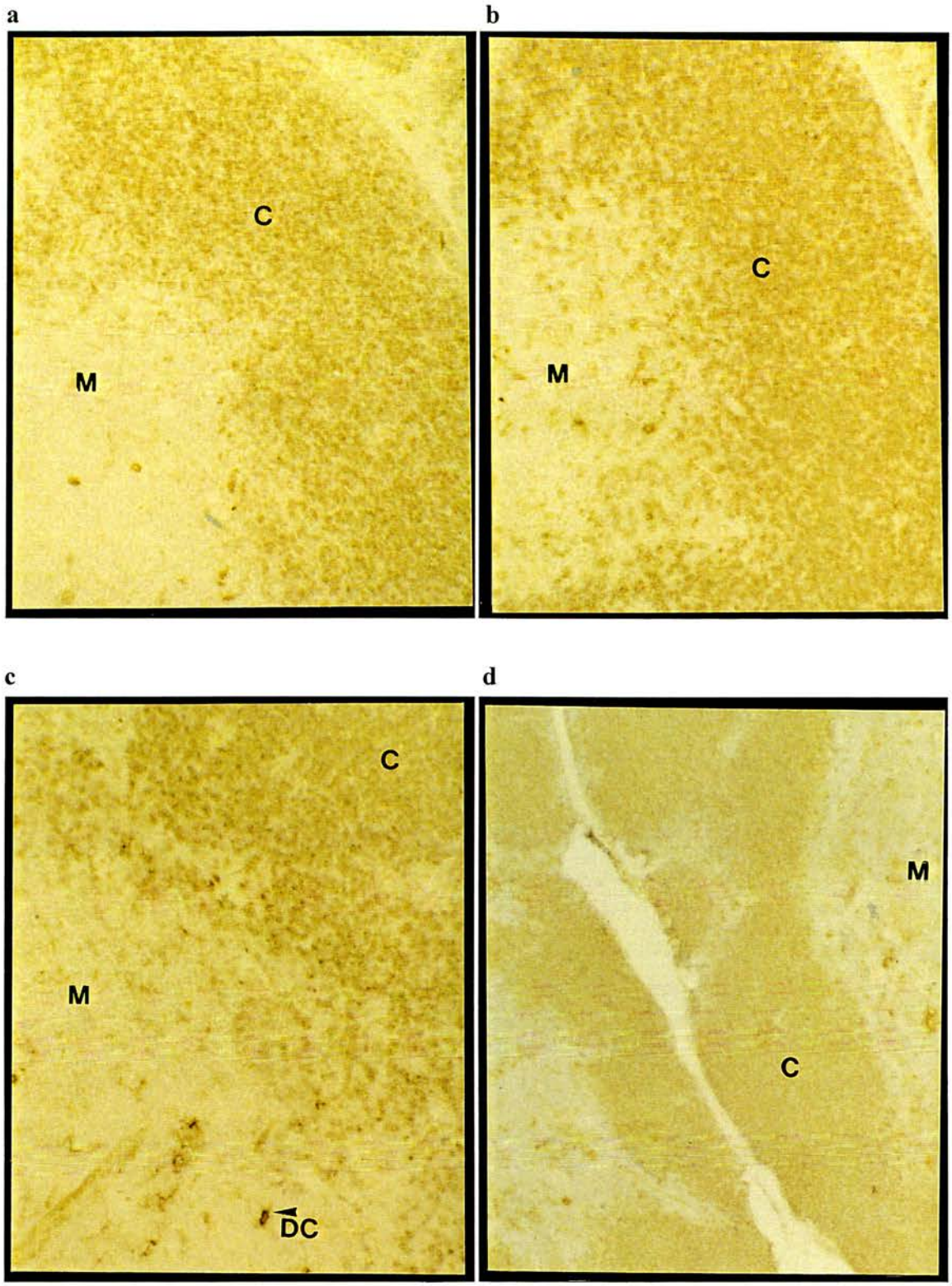


FIGURE 3.2

Immunoperoxidase stained sections of frozen mammary skin.

SBU-T6 (a and b) shows staining of epidermal Langerhans' cells (arrowheads) and dermal dendritic cells (arrows).

CC118 (c) and VPM5 (d) show staining of dermal dendritic cells only (arrowheads).

Magnification x400.

FIGURE 3.2

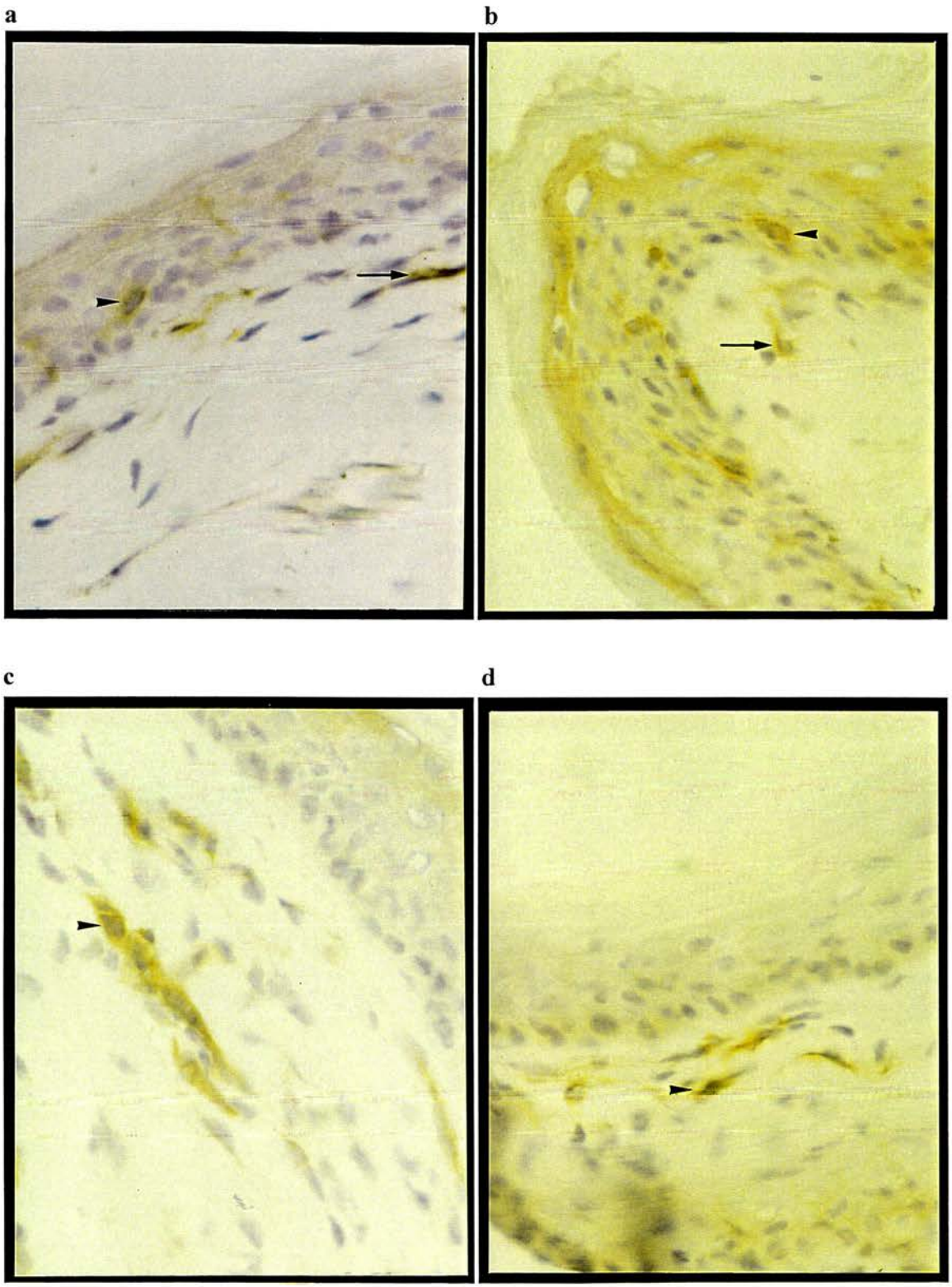


FIGURE 3.3

Immunoperoxidase stained sections of frozen spleen showing red pulp (**RP**), white pulp (**WP**) and marginal zone (**MZ**).

SBU-T6 (a), CC118 (b) and CC43 (c) show staining of mantle zone B cells within the germinal centre (arrowheads) and scattered red pulp constituents.

Magnification a, b - x250.

Magnification c - x400.

FIGURE 3.3

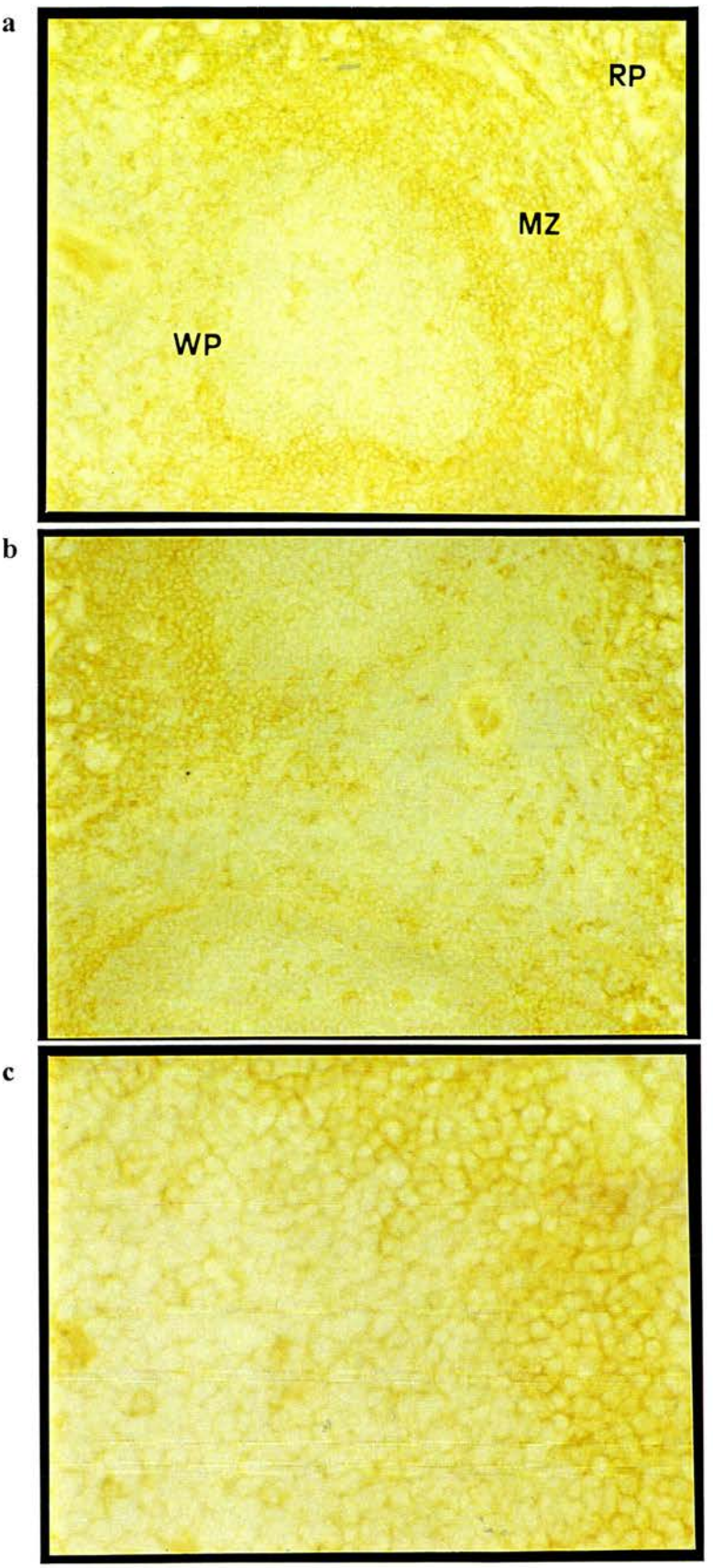


FIGURE 3.4

Immunoperoxidase stained sections of frozen lymph node (a, b and c) and liver (d).

(a) CC118 showing staining restricted to mantle zone B cells (**MZ**) within the germinal centre. Only isolated cells are stained within the follicle (**F**). Magnification x100.

(b) SBU-T6 showing staining of dendritic cells within the lymph node paracortex (arrowheads). Magnification x250.

(c) CC20 showing staining of subcapsular and paracortical dendritic cells (arrowheads). Magnification x250.

(d) SBU-T6 showing staining of Kupffer cells within the hepatic parenchyma (arrowhead). Magnification x400.

FIGURE 3.4

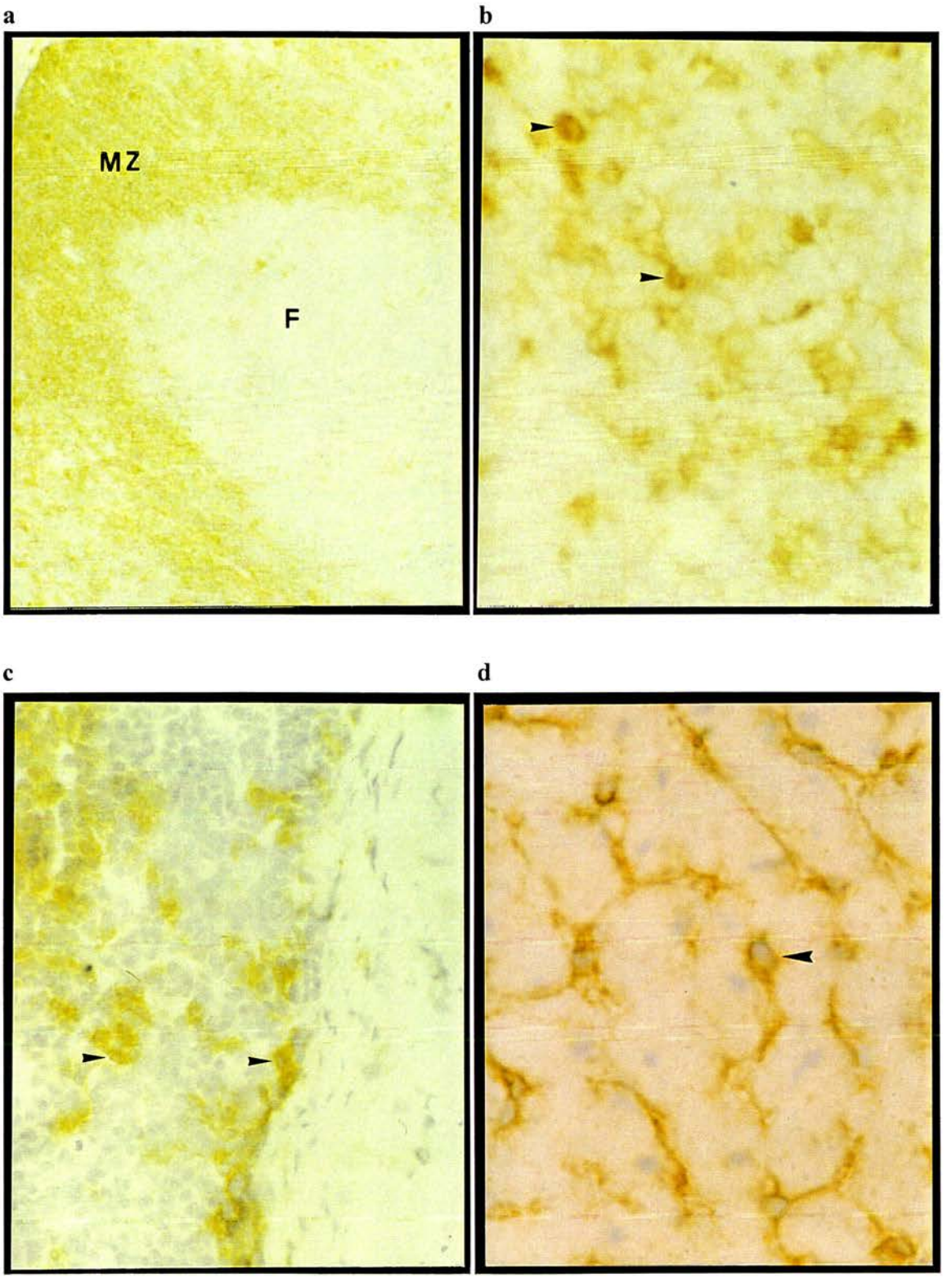


FIGURE 3.5

Immunoperoxidase stained sections of frozen small intestine (a and b), jejunal Peyers patch (c) and colonic lymphatic tissue (d).

(a) CC118 and (b) SBU-T6 show intense staining of lamina propria macrophages (arrowheads). Magnification x250.

(c) SBU-T6 staining lymphocytes in the dome region of the Peyers patch (**PP**) (arrowhead). Magnification x100.

(d) CC118 staining of dome region lymphocytes within a follicle (**F**) in colonic lymphatic tissue. Magnification x100.

FIGURE 3.5

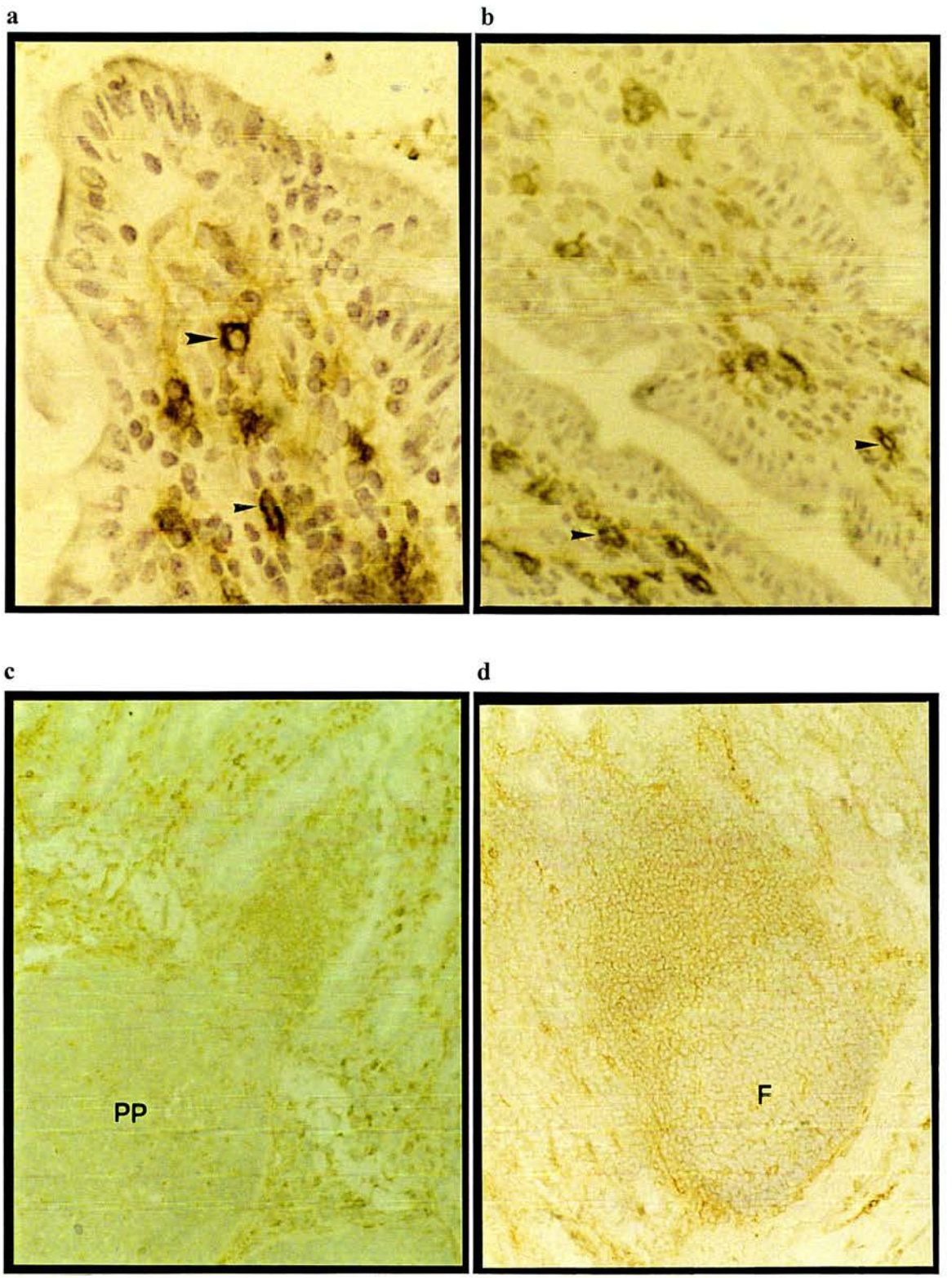


FIGURE 3.6

Immunoperoxidase stained sections of frozen small intestine from late foetal lamb.

(a) CC20 and (b) CC14 show staining of cells lining the intestinal crypts.

(c) SBU-T6 shows staining of lamina propria macrophages.

(d) VPM53 negative control.

Magnification x400.

FIGURE 3.6

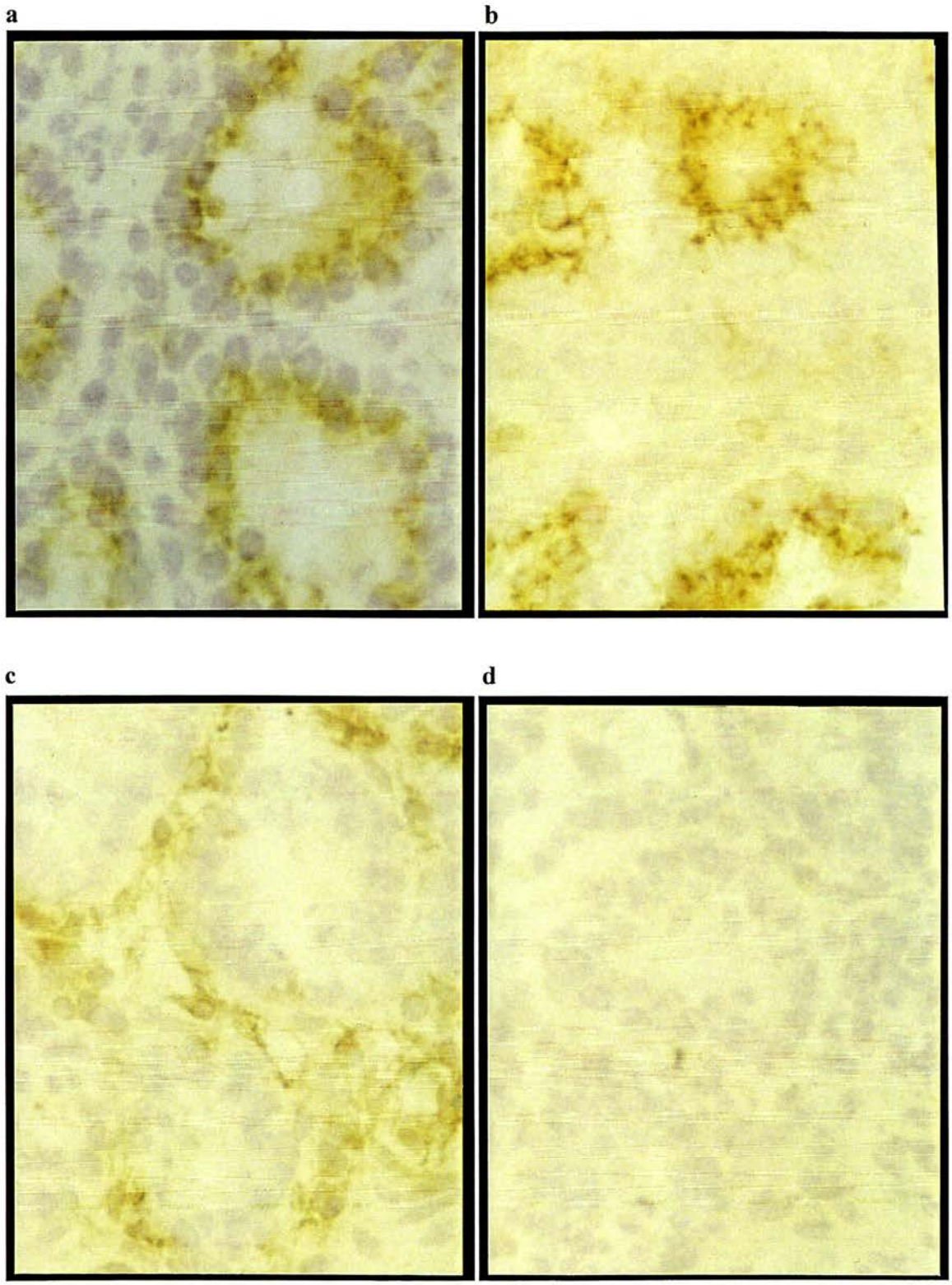


FIGURE 3.7

Immunoperoxidase stained sections of frozen small intestine from late foetal lamb showing ileal Peyers patches.

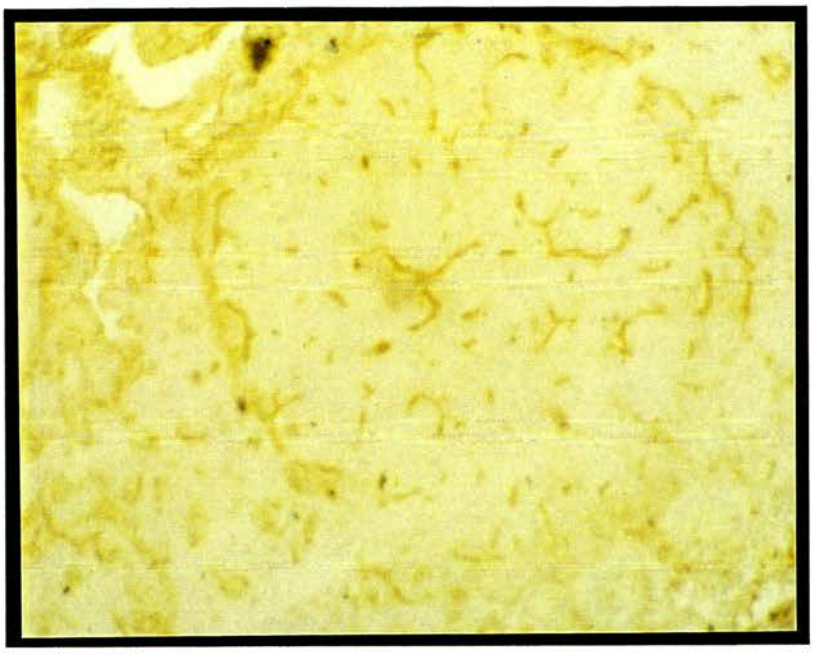
SBU-T6 (a) and CC20 (b) show staining of dendritic cells within the lymphoid tissue.

Magnification (a) - x250.

Magnification (b) - x400.

FIGURE 3.7

a



b

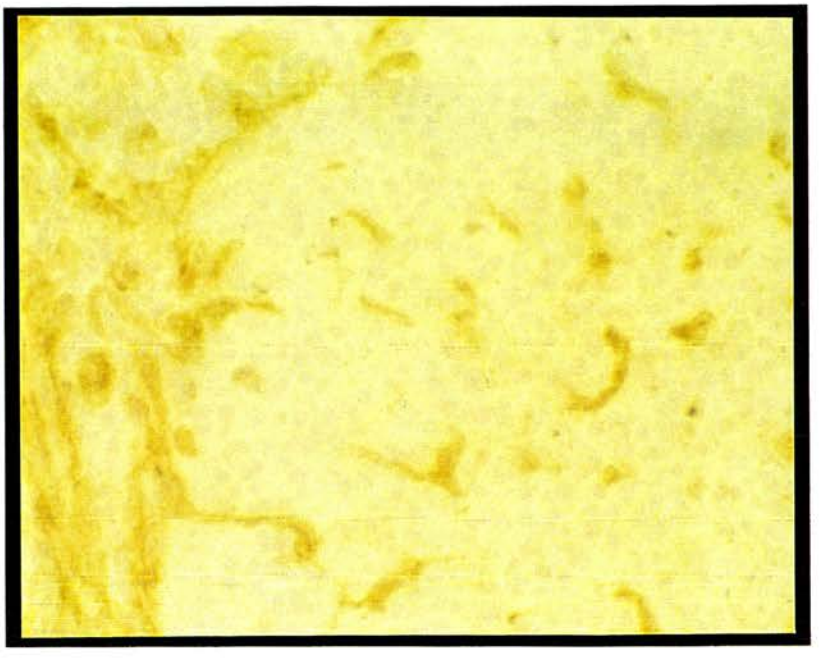


FIGURE 3.8

Immunoperoxidase stained sections of frozen cerebellum.

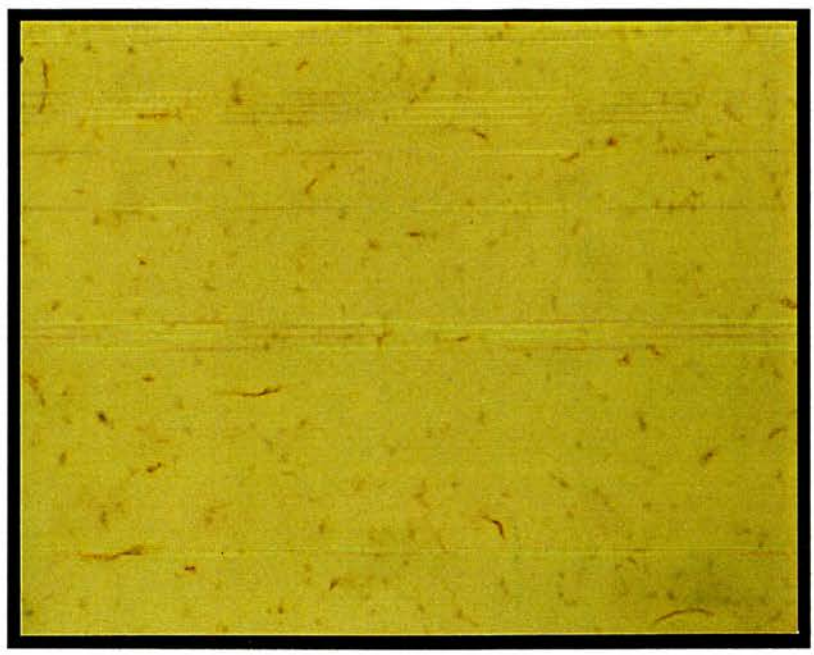
(a) SBU-T6 and (b) CC118 show staining of microglial cells within the medulla (arrowheads).

Magnification (a) - x100.

Magnification (b) - x400.

FIGURE 3.8

a



b

FIGURE 3.9

Immunostained cytosmears of afferent lymph dendritic cells (ADCs) (a and b) and mammary macrophages (c).

(a) SBU-T6 and (b) CC20 staining of ADCs.

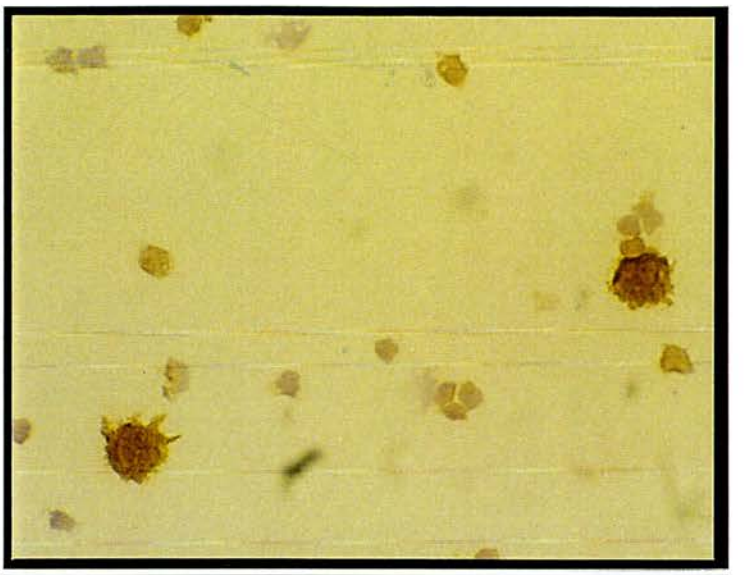
(c) SBU-T6 staining of mammary macrophages.

Magnification (a) - x250.

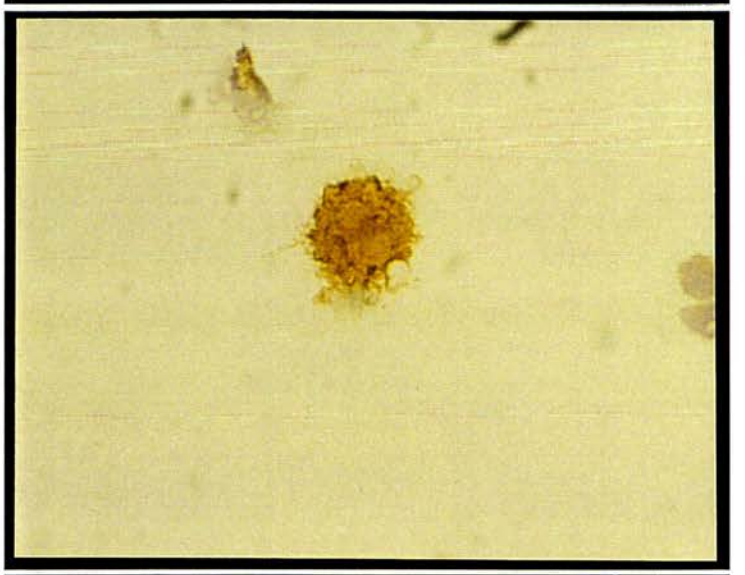
Magnification (b) and (c) - x400.

FIGURE 3.9

a



b



c

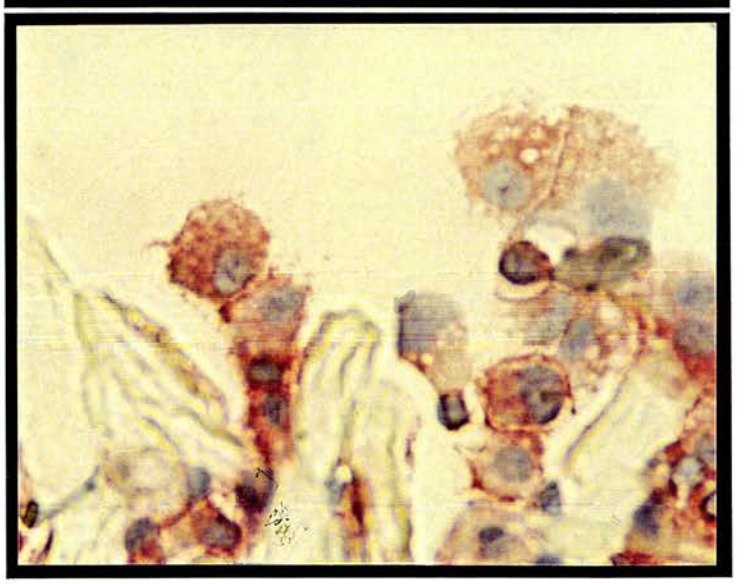


FIGURE 3.10

Immunostained sections of frozen small intestine from a sheep with lepromatous paratuberculosis.

(a) CC118 and (b) SBU-T6 show staining of lymphoid cell aggregates within follicles in the lamina propria.

(c) and (d) CC14 show staining of focal clusters of cells within lymphoid aggregates.

Magnification (a), (b) and (c) - x100.

Magnification (d) - x400.

FIGURE 3.10

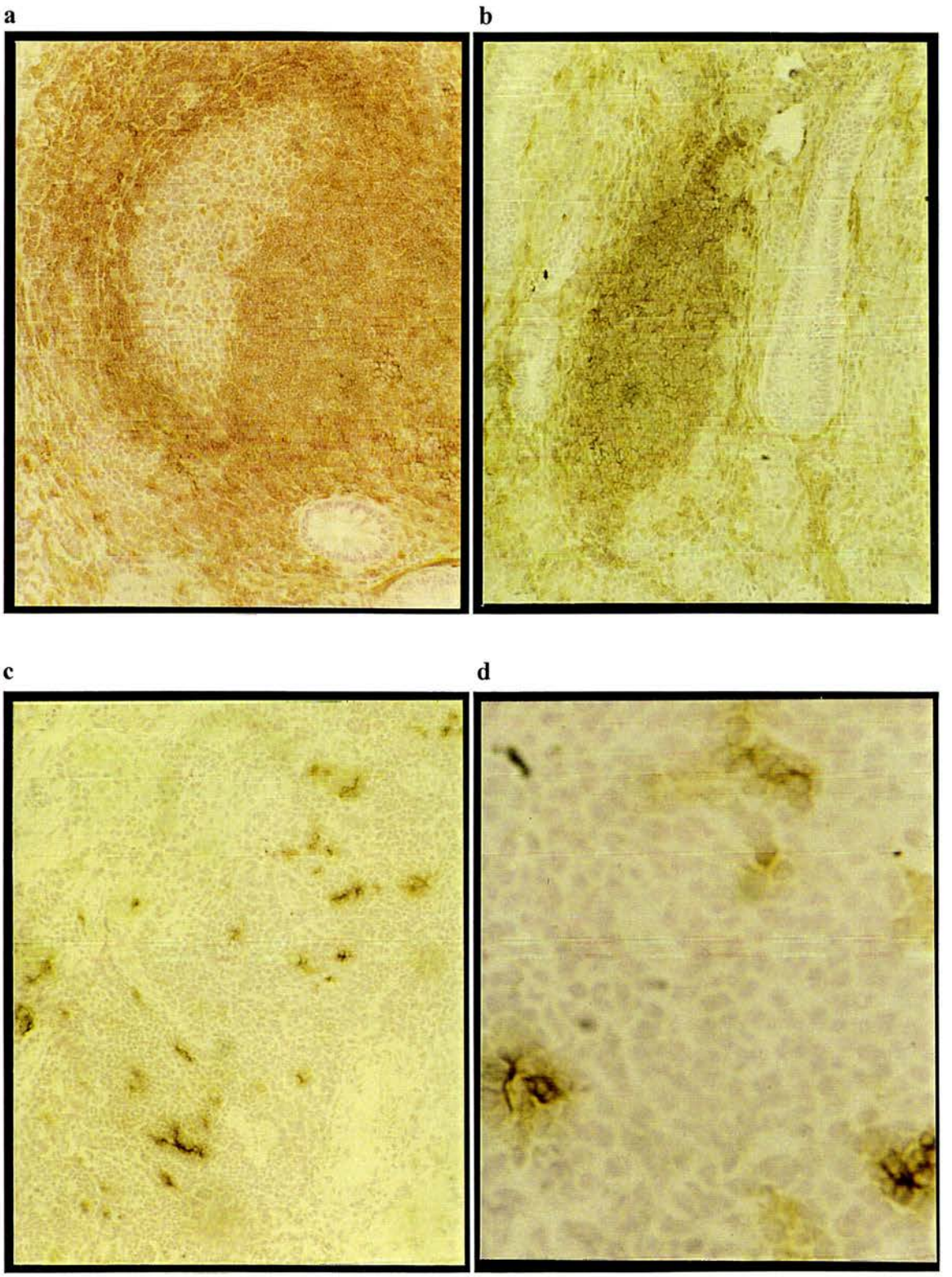


FIGURE 3.11

Immunostained sections of frozen small intestine from a sheep with tuberculoid paratuberculosis.

CC118 (a) and SBU-T6 (b) show intense staining of lamina propria macrophages.

CC14 (c) and (d) shows staining on the apical surface of villous epithelial cells (c) and within crypts (d).

Magnification - x250.

FIGURE 3.11

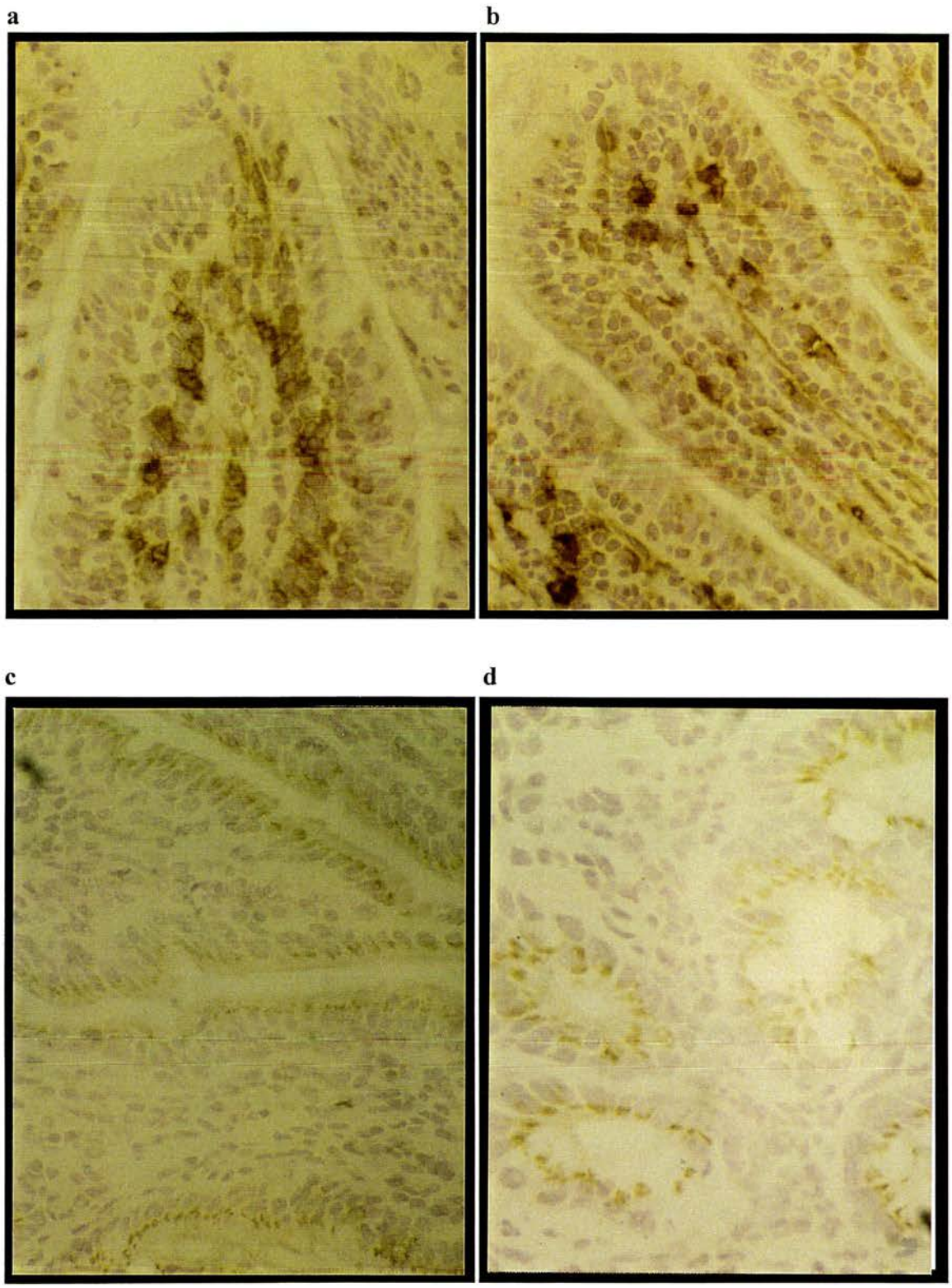


TABLE 3.1

Table summarising the results of immunostaining using anti-CD1 mAbs. On the basis of this staining, the mAbs are divided into:

Group 1 (CC13, CC14, CC20, CC40, CC90, CC132, CC122, TH97A and VPM5).

Group 2a (SBU-T6).

Group 2b (CC43, CC118).

TABLE 3.2

Table summarising the results of FACS analysis using anti-CD1 mAbs. The mAbs are grouped as above.

TABLE 3.1

TISSUE		GROUP 1	GROUP 2a	GROUP 2b
SKIN	Dermal dendritic cells	+	+	+
	Epidermal Langerhans cells	-	+	-
THYMUS	Cortical thymocytes	+	+	+
	Medullary dendritic cells	+	+	+
LYMPH NODE	Paracortical dendritic cells	+	+	+
	B cells	-	+	+
SPLEEN	White pulp dendritic cells	+	+	+
	Marginal zone B cells	-	+	+
	Red Pulp Macrophages	-	+	+
INTESTINE	Lamina propria macrophages	-	+	+
	Crypt epithelium	+(AGE RELATED)	-	-
LIVER	Kupffer cells	-	+	+
CNS	Brain, spinal cord	-	+	+

TABLE 3.2

CELL TYPE		GROUP 1	GROUP 2a	GROUP 2b
THYMOCYTES		+	+	+ (LOWER INTENSITY)
PERIPHERAL BLOOD	B cells	-	+	+
	Monocytes	-	+	+
EFFERENT LYMPH	B cells	-	+	+
AFFERENT LYMPH	B cells	-	+	+
	Dendritic cells	+	+	+ (LOWER INTENSITY)

3.2.2 Flow Cytometry

Flow cytometry was carried out on resting cell populations to establish the location of constitutive CD1 expression as recognised by the group 1 and group 2 mAbs.

Thymocytes

The results of flow cytometry analysis using group 1 and group 2 mAbs on thymocytes are shown in Figure 3.12. All the group 1 mAbs and SBU-T6 consistently stain 70% of thymocytes. In contrast, CC43 and CC118 stain 35% of thymocytes with a lower intensity.

CD1 has been shown in other species to exhibit reciprocal expression to MHC class I within the thymus. In order to confirm this reciprocal expression within sheep thymus, double staining was carried out using SBU-T6 and VPM19 (MHC class I specific). The result of this experiment is shown in Figure 3.13 and demonstrates the mutually exclusive nature of the thymocyte populations expressing CD1 and MHC class I.

Dendritic Cells

Single staining of afferent lymph dendritic cells with group 1 and group 2 mAbs is shown in Figure 3.14. All mAbs stain approximately 70% of DCs including CC43 and CC118 although these latter mAbs demonstrate a lower intensity of staining.

Peripheral Blood Lymphocytes

Single staining results using gate 1 or gate 2 are shown in Figure 3.15. These results show the increase in staining that occurs when the gate is enlarged to encompass large lymphocytes and monocytes.

Double staining was carried out using the group 2 mAbs in conjunction with B cell markers and a MHC class II marker (VPM46). The results are shown in Figure 3.16 and demonstrate that these antibodies recognise a non-B cell class II positive cell when the larger gate is used. This was further investigated using the mAb VPM65 which recognises the ovine homologue of human CD14 and can thus be used as a

monocyte marker. This demonstrates that the group 2 mAbs recognise the majority of peripheral blood monocytes.

In order to show that these antibodies were not recognising T cells, double staining was carried out using mAbs for CD4, CD8 and γδ T cells. The results are shown in figure 3.17 and confirm the absence of CD1 from these cell populations.

Afferent And Efferent Lymphocytes

Single and double staining results are shown in Figure 3.18 and 3.19. As expected, CC118 and SBU-T6 recognise B cells in afferent and efferent lymph (identical results were obtained using CC43). The apparently lower percentage of cells stained in afferent lymph is a result of the fact that VPM30 stains activated T cells in addition to B cells. To confirm this, double staining was also carried out using the B cell marker DU2104 and SBU-T6. This is shown in Figure 3.19g. This demonstrates that SBU-T6 recognises virtually all B cells in afferent lymph.

It is apparent therefore that the group 1 and group 2 mAbs have distinct patterns of reactivity with cells in blood and lymph which parallels the results obtained by immunostaining. The flow cytometry results also allow division of the group 2 mAbs into group 2a (SBU-T6) which stains thymocytes and dendritic cells in an identical way to the group 1 mAbs and group 2b (CC43 and CC118) which stain thymocytes and dendritic cells at lower intensity and in the case of thymocytes stain a lower percentage (although this is not seen on immunostaining of thymus sections - Figure 3.1a). The flow cytometry results are summarised in Table 3.2.

3.3 Discussion

The results obtained with the group 1 mAbs were to a certain extent expected in that they demonstrated recognition of a molecule consistent with human CD1b i.e. cortical thymocytes and dendritic cells in various tissues. The recognition by these mAbs of CD1 on crypt epithelial cells within the intestine represents the only situation where group 1 mAbs recognise a cell type that the group 2 mAbs do not.

FIGURE 3.12

Flow cytometry analysis of sheep thymocytes.

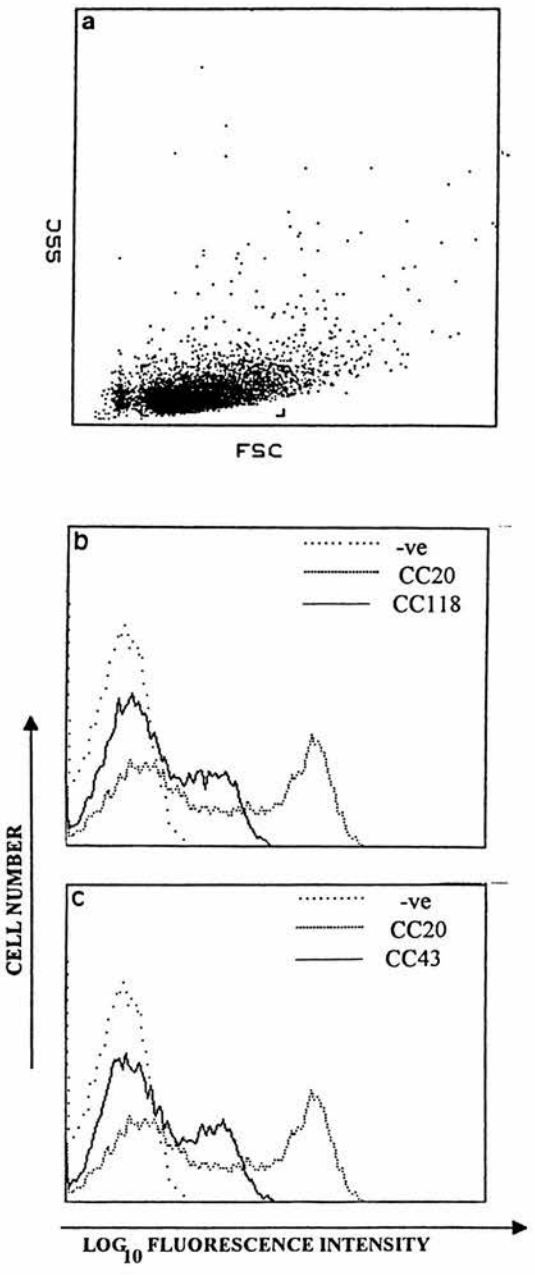
(a) Forward and side scatter profile of thymocytes showing analytical region.

(b) Frequency histograms of thymocytes within the analytical region stained with negative control VPM53 (. . . .), CC20 (.....) and CC118 (____).

(c) Frequency histograms of thymocytes within the analytical region stained with negative control (. . . .), CC20 (.....) and CC43 (____).

(d) Table showing percentage of cells stained when compared to the control and the mean fluoresence intensity of the stained population.

FIGURE 3.12



d

MAB	MFI	% STAINED
CC118	97	36%
CC43	96	38%
CC20	120	71%

FIGURE 3.13

Two colour staining of sheep thymocytes using the analytical region defined in Figure 3.12a.

(a) Negative control dot plot using biotinylated NMS (FL2) and VPM53 (FL1). The negative control was used to set a quadrant as shown.

(b) Dot plot showing VPM19 (Class I) staining on FL1 and SBU-T6 staining on FL2.

FIGURE 3.13

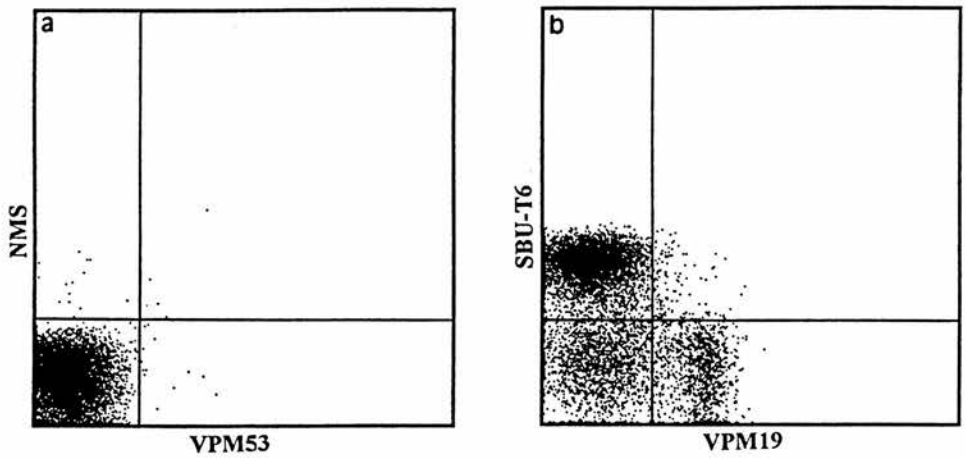


FIGURE 3.14

Flow cytometry analysis of sheep afferent lymph dendritic cells.

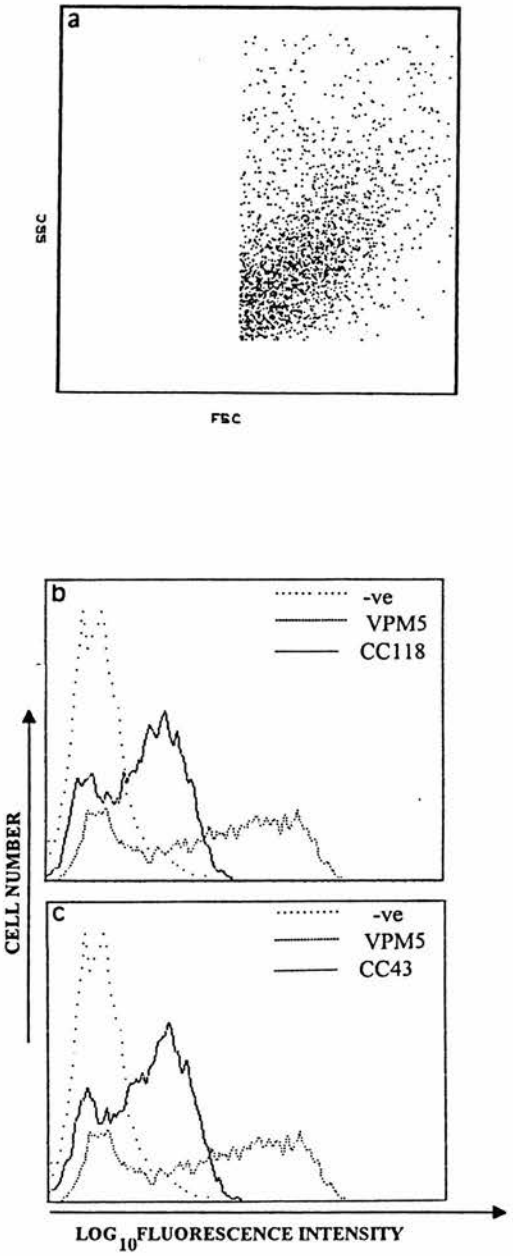
(a) Forward and side scatter profile of dendritic cells within the analytical region.

(b) Frequency histograms of dendritic cells stained with negative control (. . . .), VPM5 (.....) and CC118 (____).

(c) Frequency histograms of dendritic cells stained with negatve control (. . . .), VPM5 (.....) and CC43 (____).

(d) Table showing percentage cells of stained when compared to the control and the mean fluoresence intensity of the stained population.

FIGURE 3.14



d

MAB	MFI	% STAINED
CC118	77	69%
CC43	77	71%
VPM5	156	76%

FIGURE 3.15

Flow cytometry analysis of sheep peripheral blood mononuclear cells (PBMs).

(a) and (d) Forward and side scatter profile of PBMs showing analytical regions 1 (a) and 2 (d).

(b) and (c) Frequency histograms of PBMs stained with negative control (. . . .) and SBU-T6 (____) within region 1 (b) and region 2 (c).

(e) and (f) Frequency histograms of PBMs stained with negative control (. . . .) and CC118 (____) within region 1 (e) and region 2 (f).

FIGURE 3.15

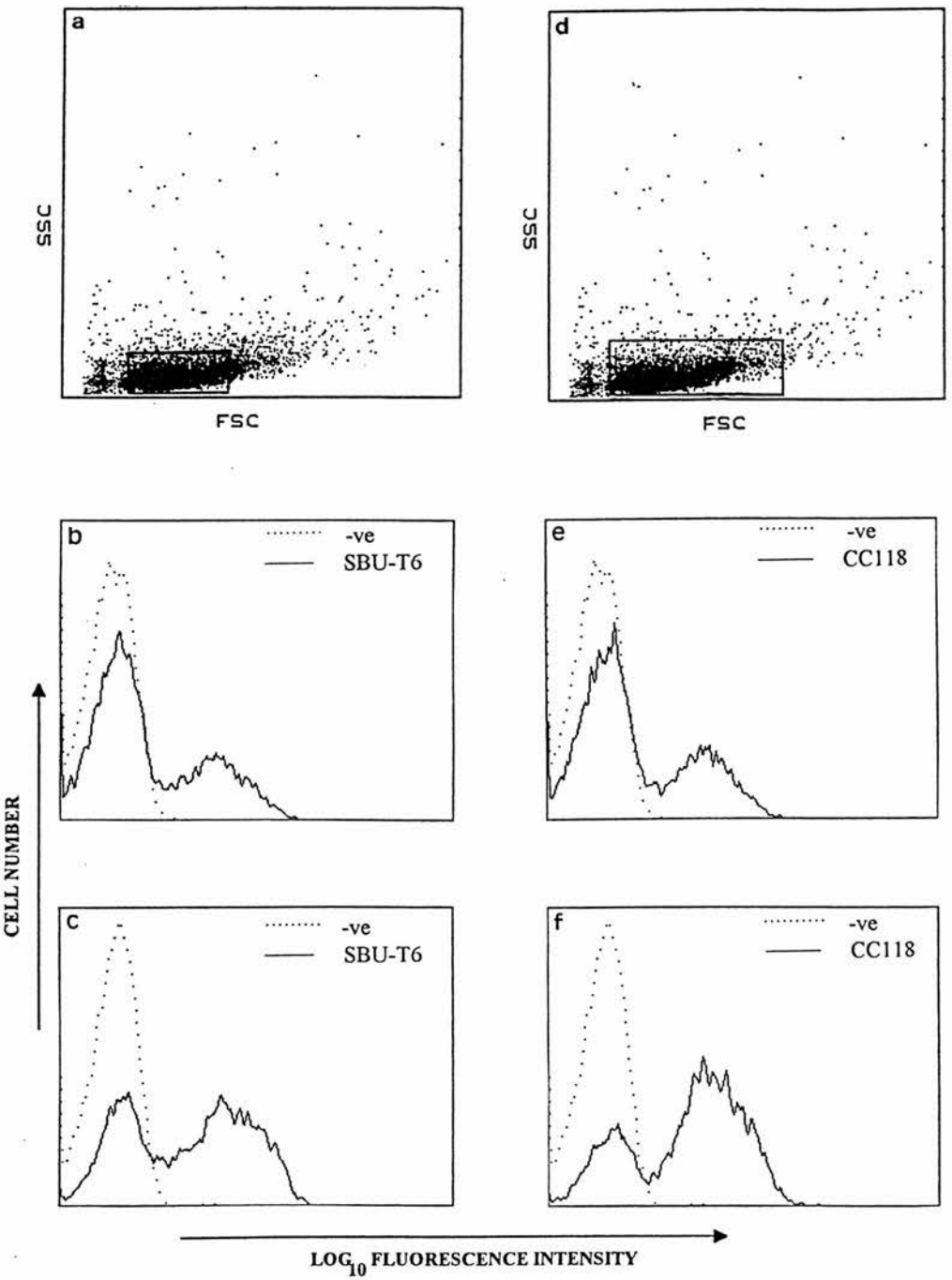

a
SSC
FSC
d
SSC
FSC
b
-ve
SBU-T6
e
-ve
CC118
c
-ve
SBU-T6
f
-ve
CC118
CELL NUMBER
LOG10 FLUORESCENCE INTENSITY

FIGURE 3.16

Two colour staining of sheep PBMs using the analytical region defined in Figure 3.11d.

(a) Negative control dot plot using biotinylated NMS (FL2) and VPM53 (FL1).

(b) and (c) Dot plots showing CC20 staining on FL1 with VPM30 (b) or VPM65 (c) staining on FL2.

(d), (e), (f) Dot plots showing SBU-T6 staining on FL1 with VPM46 (d), VPM30 (e) or VPM65 (f) staining on FL2.

(g), (h), (i) Dot plots showing CC118 staining on FL1 with VPM46 (g), VPM30 (h) or VPM65 (i) staining on FL2.

FIGURE 3.16

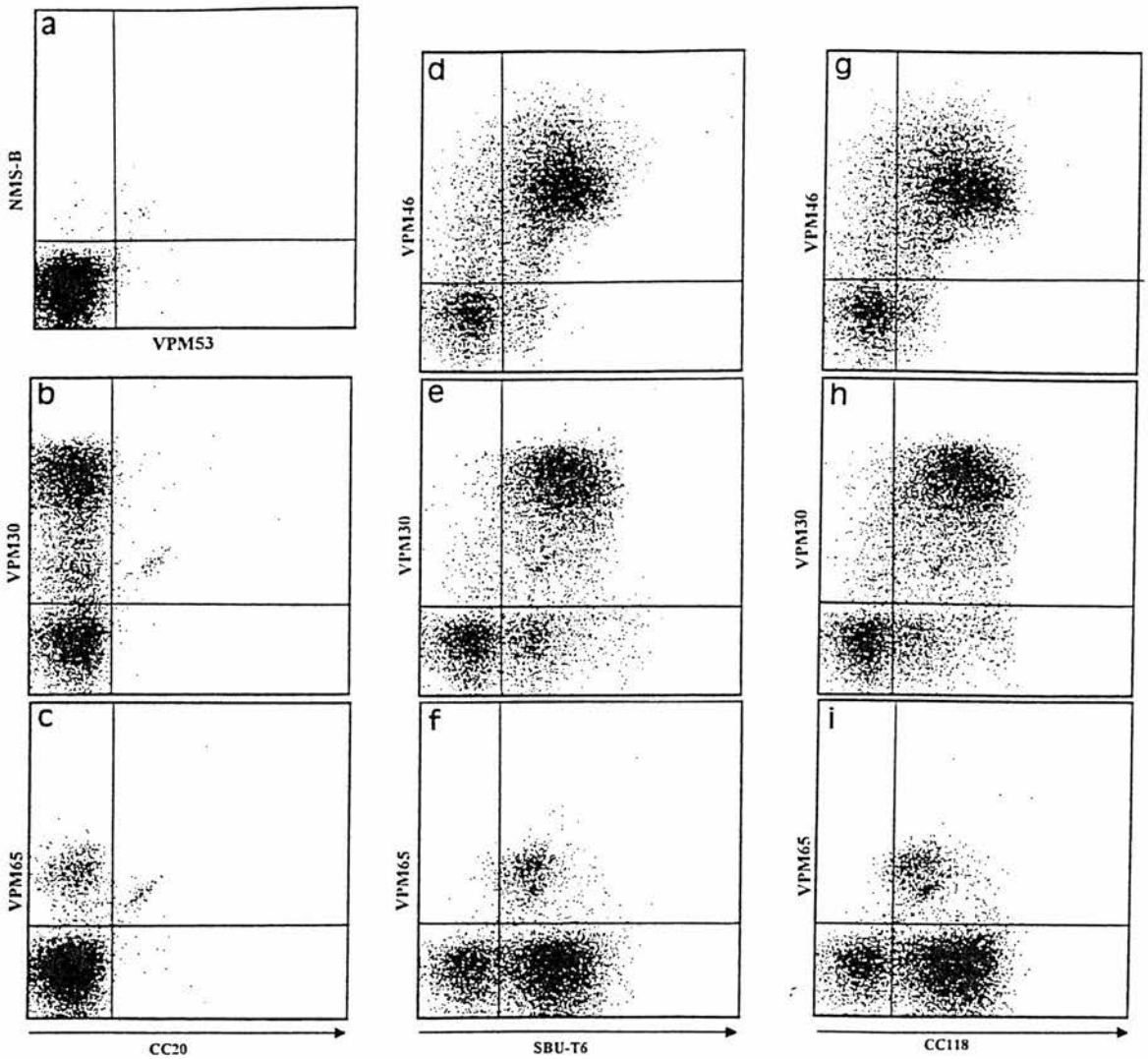

a
NMS-B
VPM53
b
VPM30
c
VPM65
CC20
d
VPM146
e
VPM130
f
VPM65
SBU-T6
g
VPM146
h
VPM130
i
VPM65
CC118

FIGURE 3.17

Two colour staining of sheep PBMs.

(a) Forward and side scatter profile of PBMs showing analytical region.

(b) Negative control dot plot using biotinylated NMS (FL2) and VPM53 (FL1).

(c), (d), (f) Dot plots showing SBU-T6 staining on FL2 with DU2104 (c), SBU-T8 (d) and CC15 (f) on FL1.

(e) Dot plot showing SBU-T6 staining on FL1 with SBU-T4 on FL2.

FIGURE 3.17

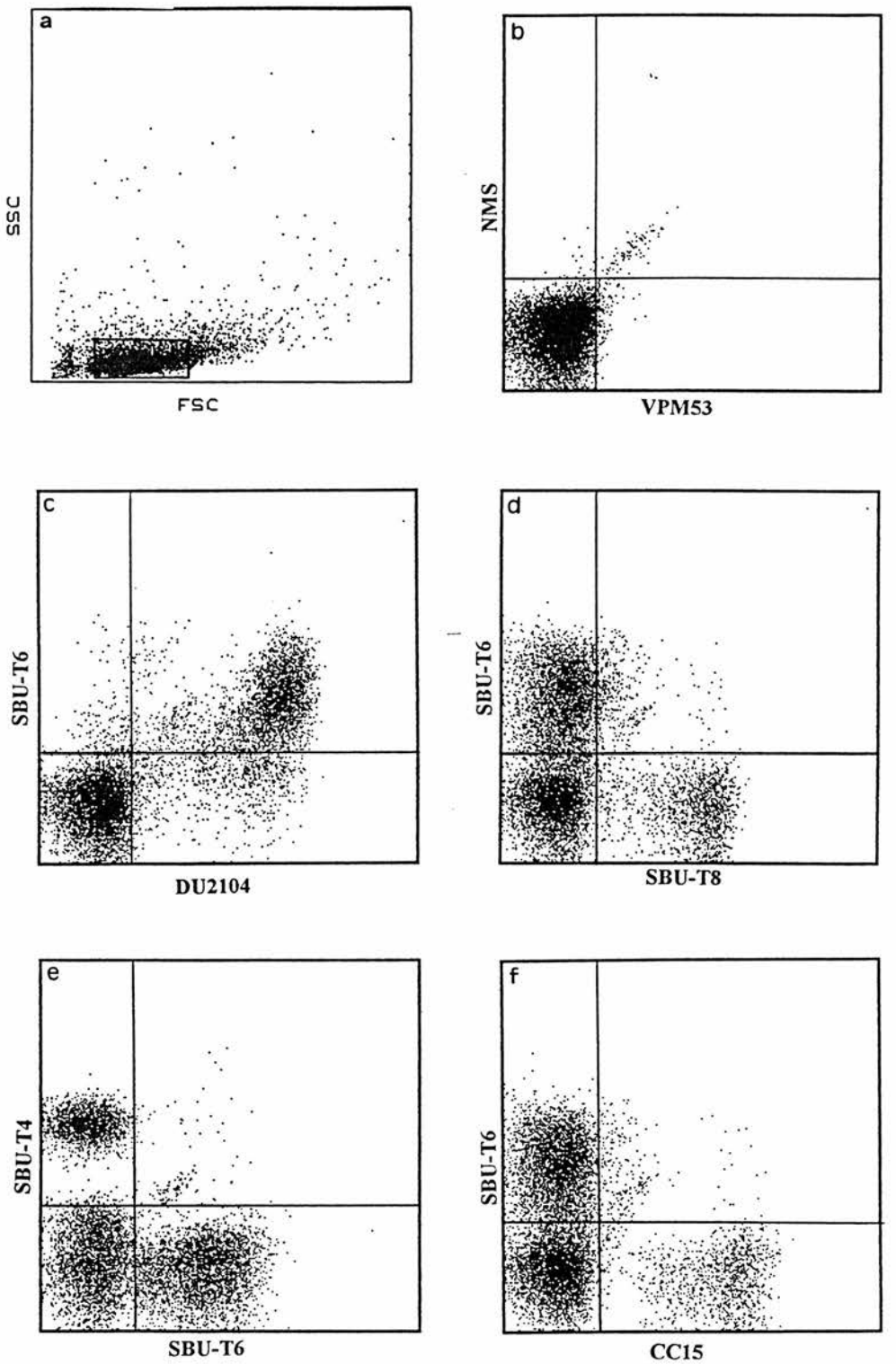

a
SSC
FSC
b
NMS
VPM53
c
SBU-T6
DU2104
d
SBU-T6
SBU-T8
e
SBU-T4
SBU-T6
f
SBU-T6
CC15

FIGURE 3.18

Frequency histograms and two colour staining of sheep efferent lymph lymphocytes.

(a) Forward and side scatter profile of efferent lymphocytes showing analytical region.

(b) Frequency histograms of lymphocytes within the region stained with negative control (. . . .), CC118 (.....) and SBU-T6 (____).

(c) Negative control dot plot using biotinylated NMS (FL2) and VPM53 (FL1).

(d), (e), (f) Dot plots showing VPM30 staining on FL2 with VPM53 (d), CC118 (e) and SBU-T6 (f) staining on FL1.

FIGURE 3.18

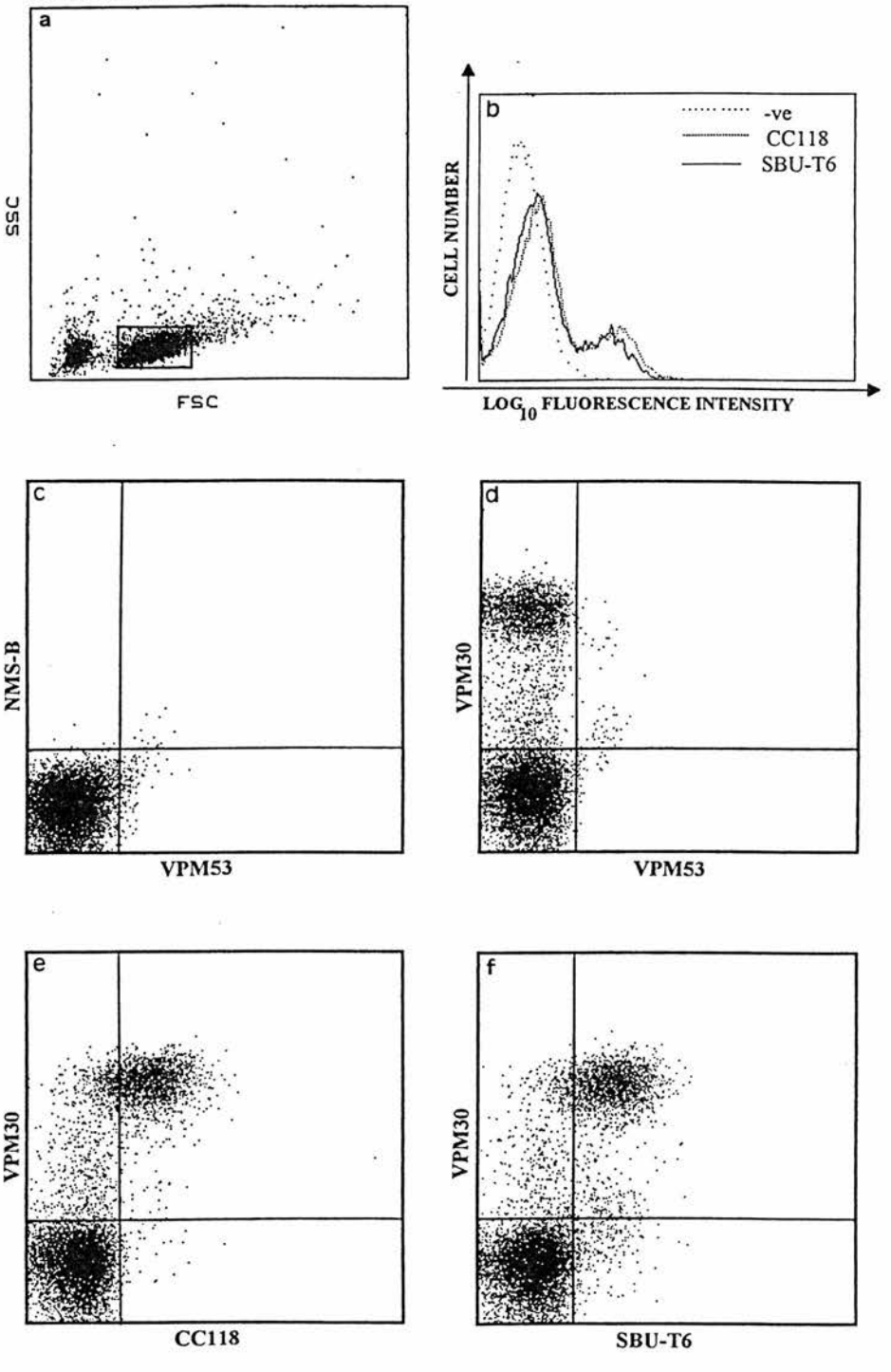

a
SSC
FSC
b
-ve
CC118
SBU-T6
CELL NUMBER
LOG10 FLUORESCENCE INTENSITY
c
NMS-B
VPM53
d
VPM30
VPM53
e
VPM30
CC118
f
VPM30
SBU-T6

FIGURE 3.19

Frequency histograms and two colour staining of sheep afferent lymph lymphocytes.

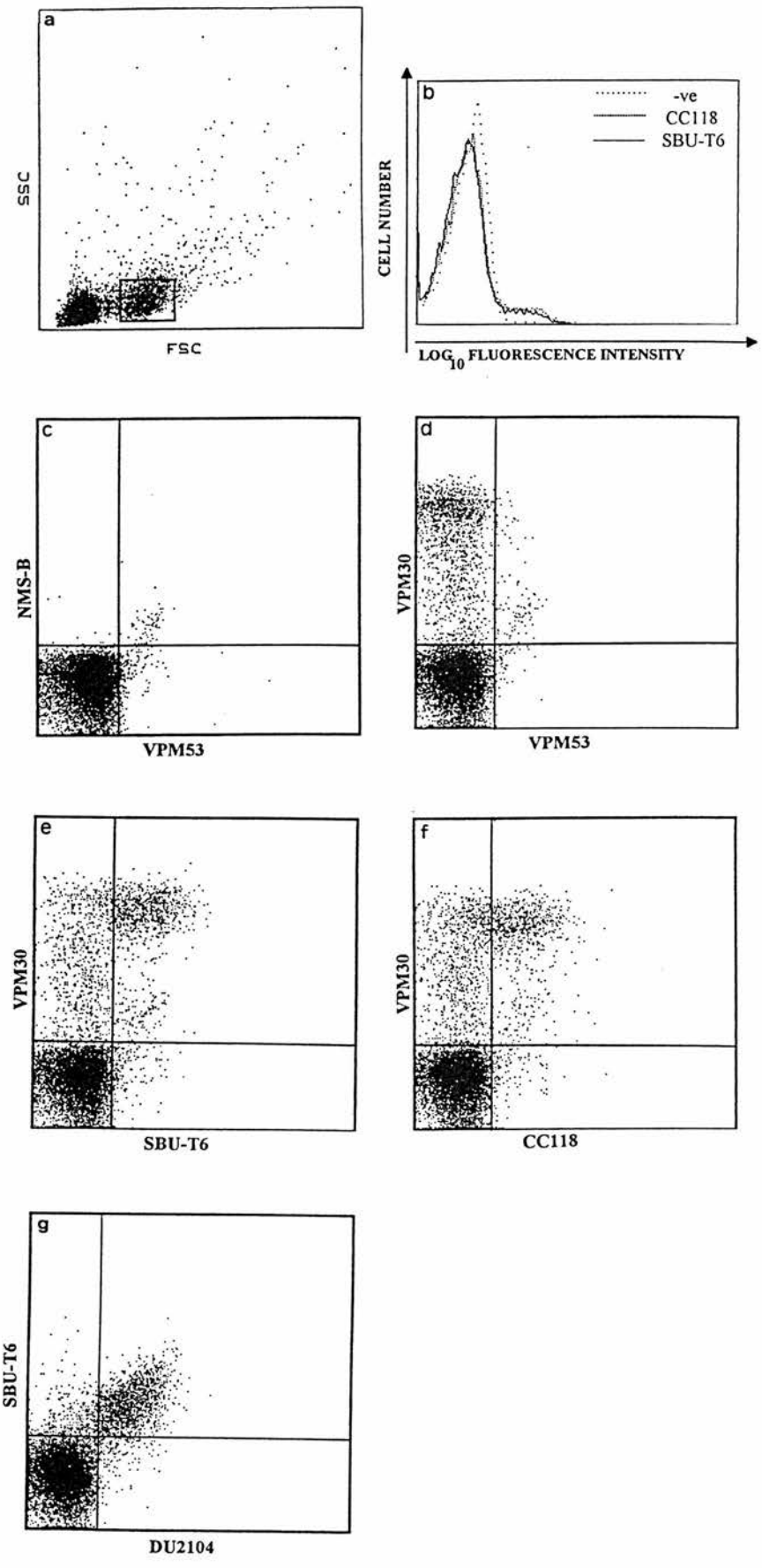
(a) Forward and side scatter profile of afferent lymphocytes showing analytical region.

(b) Frequency histograms of lymphocytes within the region stained with negative control (. . . .), CC118 (.....) or SBU-T6 (____).

(c) Negative control dot plot using biotinylated NMS (FL2) and VPM53 (FL1).

(d), (e), (f) Dot plots showing VPM30 staining on FL2 with VPM53 (d), CC118 (e) and SBU-T6 (f) staining on FL1.

FIGURE 3.19


a
SSC
FSC
b
-ve
CC118
SBU-T6
CELL NUMBER
LOG10 FLUORESCENCE INTENSITY
c
NMS-B
VPM53
d
VPM30
VPM53
e
VPM30
SBU-T6
f
VPM30
CC118
g
SBU-T6
DU2104

The group 2 mAbs demonstrated a much wider pattern of reactivity than is associated with any of the recognised CD1 isotypes in humans. The major notable features of the group 2 mAbs are:

- recognition of the majority of circulating B cells (in contrast to the subset of B cells recognised in humans).
- recognition of peripheral blood monocytes.
- recognition of tissue macrophages in various organs.

The remainder of this discussion will focus on the potential significance and possible functional implications of CD1 expression as demonstrated by the mAbs used in these experiments.

3.3.1 CD1 Expression by Antigen Presenting Cells

3.3.1.1 Dendritic cells

The presence of CD1 on dendritic cells, Langerhans' cells and a subset of B cells is well recognised. The presence of all isotypes of CD1 on dendritic cells is a consistent finding. Considering the key role of these cells in the immune response as described in Chapter 1, it is logical to conclude that if CD1 has a functional role then it is likely to have some involvement in DC function - potentially this could be envisaged as a role in presentation of antigen.

Functional studies using sheep afferent lymph dendritic cells have shown that DCs in resting lymph are characterised by uniform CD1 expression however, after both primary and secondary immune responses, there are detectable alterations in CD1 expression (Hopkins et al. 1989; Coughlan et al. 1996). Following secondary antigen challenge (by OVA) *in vivo* CD1 expression is significantly reduced 2-8 hours post challenge but by 24 hours levels become elevated compared with pre-challenge values (Coughlan et al. 1996). This modulation occurs in parallel with modulation of certain other cell surface markers including MHC class II and would be consistent with a role for CD1 in the immune response.

3.3.1.2 B cells

Whilst CD1 expression on dendritic cells is well recognised, the expression of ovine CD1 described here i.e. monocytes, macrophages and the majority of circulating B cells differs from mouse and man.

CD1 as recognised by the group 2 mAbs is found on the majority of circulating and afferent and efferent lymph B cells. In addition B cells in defined anatomical locations in the peripheral lymphoid tissues express CD1. In peripheral lymphoid tissues, mantle zone lymphocytes are considered to be resting B cells; plasma cells terminally differentiated B cells and germinal centre cells actively proliferating B cells (Hsu and Jaffe, 1984).

Marker expression is highly variable among different stages of B cell activation and it is difficult to study phenotypic changes associated with B cell maturation as there is no bursa analogue in mammals (although ileal Peyers patches in sheep do have B lymphopoietic capacity). Relevant to this point is the finding that B cells within the Peyers patches in both foetal and adult intestine are not stained by group 1 or group 2 mAbs (Figures 3.5 and 3.7) implying that CD1 does not play a similar role in the ontogeny of B cells as is proposed for T cells.

In peripheral blood, the pattern of CD1 expression on ovine B cells differs from the human situation. In humans, CD1c and CD1d are expressed on B cells (Blumberg et al. 1991; Calabi et al. 1991). CD1c is expressed on approximately 90% of B cells at birth although subsequently the percentage of positive B cells decreases with age. CD1c has also been shown to be expressed on B cells from children suffering from severe combined immunodeficiency (SCID).

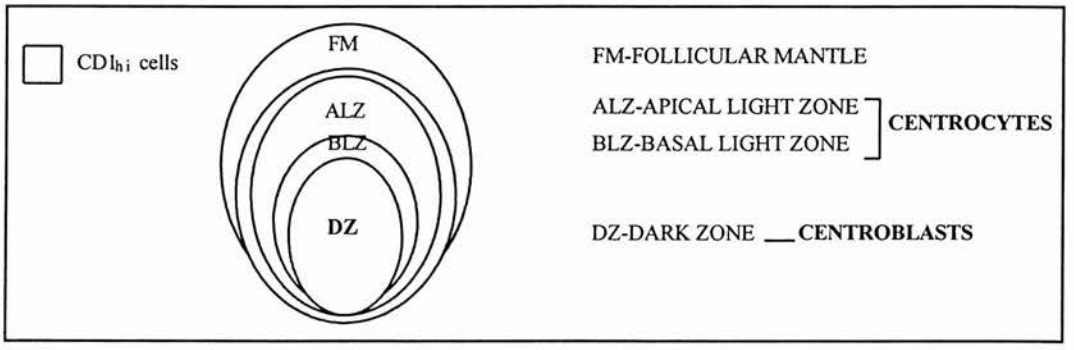
The group 2 mAbs consistently stain the majority of B cells in peripheral blood in adult sheep in contrast to the small percentage of cells which are recognised in human adults.

The secondary lymphoid organs i.e. the lymph nodes, spleen and gut associated lymphoid tissue are the site of initial contact with antigen for B cells. The B cell areas within this tissue are the lymphoid follicles. Primary lymphoid follicles are

found prior to antigen challenge and consist of a network of FDCs and recirculating B cells.

Secondary lymphoid follicles are characterised by the presence of germinal centres (see below). During the exponential growth of blast cells as the germinal centre is formed, the small recirculating B cells are excluded from the follicle centre and form the follicular mantle (Liu et al. 1992).

The following is a diagrammatic representation of the structure of a secondary follicle with the follicular mantle either entirely surrounding the GC or forming a cap over it.



Results of immunohistology demonstrate a distinctive pattern of staining with the group 2 mAbs in the follicular mantle in germinal centres in both lymph nodes and spleen. Thus CD1 expression is restricted to the small recirculating B cells of the mantle and is lost as the B cells develop through centroblast and centrocyte stages.

Within the spleen, the white pulp consists of sheaths of lymphocytes (mainly T cells) surrounding the central arteries forming the periarterial lymphoid sheaths (PALS) and lymphoid nodules (mainly B cells) which can form germinal centres as in lymph nodes. Group 2 CD1 expression is observed here as in lymph nodes. The red pulp consists of blood filled sinusoids and cords of cells between the sinusoids. These cords contain macrophages, monocytes and lymphocytes in addition to blood elements. Group 2 mAbs stain scattered red pulp constituents and also cells within the marginal zone which consists of a layer surrounding the PALS and B cell follicles predominantly composed of intermediate sized lymphocytes and macrophages.

Thus it is apparent that in lymphoid follicles within peripheral lymphoid tissue i.e. lymph node and spleen, as the B cells develop into centroblasts and centrocytes following contact with antigen, there is a marked down-regulation of CD1 expression. The significance of this observation is unclear. However loss of cell surface expression following cell activation is a feature of a number of surface molecules including MHC class II and it is possible that CD1, having performed its 'function' in the resting cell, is not required as the cell becomes activated.

3.3.1.3 Monocytes/ Macrophages

One of the major differences between CD1 expression in the sheep and in humans is the constitutive expression on monocytes/ macrophages of the antigens defined by the group 2 mAbs in the sheep.

Although CD1 expression is not constitutive on human monocytes, expression of CD1a-c has been induced by stimulation with the cytokines GM-CSF and IL-4 (Porcelli et al. 1992) and CD1c expression has been induced by GM-CSF alone (Kasinrerk et al. 1993). Cytokines which induce MHC class I and class II expression e.g. IFNγ, do not however induce CD1 expression (Kasinrerk et al. 1993).

In humans, CD1a expression has been shown on certain monocytic leukaemias (Misery et al. 1992). Possible explanations for expression by these neoplastic cells include deregulated expression of the CD1a gene or the presence of a neoplastic cell population representative of blast cells at a stage in ontogeny common to both monocytes and Langerhans' cells.

Considering the expression of the molecule recognised by these mAbs on monocytes, it is perhaps not surprising that they also recognise tissue macrophages - again this pattern of staining is one which has not been described in other species. Recognition of CNS microglial cells by the group 2 mAbs represents the first description of CD1 expression within the CNS.

Constitutive expression of CD1 by the majority of ovine monocytes and macrophages may indicate the presence of a different CD1 isotype on these cells. If this is true then expression of this different isotype on ovine B cells could explain the

inconsistency between the percentage of B cells which stain positive for CD1 in humans compared to sheep.

3.3.2 CD1 In The Intestine

Group 1 mAbs

An unexpected finding was the presence of CD1 expression on foetal intestinal epithelium and also on cells lining the crypts. This is a feature restricted to the group 1 mAbs and indeed represents the only situation where group 1 mAbs stain a cell population that the group 2 mAbs do not.

In humans and mice, CD1 is expressed on intestinal epithelium, however, this is a feature of the CD1d isotype and is not found with the human CD1b isotype. Experiments in rodents using *in situ* hybridization to detect mRNA in this region (Lacasse et al. 1992), showed that Paneth cells at the base of the crypts contain CD1 mRNA. CD1 expression in this region in late foetal lambs may have a role in mucosal immunity. As described in Section 1B.1.2, the murine non-classical class I molecule, TL is expressed on intestinal epithelial cells and has been proposed as a restriction element for populations of T cells within this area with invariant TCRs. CD1 in the sheep may therefore play a similar role although it is surprising to find this pattern of expression with molecules recognised by the group 1 mAbs (i.e.CD1b-like) when comparisons to other species would indicate that intestinal epithelial CD1 expression is normally a feature of the CD1d isotype.

One alternative and purely speculative suggestion is that CD1 in this region could play a role in the neonate analogous to the rat neonatal receptor for Ig (Section 1B.1.3).

Group 2 mAbs

The group 2 mAbs recognise cells within the lamina propria of the intestine which resemble lamina propria macrophages. In addition, intense staining of certain areas of the gut associated lymphoid tissue has been demonstrated. Again this is consistent with the staining seen within lymphoid follicles in lymph node and spleen.

The presence the group 2 CD1 molecule(s) within the lamina propria and B cell areas of the intestine is consistent with its presence on macrophages and B cells as described above. In cattle, CC43 and CC118 have been shown to stain intraepithelial cells within the intestine - this was not seen using these mAbs in sheep.

3.3.3 Modulation of CD1 expression in disease

It is apparent from Figures 3.10 and 3.11 that expression of both group 1 and group 2 CD1 molecules is modulated during mycobacterial infection. The pattern of reactivity observed with the the group 2 mAbs (Figures 3.10 and 3.11 (a) and (b)) reflects that seen in normal tissue i.e. staining of macrophages and regulated expression within B cell areas.

The alterations in group 1 CD1 expression which occur in tissue infected with mycobacteria is interesting in view of the recent demonstration that T cells can recognise mycobacterial antigens in the context of CD1b (Beckman et al. 1994, Sieling et al. 1995). The focal clusters of CD1b positive cells which are seen in Figures 3.10c and 3.10d could represent antigen presenting cells which have up-regulated their CD1 levels in order to present the mycobacterial antigen to T cells. It is possible that the macrophages which are present in the lamina propria (Figures 3.11a and 3.11b) become activated as a result of the infection and elaborate cytokines which result in induction of CD1 within epithelial cells. However, although this staining is apparently specific for the group 1 mAbs, it is intracellular and as such may be artefactual although no such intracytoplasmic staining was observed in controls or with group 2 mAbs. A recent report by Sugita and colleagues (1996) describes localization of CD1b to endocytic compartments. Assuming that the staining observed in the intestine with the group 1 mAbs is not an artefact, it may represent internalized CD1 that has been targeted to these endosomal compartments.

3.3.4 Summary

In summary therefore, it has been shown that all the 'professional' antigen presenting cells in the sheep (i.e. B cells, monocytes, macrophages and dendritic cells) constitutively express members of the CD1 family. The fact that CD1 expression is

restricted to APCs (with the exception of cortical thymocytes and intestinal epithelial cells) implies a role for this molecule on these cells. In addition, the existence of a CD1 molecule with a much wider distribution than CD1 molecules in man and mouse may imply that sheep have retained or acquired a functional requirement for this CD1 isotype which does not exist in man or mouse.

CHAPTER 4

CHARACTERISATION OF OVINE CD1 ANTIGENS

4.1 Introduction

A variety of biochemical studies have been carried out on human and rodent CD1 antigens and to a lesser extent on ruminant CD1. Surface iodination and subsequent immunoprecipitation with anti-CD1 mAbs demonstrated the different α chain molecular weights associated with the different human CD1 isotypes (see Chapter 1). In ruminants, the majority of mAbs immunoprecipitate a 46kd α chain consistent with CD1b in humans. Exceptions to this are the putative anti-CD1 mAbs, CC43 and CC118, which have been shown to immunoprecipitate an α chain of 43kd in cattle (Howard et al. 1993a). The biochemical nature of the CD1 antigens has been further studied utilising the techniques of 2-dimensional electrophoresis, deglycosylation and NH_2-terminal sequencing.

Of the techniques listed above, only immunoprecipitation has been carried out using anti-CD1 mAbs in sheep. In order to further characterise the antigens recognised by the group 1 and group 2 mAbs (as classified in Chapter 3), the techniques of immunoprecipitation, two dimensional electrophoresis and Western blotting were employed. Subsequently, affinity purification of antigens was carried out allowing ELISA studies and NH_2-terminal sequencing of the CD1 proteins. These studies were considered to be of particular interest in view of the possible existence of a novel CD1 isotype(s) as defined by flow cytometry and immunostaining data.

mAbs

For the majority of the studies described below, comparisons are made between group 1 and group 2 mAbs. SBU-T6 and CC118 are used as group 2a and group 2b mAbs respectively. CC20, which has been shown to recognise COS cells transfected with human CD1b (Howard et al. 1993b), and CC14 are used as representatives of group 1 mAbs.

4.2 Results

4.2.1 Immunoprecipitation

Immunoprecipitation of antigens from I^{125} labelled thymocytes was performed with all the mAbs. Previous studies in the sheep have shown the existence of a 46kd α chain with all the mAbs used (Dutia and Hopkins, 1991). A similar situation exists in cattle with the exception of CC43 and CC118 which immunoprecipitate a 43kd heavy chain (Howard et al. 1993). Figure 4.1a shows the results of immunoprecipitation using the ELISA plate method with a panel of anti-CD1 mAbs. Under these conditions, all the mAbs immunoprecipitated a 46kd heavy chain in association with β2 microglobulin with the exception of CC43 and CC118 which failed to immunoprecipitate using this method.

In order to enhance any signal with these two mAbs, a similar technique utilising streptavidin coated ELISA plates to provide an additional amplification step was performed. Figure 4.1b shows the results of this experiment and demonstrates that CC43 and CC118 immunoprecipitate an α chain of the same 46kd size as SBU-T6.

4.2.2 Western Blotting and Two-Dimensional Electrophoresis

Western blotting was carried out to establish whether any of the available mAbs recognised antigen blotted onto nitrocellulose. Fresh thymocytes were lysed, fractionated on a 12% SDS-polyacrylamide gel and blotted onto nitrocellulose. Strips of the blot were subsequently incubated with individual mAbs. Figure 4.2a shows that CC20 recognises a 46kd antigen. None of the other antibodies recognised blotted antigen with the exception of SBU-T6 which gave a weak signal at 46kd when blots were developed using enhanced chemiluminescence (ECL) (Figure 4.2b).

In order to further investigate the protein species recognised by the mAbs, the technique of two dimensional (2-D) electrophoresis was used. In particular, a major aim was to establish any obvious differences between the species recognised by the group 1 and group 2 mAbs. Fresh thymocytes were lysed in sample buffer and run on a two-dimensional gel as described in section 2A.9. The gel was subsequently blotted onto nitrocellulose and developed with CC20. Initially, non equilibrium pH gradient

FIGURE 4.1

Immunoprecipitation of CD1 antigens.

(a) Autoradiograph of surface I^{125} labelled thymus lysate immunoprecipitated with a panel of anti-CD1 mAbs and run on a 5-20% non-reducing SDS-PAGE gel. Immunoprecipitation was carried out using the ELISA plate method and normal mouse serum (NMS) used as a negative control.

(b) Autoradiograph of surface I^{125} labelled thymus lysate immunoprecipitated with SBU-T6, CC43, CC118 and NMS control. Immunoprecipitation was carried out using streptavidin coated ELISA plates.

FIGURE 4.1a

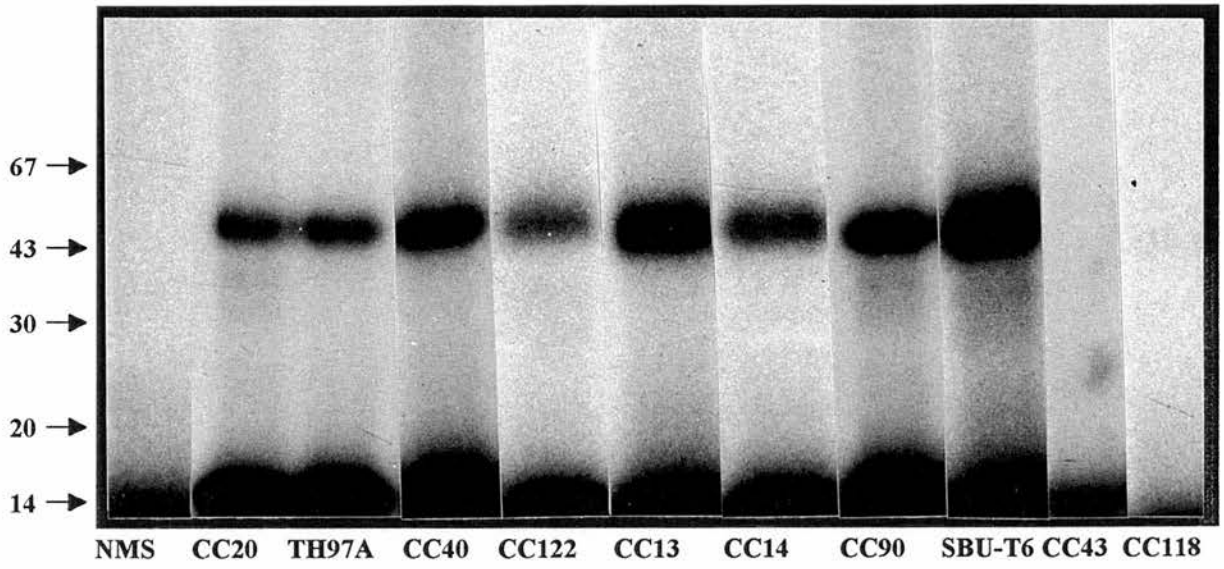


FIGURE 4.1b

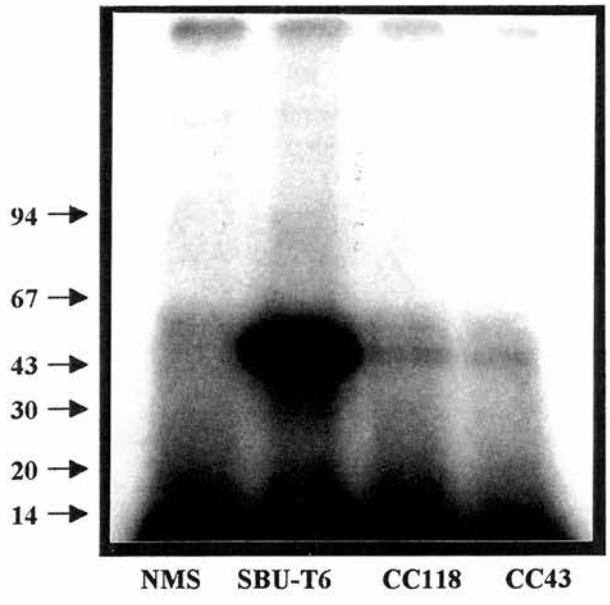


FIGURE 4.2

Western blotting of thymus lysate.

(a)Western blot of thymus lysate incubated with NMS (lane 2) or CC20 (lane 1) then developed using alkaline phosphatase.

(b)Western blot of thymus lysate incubated with NMS (lane 1) or SBU-T6 (lane 2) and developed using ECL. The faint band at 46kd is indicated by an arrow.

FIGURE 4.2a

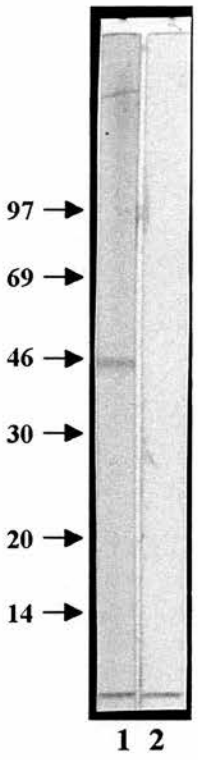
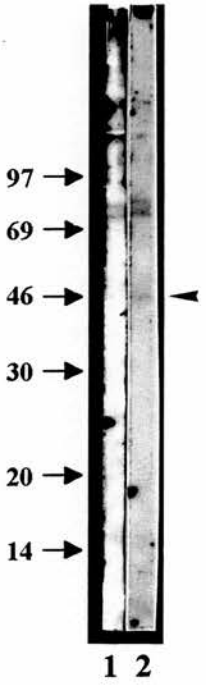


FIGURE 4.2b



electrophoresis (which allows separation of basic proteins) was used in the first dimension (pH range 3.5-10). The CD1 antigens were concentrated at the acidic end of the gel indicating that the proteins are not highly basic. Following investigation using different ampholine ranges, optimal separation and definition of proteins was obtained using isoelectric focusing in the first dimension with a pH range of 5 to 8. The result of such a blot incubated with CC20 shown in Figure 4.3a illustrating a range of protein species at 46kd and a single species at 33kd.

Repeating this experiment and incubating the blot with SBU-T6 followed by development by chemiluminescence failed to produce a defined signal thus comparison of group 1 and group 2 mAbs by this method was not possible.

A further attempt to compare the 2-dimensional pattern obtained with group 1 and group 2 mAbs was carried out by running immunoprecipitated antigen on a 2-dimensional gel. Thymocytes were surface labelled with I^{125} and lysate was immunoprecipitated as described for single dimension immunoprecipitations then fractionated on a two-dimensional gel which was subsequently dried down and exposed to X-ray film for several weeks. Figures 4.3b and 4.3c show the result of antigen immunoprecipitated with SBU-T6 and CC20 respectively and run on a 2-dimensional gel. As a result of poor intensity and definition of protein spots, a detailed comparison of the immunoprecipitates is not possible, however it is apparent that the two immunoprecipitates are broadly similar.

4.2.3 Affinity purification of CD1 antigens

Affinity columns were made as described in Section 2A.12 using the mAbs SBU-T6, CC14 and CC118. Following elution of these columns, the resulting purified antigen was used for ELISA, blotting and NH_2-terminal sequencing.

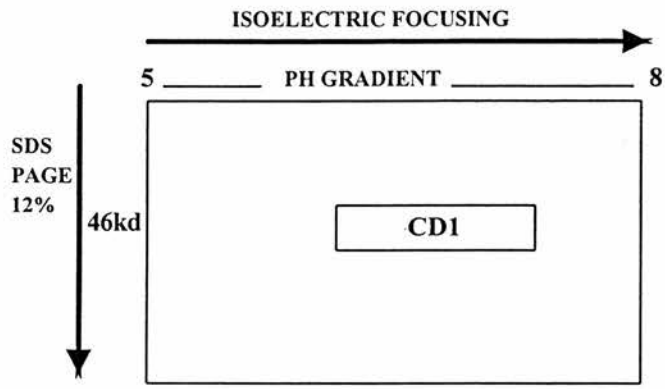
4.2.3.1 ELISA

The three purified antigens were coated onto ELISA plates and tested for reactivity with a range of CD1 mAbs. The results are shown in Table 4.1. An irrelevant mAb was also used as a control in these experiments. A negative result was considered to be one which was less than the mean of the control plus three times the standard

FIGURE 4.3

Two-dimensional electrophoresis.

Optimal conditions for analysis of CD1 in two-dimensional electrophoresis are illustrated below. These conditions were used in the blot and immunoprecipitations shown in Figure 4.3



(a) 2-D IEF blot of thymocyte lysate incubated with CC20 then developed using alkaline phospatase. This demonstrates 9 spots at 46kd with one smaller spot at 35kd.

(b) and (c). Autoradiograph of I^{125} labelled thymus lysate immunoprecipitated with SBU-T6 (Figure 4.3b) or CC20 (Figure 4.3c) and run on a 2-D gel. Multiple poorly defined spots are visible at 46kd in addition to the intense signal generated by β2m at 14kd.

FIGURE 4.3a

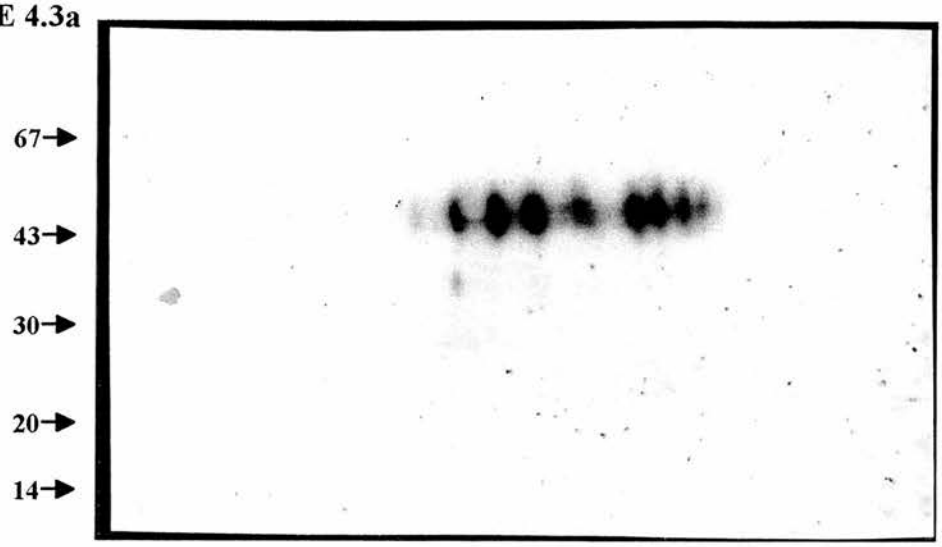


FIGURE 4.3b

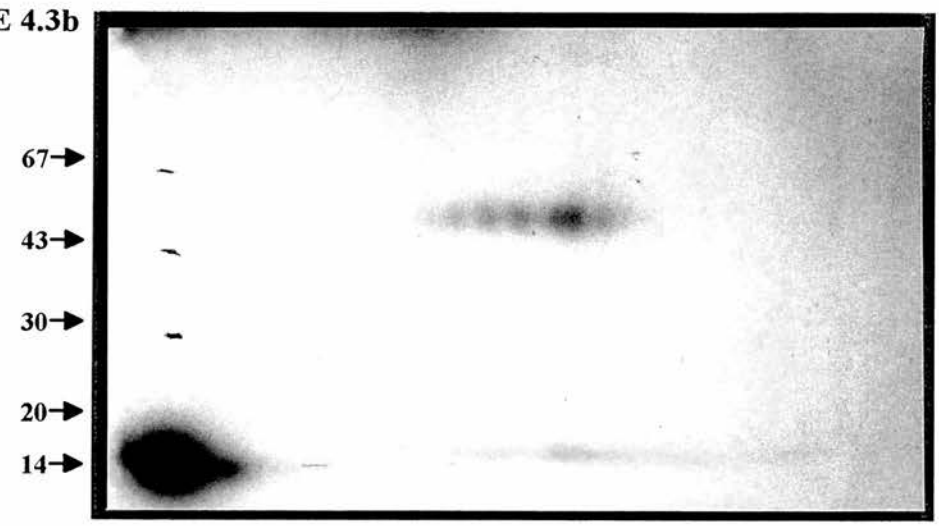


FIGURE 4.3c

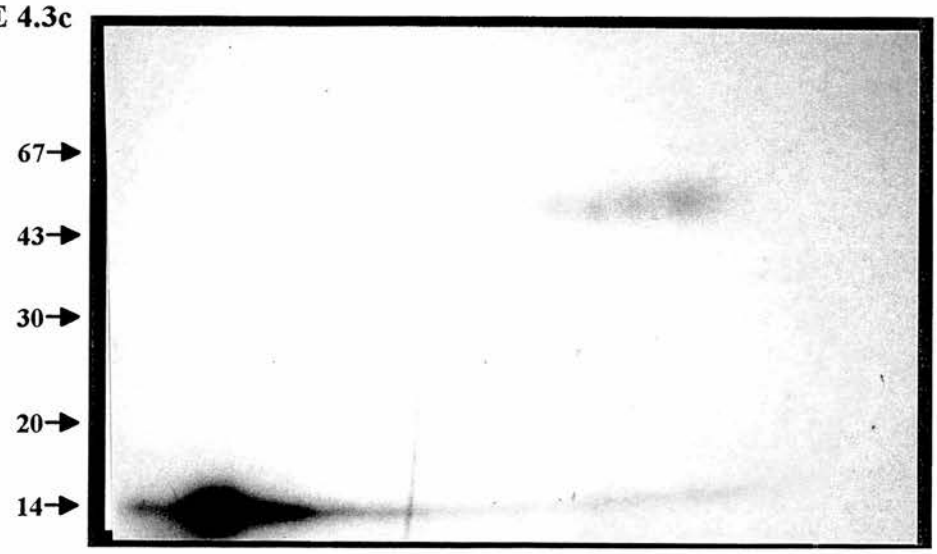


TABLE 4.1

Table showing ELISA data. Immunopurified CC14, SBU-T6 and CC118 antigens were coated onto ELISA plates and incubated with a variety of anti-CD1 mAbs. Figures shown represent the readings obtained following detection of bound antibody by indirect peroxidase technique and reading at OD_{595} on an ELISA reader. Each result is the mean (+/- standard deviation) of three experiments performed in parallel. An irrelevant mAb was also used as a control in these experiments. A negative result was considered to be one which was less than the mean of the control plus three times the standard deviation associated with that control. Using this criterion, these data show that all the mAbs recognise the CC14 and SBU-T6 antigens with the exception of CC118 ($p < 0.001$). The only mAbs to recognise the CC118 antigen are CC118 ($p = 0.011$) and SBU-T6 ($p = 0.0005$).

TABLE 4.1

	ANTIBODY						
ANTIGEN	CONTROL	CC14	CC20	CC40	CC122	CC118	SBU-T6
SBU-T6	0.159 +/- 0.023	0.739 +/- 0.040	1.484 +/- 0.066	1.131 +/- 0.014	1.153 +/- 0.036	0.216 +/- 0.023	1.733 +/- 0.041
CC14	0.176 +/- 0.028	1.609 +/- 0.072	1.745 +/- 0.015	1.908 +/- 0.063	1.722 +/- 0.029	0.215 +/- 0.032	1.882 +/- 0.018
CC118	0.205 +/- 0.016	0.184 +/- 0.020	0.214 +/- 0.019	0.218 +/- 0.023	0.219 +/- 0.028	0.424 +/- 0.037	0.483 +/- 0.025

deviation associated with that control. Using this criterion, these data show that all the mAbs recognise the CC14 and SBU-T6 antigens with the exception of CC118 ($p < 0.001$). The only mAbs to recognise the CC118 antigen are CC118 ($p = 0.011$) and SBU-T6 ($p = 0.0005$).

4.2.3.2 Western Blotting Using Purified Antigen

Deglycosylation

Having purified the antigen, and possessing a mAb from each of the groups which blotted (i.e. CC20 and SBU-T6) further blotting experiments were carried out using the purified CC14 and SBU-T6 antigens as protein on the blot.

Figure 4.4 shows the result of an experiment in which immunopurified untreated antigen (lanes 1 and 3) and antigen treated with the enzyme N-glycosidase F (lanes 2 and 4) were fractionated by SDS-PAGE. The gel was blotted onto nitrocellulose, incubated with CC20 then developed using ECL. This demonstrates that both the CC14 antigen (lanes 1 and 2) and the SBU-T6 antigen (lanes 3 and 4) possess a protein backbone of 33kd.

SBU-T6 - Blotting

Subsequent experiments using SBU-T6 to blot revealed some unexpected results. Figure 4.5 shows the results of a non-reducing gel of immunopurified, acetone precipitated CC14 and SBU-T6 antigen incubated with SBU-T6 and developed using ECL. Whilst recognising the expected 46kd band on the CC14 antigen, major bands of approximately 33kd and 66kd were observed on the SBU-T6 antigen in addition to a much weaker band at the expected size of 46kd.

The nature of the 33kd and 66kd bands were further investigated by running samples in non-reducing sample buffer alongside samples prepared in reducing buffer. Figure 4.6a shows the result of blotting immunopurified CC14 antigen (lanes 1 to 4) and SBU-T6 antigen (lanes 5 to 8) followed by incubation in SBU-T6 and development using ECL. Lanes 1 and 5 contain unprecipitated antigen in non-reducing buffer; lanes 2 and 6 unprecipitated antigen in reducing buffer; lanes 3 and 7 precipitated antigen in non-reducing buffer and lanes 4 and 8, precipitated antigen in reducing

buffer. The result demonstrates that, as before, SBU-T6 only recognises a 46kd band on the CC14 antigen. With the SBU-T6 antigen however, the additional 33kd band which is recognised (lane 5) forms a dimer when the antigen is acetone precipitated (lane 7). The strength of the signal generated by the antigen in lane 7 has resulted in poor definition of individual bands and as such the 67kd antigen is indicated with an arrow. That the larger molecular weight antigen which appears following acetone precipitation is a dimer of the 33kd antigen is confirmed in lane 8 when the use of a reducing buffer reduces the antigen to 33kd. The lack of a visible 33kd band in lane 6 is probably the result of loading a smaller amount of antigen due to the use of a less concentrated sample buffer.

The nature of the SBU-T6 antigen was further investigated by blotting the SBU-T6 antigen onto nitrocellulose then incubating one strip in CC20 (Figure 4.6b - lane 1) and incubating another strip in 'Protogold' protein detection solution (Figure 4.6b - lane 2). This shows that indeed the major protein present in the SBU-T6 antigen is at 33kd but this protein does not contain the epitope which is recognised by CC20.

In order to confirm that the 33kd antigen did not represent a deglycosylated form of the 46kd antigen, precipitated SBU-T6 antigen was deglycosylated and run on a gel as above. Figure 4.6c shows the results of this experiment. Lane 1 contains precipitated untreated antigen; lane 2 contains precipitated and deglycosylated antigen and lane 3 contains precipitated, deglycosylated and reduced antigen. This shows that treatment of the SBU-T6 antigen with N-glycosidase-F reduces the size of the 33kd antigen to 25kd thus demonstrating that the 33kd antigen is glycosylated. The 46kd band only appears very faintly on this blot. The reason for this is not certain however it was a general finding that antigen purified on different occasions using the affigel column exhibited differences in the relative quantities of 33kd and 46kd antigen.

FIGURE 4.4

Deglycosylation of CD1 antigen.

Western blot of immunopurified CC14 antigen (lanes 1 and 2) and SBU-T6 antigen (lanes 3 and 4). In lanes 2 and 4 the antigen has been deglycosylated. The blot was incubated in CC20 and developed using ECL. This blot shows that the peptide backbone of both these antigens as rcognised by CC20 is 33kd.

FIGURE 4.5

Western blot of acetone precipitated immunopurified CC14 antigen (lane 1) and SBU-T6 antigen (lane 2) incubated with SBU-T6 and developed using ECL. This shows that a band of 46kd is recognised on CC14 antigen but on SBU-T6 antigen, the major band is at 67kd with less intense bands at 46 kd and 30-33kd.

FIGURE 4.4

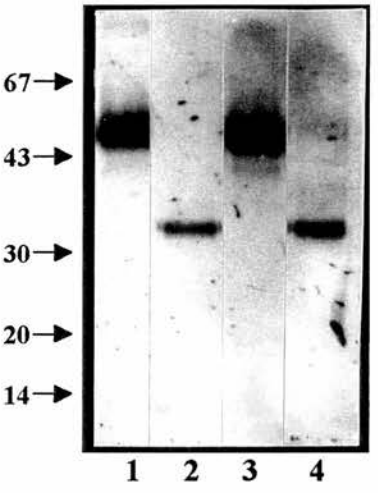


FIGURE 4.5

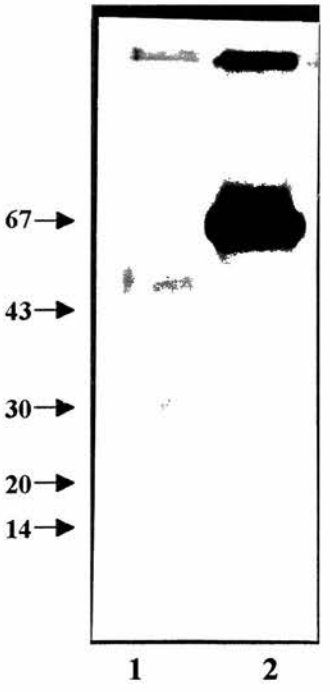


FIGURE 4.6

(a) Western blot of immunopurified CC14 antigen (lanes 1 to 4) and SBU-T6 antigen (lanes 5 to 8) incubated with SBU-T6 and developed using ECL. Lanes 1 and 5 contain unprecipitated antigen; lanes 2 and 6 contain unprecipitated, reduced antigen; lanes 3 and 7 contain precipitated antigen; lanes 4 and 8 contain precipitated, reduced antigen. In addition to showing the different molecular weight antigens recognised by SBU-T6 on immunopurified SBU-T6 antigen in addition to the 46kd antigen recognised on the CC14 antigen, this blot also shows that precipitation of the SBU-T6 antigen results in the formation of a homodimer of the 33kd antigen.

(b)

Lane 1 - Western blot of SBU-T6 antigen incubated in CC20 and developed using alkaline phosphatase.

Lane 2 - The same blot as in lane 1 but incubated in protein detection solution. This shows the presence of two proteins - one at 46kd and a more prominent one at 30-33kd. CC20 does not recognise an epitope on this lower molecular weight protein.

(c)

Western blot of SBU-T6 antigen incubated in SBU-T6 and developed using ECL. In lane 1, the antigen is precipitated; in lane 2, precipitated and deglycosylated and in lane 3, precipitated, deglycosylated and reduced.

This shows that the smaller molecular weight protein recognised by SBU-T6 is glycosylated as its size decreases from 33kd to 25kd following deglycosylation.

FIGURE 4.6a

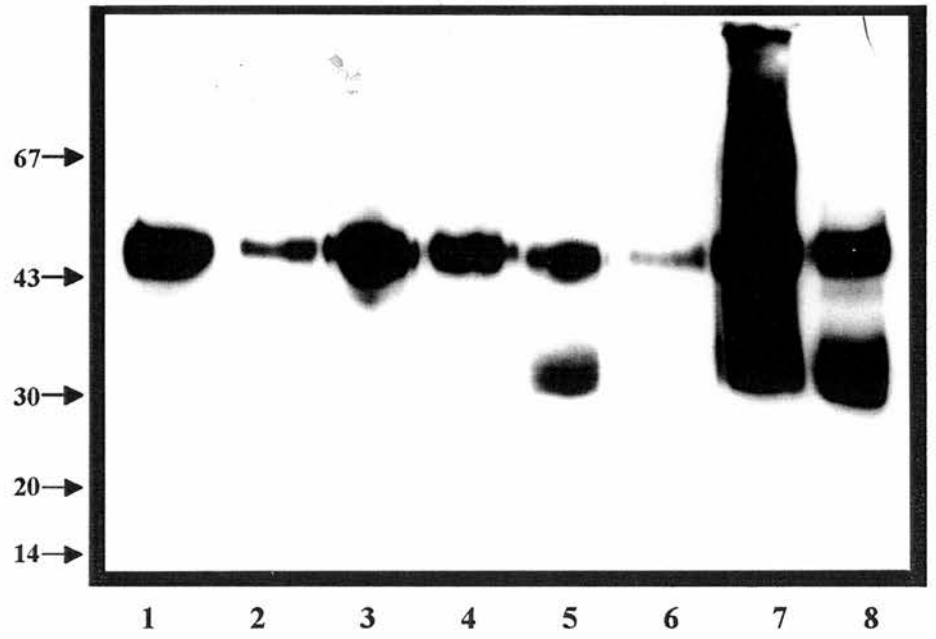


FIGURE 4.6b

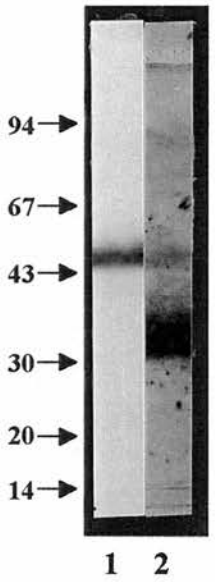
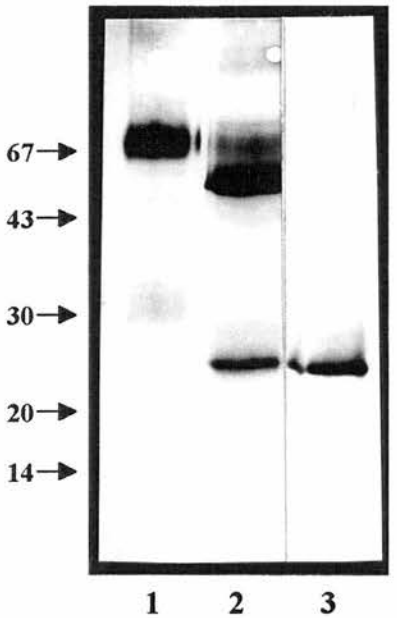


FIGURE 4.6c



4.2.3.3 NH_2-terminal sequencing

The main reason however for purifying these antigens was to obtain the NH_2-terminal amino-acid sequence - obviously this was of particular importance for the SBU-T6 antigen.

The antigens were ethanol precipitated and fractionated by SDS-PAGE prior to wet-blotting onto 'ProBlott' membrane as described in section 2A.15. After blotting, the gel was stained in Coomassie Brilliant Blue to check transfer of proteins to the membrane. Figure 4.7 demonstrates a stained gel of SBU-T6 antigen which had retained a visible amount of protein on the gel and shows the presence of the 33kd and 67kd bands which are recognised by SBU-T6 on a blot of purified antigen (Figure 4.5).

On repeating this experiment, the 33kd and 67kd bands were again the most prominent although a weaker band was present at 46kd (not shown). The 46kd band and the 67kd band were excised from dried membrane and sent for sequencing. The 46kd band yielded two proteins and as a result the sequences were somewhat ambiguous. The most likely sequences of the two proteins (based on the subsequent sequence of the 67 kd band) are shown in Figure 4.8a. Sequence 1 matches the first 13 residues of the 67 kd sequence. A search of the database failed to reveal any significant homology of sequence 2 to other proteins.

The 67kd band revealed a 29 amino-acid sequence shown in Figure 4.8b. When compared to known CD1 sequences of different species, this sequence revealed closest homology (66%) to the predicted amino-acid sequence of human CD1E in this region.

The NH_2-terminal sequence of the antigen recognised by CC14 was also obtained using the same method. Following blotting of the antigen and drying of the membrane, only a 46kd band was visible - this was excised and sent for sequencing . 13 amino-acids were obtained for the CC14 sequence and over this region there was 100% homology to the predicted amino-acid sequence of the ovine CD1 clones SCD1B-42 and SCD1B-52 (Figure 4.9) and 85% homology to SCD1A25 which is the only other ovine CD1 clone for which sequence is available in this region

FIGURE 4.7

Photograph of a gel following SDS-PAGE of precipitated SBU-T6 antigen and staining with Coomassie Brilliant blue. Lane 1 shows diffuse bands at 65-70kd and 30-35kd. Lane 2 shows marker bands. This gel demonstrates the size of the major protein species obtained following precipitation.

FIGURE 4.7

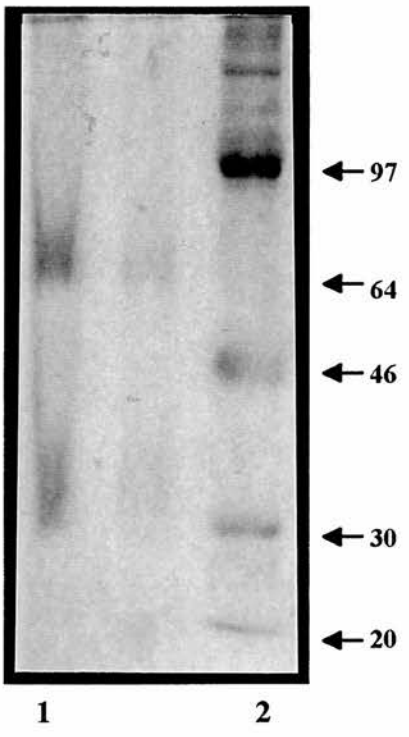


FIGURE 4.8a

NH_2-terminal sequencing results from the band excised at 46kd following SDS-PAGE of immunopurified, precipitated SBU-T6 antigen. Two protein sequences were detected resulting in some ambiguity in residues. However, utilizing the sequence obtained for the 67kd band (Figure 4.8b), the individual sequences 1 and 2 were deduced.

FIGURE 4.8a

46kd band

SEQUENCE 1	E E S P S F R L I Q I S S F A

SEQUENCE 2	X D Q G Q F V L H Q K

FIGURE 4.8b

N-terminal sequencing results from the 67kd band. 33 residues were obtained and the sequence is compared known human, sheep and mouse sequences. The highest degree of homology of this region is with human CD1E (66%).

CD1 CONSERVED RESIDUES

FIGURE 4.8b
67 kd band

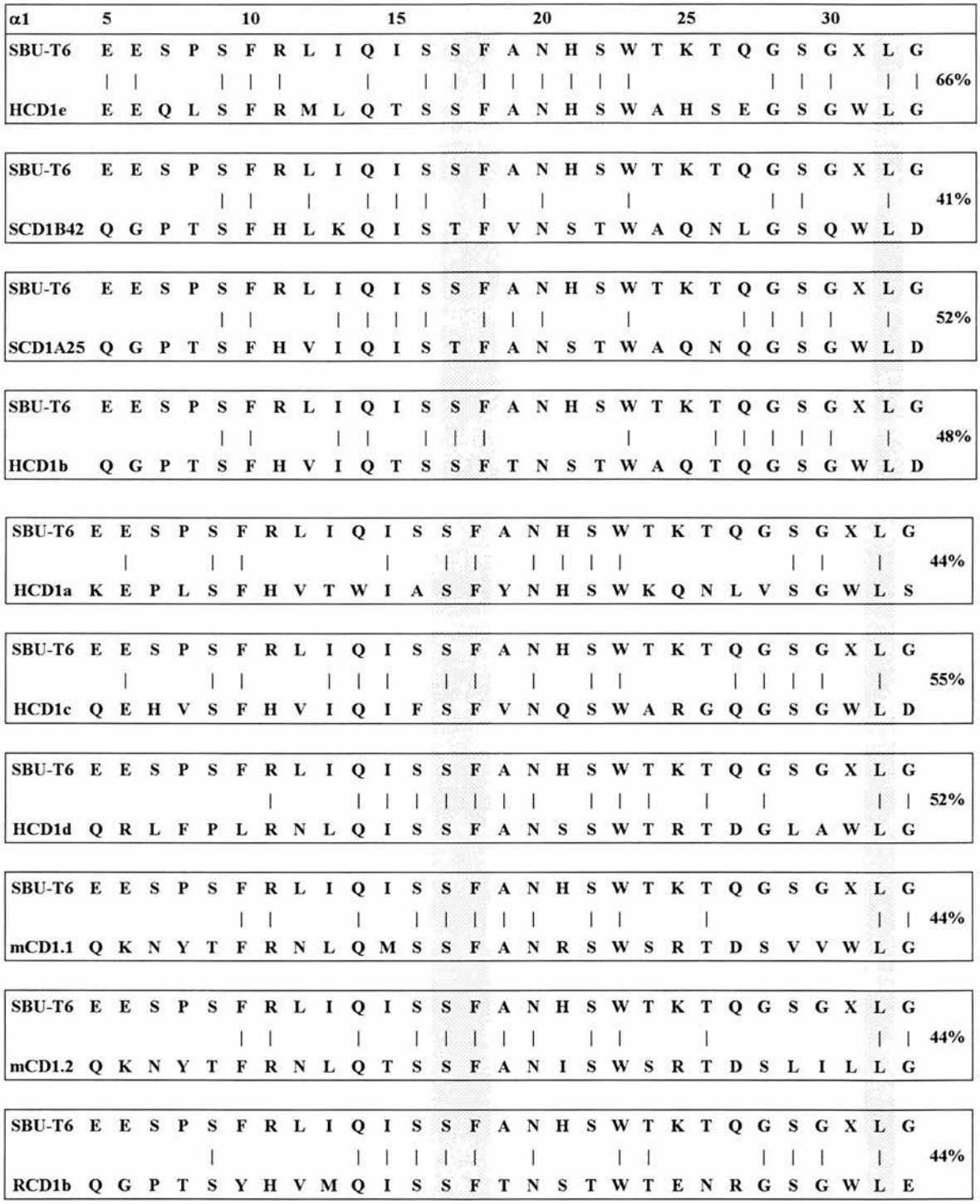


FIGURE 4.9

NH_2-terminal sequencing of the 46kd band obtained following SDS-PAGE of immunopurified and precipitated CC14 antigen. The sequence is compared to sheep sequences and human CD1B. This demonstrates 100% identity of this antigen with the predicted amino-acid sequence of the protein encoded by SCD1B-42/ SCD1B5-52 over this region.

FIGURE 4.9

α1	3		5					10				15		
CC14	V	F	Q	G	P	T	S	F	H	L	K	Q	I	
	|	|	|	|	|	|	|	|	|	|	|	|	|	100%
SCD1B-42/ SCD1B-52	V	F	Q	G	P	T	S	F	H	L	K	Q	I	

CC14	V	F	Q	G	P	T	S	F	H	L	K	Q	I	
	|	|	|	|	|	|	|	|	|			|	|	85%
SCD1A25	V	F	Q	G	P	T	S	F	H	V	I	Q	I	

Immunopurified CC118 antigen was also sent for sequencing however sequence was not obtainable due to N-terminal blockage.

4.3 Discussion

Biochemical studies on CD1 antigens

The results of immunoprecipitation reveal that the mAbs CC43 and CC118 immunoprecipitate an α chain of the same molecular weight as the other CD1 mAbs i.e. 46kd. This is in contrast to the situation in cattle where these mAbs immunoprecipitate a 43 kd α chain (Howard et al. 1993). Results obtained using I^{125} immunoprecipitates in 2-dimensional electrophoresis did not yield a signal of required definition or intensity to allow detailed comparison between the molecules recognised by group 1 and group 2 mAbs. Despite this, it would appear that there is no major difference between the patterns of protein species generated by 2-dimensional electrophoresis. In addition, the results demonstrate that the ovine CD1 α chain consists of a similar number of protein species when compared to murine and human CD1 when analysed in this fashion (Van Agthoven and Terhorst, 1982; Mosser et al. 1991) i.e 9 or 10 different protein spots within an IEF range 5 to 8.

The finding that SBU-T6 recognises an epitope present on a 33kd CD1 antigen (and its 67kd homodimer) is unexpected as there is no evidence for immunoprecipitation by this antibody of a molecule of this size. This may reflect the fact that the immunoprecipitations were carried out using surface labelled cells whereas antigen purification allows the mAb access to both intracellular and extracellular proteins. Although there have been no specific reports of CD1 antigens existing in forms outwith the 43-49kd range, the fact the CD1 genes are known to undergo alternative splicing would be consistent with the existence of different molecular weight isoforms (see Chapter 7). Boumsell and colleagues (1989) present immunoprecipitates which clearly show bands at 30kd and approximately 60kd although no controls are shown and this is not commented on by the authors. In

addition, immunoprecipitation of rat spleen cells with anti-CD1 mAbs revealed a 65kd band and a 29kd band in addition to the 'expected' 45kd band.

It is possible that the 33kd antigen could represent an intracellular form of the antigen which would explain why the mAb does not immunoprecipitate this isoform from surface labelled cells. An additional explanation involves the possibility of the 33kd antigen representing the product of an alternatively spliced CD1 gene. SCD1B-52 contains a precise α3 deletion and, as discussed in Chapter 7, alternative splicing is known to be a feature of the CD1 genes. Indeed, a deletion of any one of the external domains could equate to a molecule of this size. Caveats to this theory however are the inability of SBU-T6 to immunoprecipitate a 33kd antigen and in addition, the deglycosylation data shown in Figure 4.6c clearly shows that the 33kd antigen is not glycosylated to the same extent as the 46 kd antigen i.e. the 46kd band is reduced by 13kd following deglycosylation whereas the 33kd band is reduced by 8kd. Since all the potential glycosylation sites are contained within the α1 and α2 domains, a molecule lacking the α3 domain would therefore be expected to demonstrate the same extent of glycoslyation as a complete protein. It is possible however that a combination of alternative splicing and differential glycosylation could combine to produce the 33kd band. Relevant to this discussion is the demonstration by Balk et al (1994) that CD1d can exist as a 37kd protein with no N-linked carbohydrate.

Another possible explanation for this result is that the smaller protein represents a proteolytic product of the intact 46kd antigen. Relevant to this hypothesis is the evidence that the mAbs 1H1 and 3C11 (which recognise mCD1.1) immunoprecipitate a 37kd band in addition to the 'expected' 48kd α chain from murine cells (Bleicher et al. 1990). Also, the mAb 1H1 immunoprecipitated species of 35, 28 and 17kd from CD1d transfected cells in addition to the 48kd major protein (Blumberg et al. 1991). This is perhaps the most likely explanation for this phenomenon, indeed a similar situation has been described in the murine non-classical class I antigen, TL and is referred to as the 'TL breakdown phenomenon' (Chorney et al. 1991). Pickering and Wolcott (1979) first demonstrated

degradation of the TL antigen postlysis and concluded that an autolytic mechanism was functioning and cleaving TL at a susceptible hinge region similar to that found in immunoglobulin molecules.

Explanation of these results is further complicated when considering the ELISA results. The fact that SBU-T6 is a representative of the group 2 mAbs and as such recognises an antigen with a wider distribution than the group 1 mAbs suggests that this mAb may, as previously proposed, represent a 'pan-CD1' mAb (Dutia and Hopkins, 1991). SBU-T6 recognises not only its own antigen but also the group 1 antigen and that of CC118. The group 1 mAbs recognise a group 1 antigen (CC14) and SBU-T6 antigen but not CC118. This data cannot be reconciled with SBU-T6 recognising an epitope common to all the CD1 antigens since the anti-CD1b mAbs in turn recognise SBU-T6. The cross-reactivity that is clearly occurring under these conditions may be a feature of the generally conserved nature of the CD1 antigens. The only definite conclusion to be made from the ELISA data is that CC118 recognises a different antigen from the group 1 mAbs.

NH_2-terminal sequencing results

Initial attempts at obtaining an NH_2-terminal sequence for human CD1 antigens indicated that the NH_2-terminus was blocked (Lerch et al. 1986). Subsequently however, Wang and colleagues (1988) reported the NH_2-terminal sequence of the rabbit CD1b antigen and more recently, Woolfson and Milstein (1994) have reported the NH_2-terminal sequence of a human CD1a protein. These sequences are compared with the sequences obtained using the mAbs CC14 and SBU-T6 in Figure 4.10. This indicates that the point of cleavage of the signal peptide from the mature protein differs between different isotypes in the same species and between the same isotypes in different species.

Although the CC14 sequence is a 100% match to the predicted sequence of the proteins encoded by the SCD1B-42 and SCD1B-52 genes, complete accuracy of the SBU-T6 sequence cannot be assumed as this represents the sequence of a novel protein. The initial sequence obtained for the 46kd antigen (Figure 4.8a) contained several ambiguous residues and was only clarified after obtaining the sequence of the

67kd band. In this respect, it is of note that the NH_2-terminal sequence published for rabbit CD1b (Wang et al. 1988) differs at residues 1 and 14 from the predicted sequence of the rabbit CD1b gene published later (Calabi et al. 1989) - see Figure 4.10.

When the sequence of the SBU-T6 protein is compared to other CD1 sequences of different species, it is clear that the most significant homology exists with the predicted sequence of HCD1E (Figure 4.8b). Identity to the other CD1 sequences is less than 55% in all cases across this region and of particular note is the fact that the SBU-T6 sequence is no more homologous to the known sheep CD1 sequences than to the CD1a-d isotypes in other species providing strong evidence that this is the sequence of a novel sheep CD1 isotype.

The isolation of the SBU-T6 antigen and its partial identity to the predicted amino-acid sequence of the CD1E gene was an unexpected but very interesting finding as a protein product of the CD1E gene has not yet been reported. The significance and origin of the second protein identified alongside the CD1 protein at 46 kd is uncertain and in view of the fact that no homology to other proteins could be found, it will not be discussed further.

As discussed in Chapter 1, analysis of the $\alpha 1$ and $\alpha 2$ regions of the CD1 molecules has allowed division of the CD1 molecules into two classes based on the presence of defined class specific residues. These residues are shown as boxed regions in Figure 4.11. The SBU-T6 antigen shares four class specific residues out of a possible five with the predicted sequence of human CD1E (Figure 4.11). HCD1E is the only CD1 gene to have class specific residues of glycine at positions 30 and 33 in the $\alpha 1$ domain. Considering this information together with the overall sequence homology to CD1E, it is logical to conclude that the CD1 molecule recognised by SBU-T6 may represent the sheep homologue of the antigen encoded by the CD1E gene. Although no protein product of this gene has yet been described in humans, all of the human CD1 genes have been shown to be transcribed in the MOLT-4 cell line implying that the CD1E gene is transcriptionally active and does not represent a pseudogene. An

alternative possibility is that the the antigen recognised by SBU-T6 represents a novel CD1 isotype which may be peculiar to ruminants.

FIGURE 4.10

Comparison of reported NH_2-terminal CD1 amino-acid sequences.

FIGURE 4.10

α1 domain	1				5					10					15					20					25					30				
Rabbit - NTS[1]	S	D	A	L	Q	G	P	T	S	Y	H	V	M	E	I	S	S	F															
Predicted sequence[3]	E													Q																			
Human CD1a[2]		D	G	L	K	E	P	L	S	F	H	V	T	W	I	A	S	F	Y														
Ovine CC14			V	F	Q	G	P	T	S	F	H	L	K	Q	I																		
Ovine SBU-T6					E	E	S	P	S	F	R	L	I	Q	I	S	S	F	A	N	H	S	W	T	K	T	Q	G	S	G	X	L	G

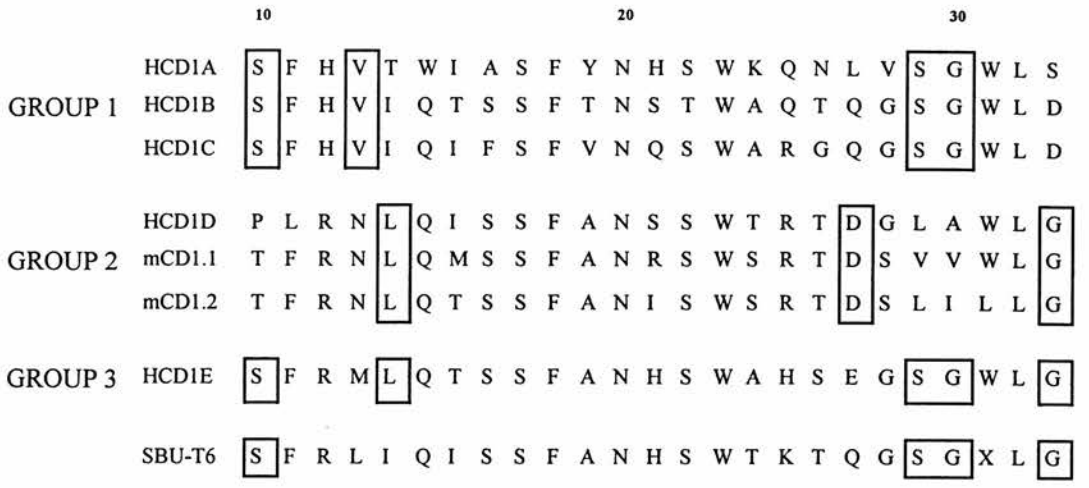
1. Wang et al., 1988.
2. Woolfson et al., 1994.
3. Calabi et al., 1989.

FIGURE 4.11

Interspecies comparison of NH_2-terminal CD1 amino-acid sequences to illustrate the existence of class specific residues. CD1a, CD1b and CD1c belong to group 1 and CD1d forms group 2. Human CD1e is in an intermediate position as it shares some class specific residues with both group 1 and group 2 proteins. With the exception of the Leucine residue at position 14, the SBU-T6 protein shares class specific residues with the predicted sequence of the HCD1E gene.

FIGURE 4.11

CLASS SPECIFIC CD1 RESIDUES



CHAPTER 5

PCR FOR THE GROUP 2 OVINE CD1 GENE(S)

5.1 Introduction

The results of the NH_2-terminal sequencing described in Chapter 4 clearly demonstrate the identification of a novel CD1 protein. The next step was to utilize this information to obtain the sequence of the gene encoding this molecule. The most logical way to achieve this was to convert an appropriate region of the amino acid sequence into nucleotide sequence which could then be used as a 5' primer along with a conserved 3' primer in a PCR to isolate the cDNA encoding this antigen.

The strong sequence conservation within the α3 region amongst the CD1 isotypes (which is present even amongst the most divergent isotypes) was exploited to generate a 3' primer capable of recognising all of the CD1 α3 regions described to date.

5.2 Design of the 5' Degenerate Primers

Several factors were taken into account in the design of the 5' degenerate primers. Where possible, stretches of protein sequence were chosen which contained the least ambiguous amino acids in terms of codon usage. Where several possibilities for codons existed, a combination of known ovine codon usage and conserved residues within the CD1 gene family was used to establish the most likely sequence coding for the amino acids in that region.

Figure 5.1 shows the region of amino acid sequence which was chosen for generation of the 5' primers and the sequence of the resultant primers P10763 and P14365.

5.3 Results

5.3.1 Thymocyte RT-PCR

The conditions utilized for all PCR reactions described in this chapter are shown in Table 5.1. As described in Chapter 2, all PCRs were 'hot-started' to reduce primer oligomerization.

FIGURE 5.1

Design of 5' degenerate primers.

(a) Region of amino-acid sequence chosen for design of the 5' degenerate primers.

(b) Sequences of the two primers derived from this region as described in the text.

FIGURE 5.1

a

SBU-T6 SEQUENCE

E E S P S F R L I Q I S S F **A N H S W T K T** Q G S G X L G

b

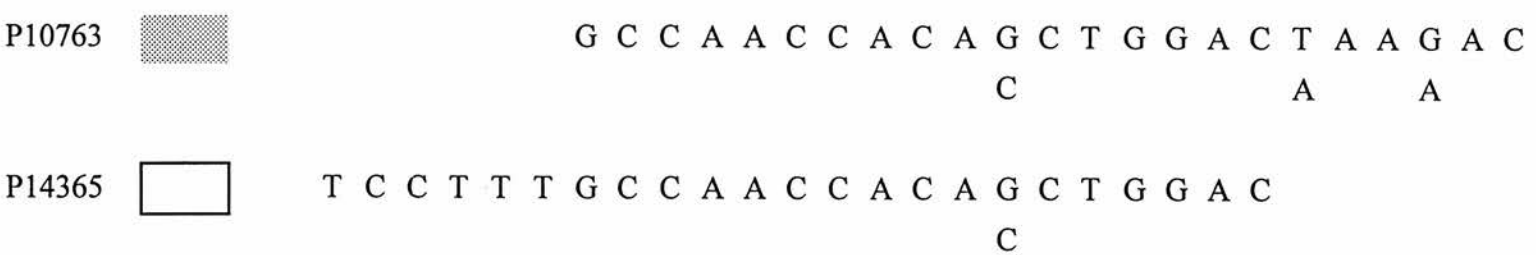


TABLE 5.1

Table outlining primer usage and cycle parameters for individual PCRs.

TABLE 5.1

SUBSTRATE					
CDNA	**PCR (NESTED)**	**PRIMERS**	**CYCLE NO.**	**CYCLE PARAMETERS**	**EXTENSION**
THYMOCYTE		P14365 P9414	35	94 - 1', 55 - 1.5', 72 - 1.5'	72 - 10'
		P10763 P9414	35	94 - 1', 55 - 1.5', 72 - 1.5'	72 - 10'
	1µl P14365, P9414	P10763 P4252	20	94 - 1', 55 - 1', 72 - 1'	72 - 10'
PBL		G5936 AP1	35	94 - 0.5', 55 - 0.5', 68 - 3'	-
	1µl G5936, AP1	P9413 AP2	20	94 - 0.5', 66 - 3'	72 - 10'
LYMPH NODE		P9414 P14365	35	94 - 1', 55 - 1.5', 72 - 1.5'	72 - 10'
		LN01 PCD2	35	94 - 1', 55 - 1.5', 72 - 1.5'	72 - 10'
	1µl LN01, PCD2	P9413 PCD2	20	94 - 1', 57 - 1', 72 - 1'	72 - 10'
		PCD1 P9414	35	94 - 1', 55 - 1.5', 72 - 1.5'	72 - 10'
	1µl PCD1, P9414	PCD3 LN02	20	94 - 1', 57 - 1', 72 - 1'	72 - 10'
	1µl PCD1, P9414	PCD3 LN03	20	94 - 1', 57 - 1', 72 - 1'	72 - 10'

cDNA was synthesized using either oligo-dT or random hexanucleotide primers as described in Chapter 2B. The cDNA was then analysed by PCR utilizing primers specific for the ATPase gene (Woodall et al. 1994). The ATPase message should be detectable in all tissues (Schull et al. 1985) and any samples which were negative for this PCR product were not utilized further.

Primers P10763 and P14365 were used in conjunction with conserved α3 region primers with the aim of obtaining clones specific for the the gene encoding the molecule recognised by SBU-T6. In addition, and in order to increase the specificity of the PCR reaction, a nested PCR was carried out utilizing alternative α3 region primers in conjunction with the degenerate 5' primers. The design of this nested PCR is illustrated in Figure 5.2. No specific PCR products were generated using either of these approaches.

5.3.2 'RACE' PCR

Having been unable to generate 5' degenerate primers capable of detecting the CD1E gene, an alternative approach, still utilizing the PCR technique was adopted. If gene specific primers could not be generated to detect the specific CD1 gene from a cell population containing all the CD1 genes (i.e. thymocytes), then a logical alternative would be to use primers from a 'conserved' region of the gene in a cell population which should only contain the gene of interest. Hence, utilizing the data showing that only the group 2 mAbs detect a percentage of peripheral blood mononuclear cells (i.e. B cells and monocytes), cDNA from this source was used as a substrate for PCR reactions to attempt to isolate the gene.

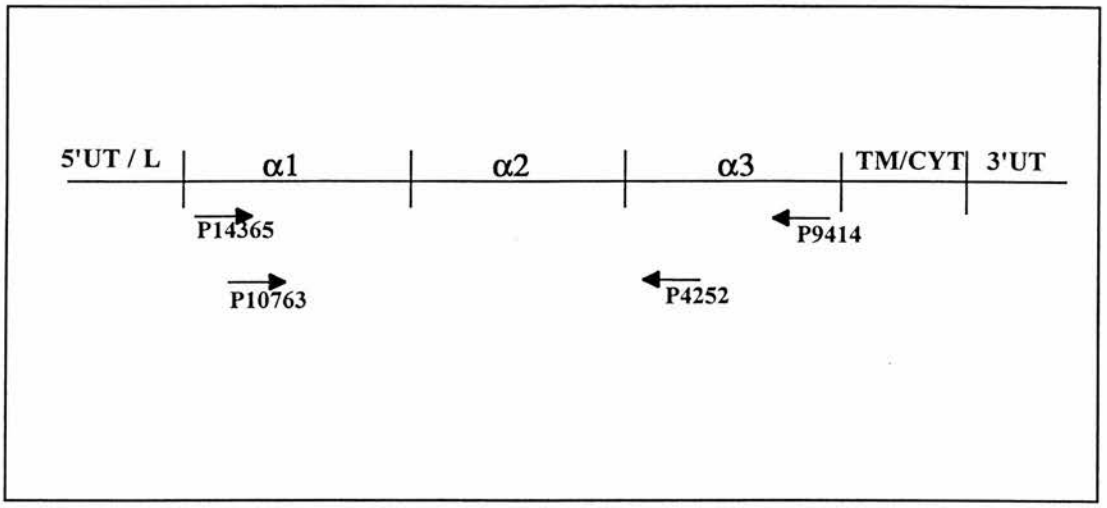
Generation of Adaptor Ligated cDNA

Total and PolyA$^+$ RNA was isolated as described in Chapter 2. The Clontech 'Marathon' cDNA kit was then used to generate double stranded cDNA which subsequently had adapters ligated onto both ends. 10% of the cDNA synthesis reaction was run on a gel alongside the manufacturers control and is shown in Figure 5.3. This demonstrates the presence of a wide size range of cDNAs. Preparation of a

FIGURE 5.2

Diagram illustrating primer usage in nested PCRs using the degenerate 5' primers in conjunction with conserved α3 region primers.

FIGURE 5.2



population of cDNA with adapters ligated at both ends was necessary as the α3 region was the only region for which a primer sequence was known. Hence an α3 specific primer could then be used as the gene specific primer in conjunction with the supplied adapter primer in a 'RACE' PCR.

A diagrammatic representation of the 'RACE' PCR technique used is shown in Figure 5.4. α3 primers were designed which were conserved across all known CD1 genes and utilised in both 3' and 5' PCR reactions.

This technique generated a product of the expected size following a nested PCR reaction utilising two α3 gene specific primers and adapter primers 1 and 2 (Figure 5.5a). The product was purified using a spinbind column and cloned as before using the TA cloning kit (Invitrogen). Sequencing of this insert revealed 90% homology to the previously cloned ovine CD1 gene SCD1B-42. This result was unexpected in that the earlier studies using mAbs to characterize CD1 expression showed that the group 1 mAbs did not react with PBLs. Thus any cells expressing 'CD1b' (i.e. dendritic cells) would be expected to be present in much smaller quantities than cells expressing the CD1e-like isotype recognised by SBU-T6.

5.3.3 Lymph node RT-PCR

For the work described in Chapter 6 to transfect cells with a cDNA library containing the gene of interest, a lymph node library contained within the vector pcDNA3 was obtained from Dr. Wayne Hein (Basel Institute for Immunology). A 1:100 dilution of this library was used as a substrate for PCR utilizing the degenerate 5' primers described above in conjunction with a conserved α3 region primer. The results of this PCR are shown in Figure 5.6a. The band which exists at approximately 700bp was estimated to be the correct size and was found consistently on repeating this PCR. A larger amount of the PCR product was therefore run on a gel and this band excised and purified using a spin bind column. The purified fragment was run on a gel to estimate quantity (Figure 5.6b) and was cloned using the TA cloning kit. 6 positive clones were analysed and restriction digests of the plasmid with EcoRI revealed that each plasmid contained the correct sized insert and 5 of the inserts

FIGURE 5.3

Agarose gel analysis of cDNA generated from mRNA using the 'Marathon cDNA kit' (Clontech).

M - marker DNA

Lane 1 - control DNA.

Lane 2 - cDNA from PBL mRNA.

Figure 5.4

Diagram illustrating strategy for 'RACE' PCR utilising gene specific primers in conjunction with supplied adapter primers 1 and 2 (AP1 and AP2).

FIGURE 5.3

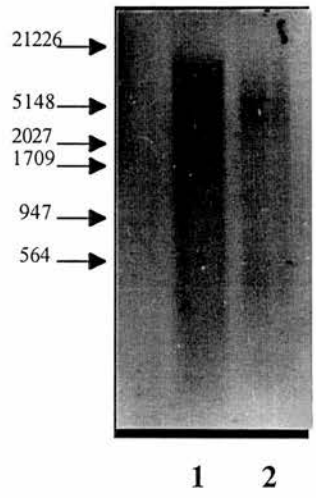


FIGURE 5.4

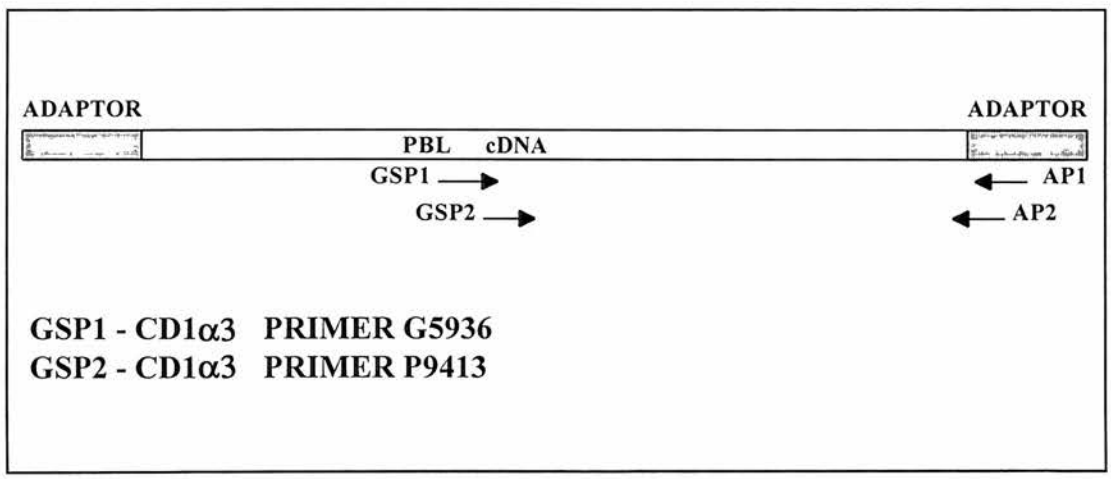


FIGURE 5.5

PCR and cloning of DNA from RACE PCR utilising primers as in Figure 5.4.

(a) - Nested PCR on adapter ligated PBL cDNA.

M - Marker DNA.

Lane 1 - First round of PCR (Primers G5936 and AP1).

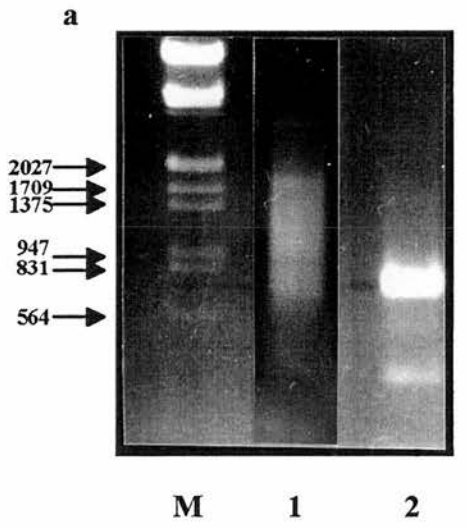
Lane 2 - Second round of PCR (Primers P9413 and AP2).

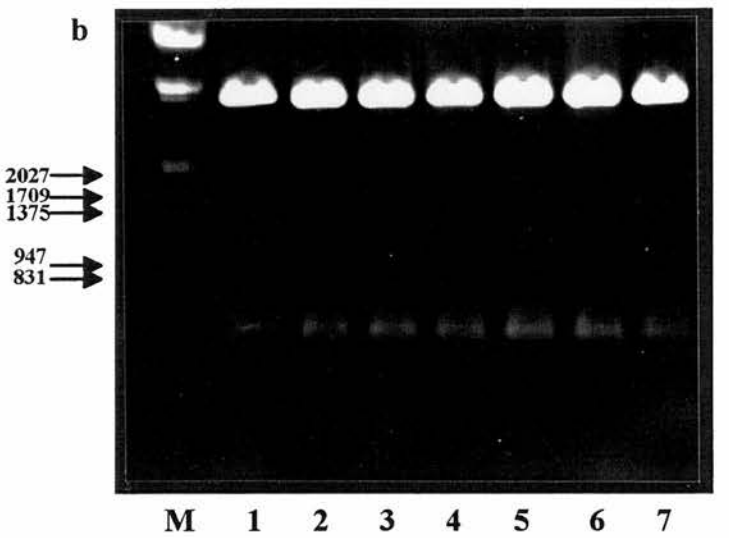
(b) - Cloning of 700bp product.

M - Marker DNA.

Lanes 1-7 - EcoRI restriction digests of plasmid from seven clones showing 700bp insert in each.

Figure 5.5


a
2027
1709
1375
947
831
564
M
1
2


b
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831
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7

contained an internal EcoRI site (Figure 5.6c). Sequencing of the insert confirmed that this was a CD1 gene which had closest homology to the CD1D genes and thus will now be referred to as SCD1D.

5.3.4 PCR for 5' and 3' ends of SCD1D

Having obtained partial sequence for the SCD1D gene, it was necessary to obtain full length sequence for this gene. The strategy adopted to perform this utilized the fact that the original sequence was obtained from a cDNA library within the vector pcDNA3. Primers were thus generated based on the sequence of this vector at both ends of the multiple cloning site and internal gene specific primers were designed based on the sequence of the segment of the SCD1D gene which was already cloned. The location of these primers and demonstration of the PCR strategy is illustrated in Figure 5.7.

Figure 5.8a shows the result of an anchored PCR for the 3' end of the gene. Lane 1 shows the products generated using primers LN01 and PCD2. Lane 2 shows the more specific result obtained by performing an anchored PCR using primers P9413 and PCD2 in a second PCR utilising DNA from lane 1 as a substrate. Since the primers used in the first and second rounds of PCR were 350bp apart, the product in lane 2 was thus the expected size and was cloned. 8 positive clones were analysed and restriction digests of the plasmid revealed that 5 contained an insert of the correct size (Figure 5.8b). Sequencing confirmed this to be the 3' end of the SCD1D gene.

Figure 5.9a shows the result of a nested PCR for the 5' end of the gene. The first round of PCR used the primers PCD1 and P9414. This PCR did not generate a specific product therefore 2 nested PCRs were subsequently carried out using this DNA as a substrate. Lane 1 shows this nested reaction utilizing primers PCD3 and LN03, lane 2 utilizing primers PCD3 and LN02. It is apparent that multiple bands are present in both reactions therefore it was necessary to perform a Southern blot of this gel and probe the DNA with internal oligonucleotides.

The result of the Southern blot is shown in figure 5.9b. This demonstrates hybridization of the probes to virtually all the bands. However the probes did not

hybridize to all the bands in lane 1 and those bands which are positive all show a size reduction in lane 2 consistent with an internal primer which was 200bp closer to the 5' end. This suggested that these bands did represent varying amounts of the 5' end of the LN1 gene. The largest band was therefore chosen for cloning. Restriction digests of purified plasmid from 8 clones are shown in Figure 5.9c. 6 of the clones contained inserts with internal EcoRI sites suggesting that this was indeed the 5' end of the gene. Sequencing of the inserts confirmed this, however the sequence obtained for the 5'UT region was not consistent with CD1 sequence and no methionine residue was evident. It thus appeared that the library was truncated at the 5' end such that sequence was only valid from residue 5 of the α1 domain (Figure 5.12).

Having obtained clones containing DNA for the majority of the gene, 4 clones for each section of the gene were amplified and purified using Qiagen midiprep columns. Sequencing of all four clones was then carried out and a consensus sequence for the gene obtained. Where nucleotide ambiguities existed, three out of four identical residues was taken as the consensus sequence.

FIGURE 5.6

PCR and cloning using lymph node cDNA.

(a) PCR on lymph node cDNA.

M - Marker DNA.

Lane 1 - Multiple bands using primers P14365 and P9414. The band at the correct size (700bp) is indicated by an arrow.

(b) DNA used for cloning. This was obtained by running a larger amount of the PCR product on a gel and excising the 700bp band from the gel. This was then purified on a spin-bind column.

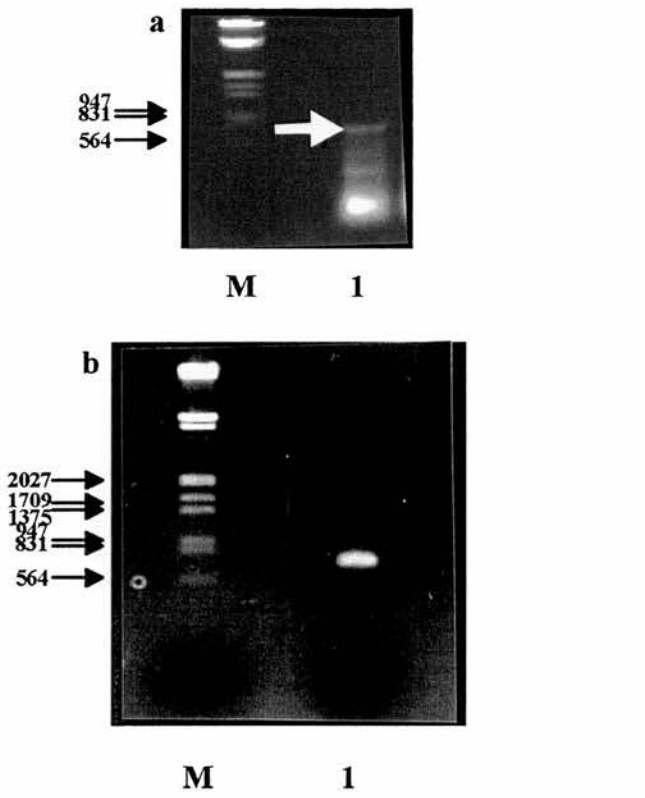
(c) EcoRI restriction digests of plasmids from six clones.

M - Marker DNA.

Lane 1 - Uncut plasmid.

Lanes 2-7 - EcoRI restriction digests of six clones showing 700bp inserts in all. With the exception of lane 5, all the inserts contain an EcoRI site.

Figure 5.6


a
947
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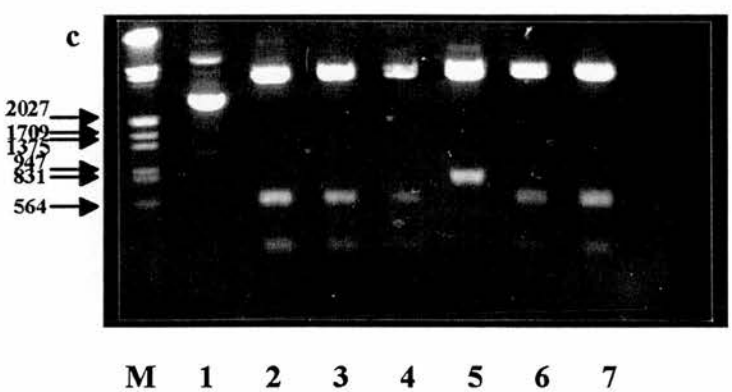
M
1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564
M
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6
7

FIGURE 5.7

Diagram illustrating the primers used and PCR strategy employed to obtain the 5' and 3' ends of the SCD1D gene.

A- Initial PCR reaction

B- Nested PCR for 3' end.

C- Nested PCR for 5' end.

Figure 5.7

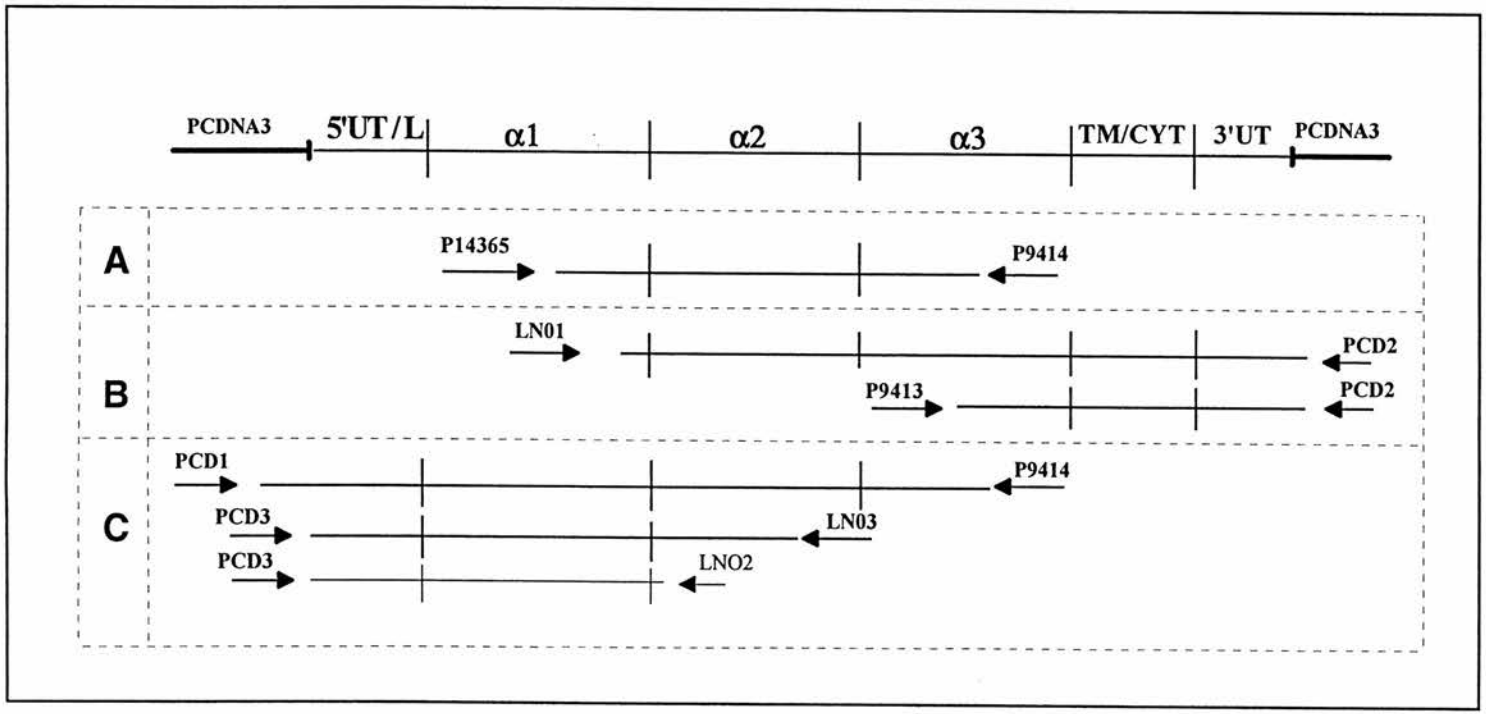

PCDNA3
5'UT/L
α1
α2
α3
TM/CYT
3'UT
PCDNA3
A
P14365
P9414
B
LN01
PCD2
P9413
PCD2
C
PCD1
P9414
PCD3
LN03
PCD3
LNO2

FIGURE 5.8

PCR and cloning of the 3' end of the SCD1D gene.

(a) Nested PCR for the 3' end.

M - Marker DNA.

Lane 1 - Multiple bands but large amounts of 1000bp product using primers LN01 and PCD2.

Lane 2 - Anchored PCR using primers P9413 and PCD2. One specific 650bp band is present.

(b) Cloning of 3' PCR product.

M - Marker DNA.

Lane 1 - Uncut plasmid.

Lanes 2-9 - EcoRI/HindIII restriction digests of plasmid from positive clones.

Lanes 2, 3, 5, 8 and 9 contain inserts of the correct size.

Figure 5.8

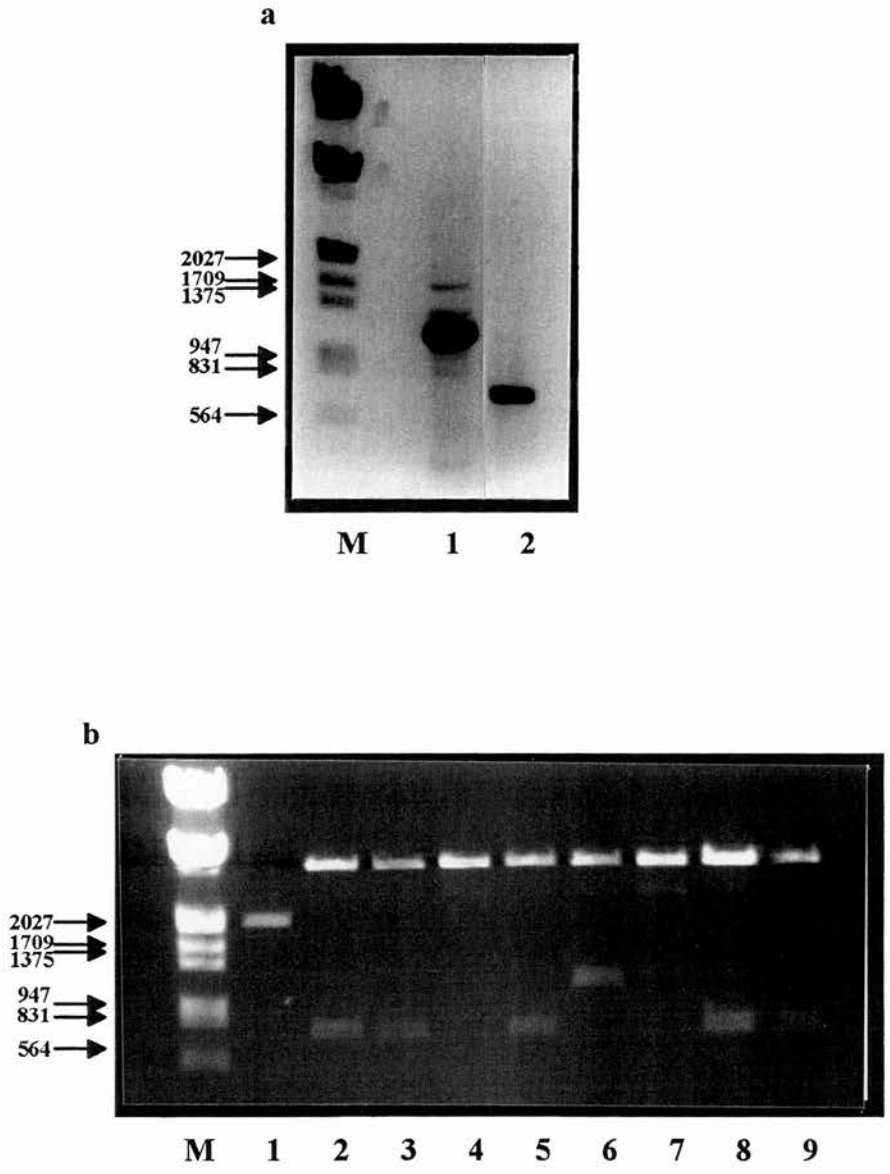

a
2027
1709
1375
947
831
564
M
1
2
b
2027
1709
1375
947
831
564
M
1
2
3
4
5
6
7
8
9

FIGURE 5.9

PCR and cloning of the 5' end of the SCD1D gene.

(a) Nested PCR utilising DNA generated with primers PCD1 and P9414 as a substrate.

M- Marker DNA.

Lane 1 - Multiple bands generated using primers PCD3 and LN03.

Lane 2 - Multiple bands generated using primers PCD3 and LN02.

(b) Southern blot of gel in Figure 5.9a probed with radiolabelled internal oligonucleotides specific for the SCD1D gene. Lanes 1 and 2 correspond to lanes 1 and 2 in Figure 5.9a.

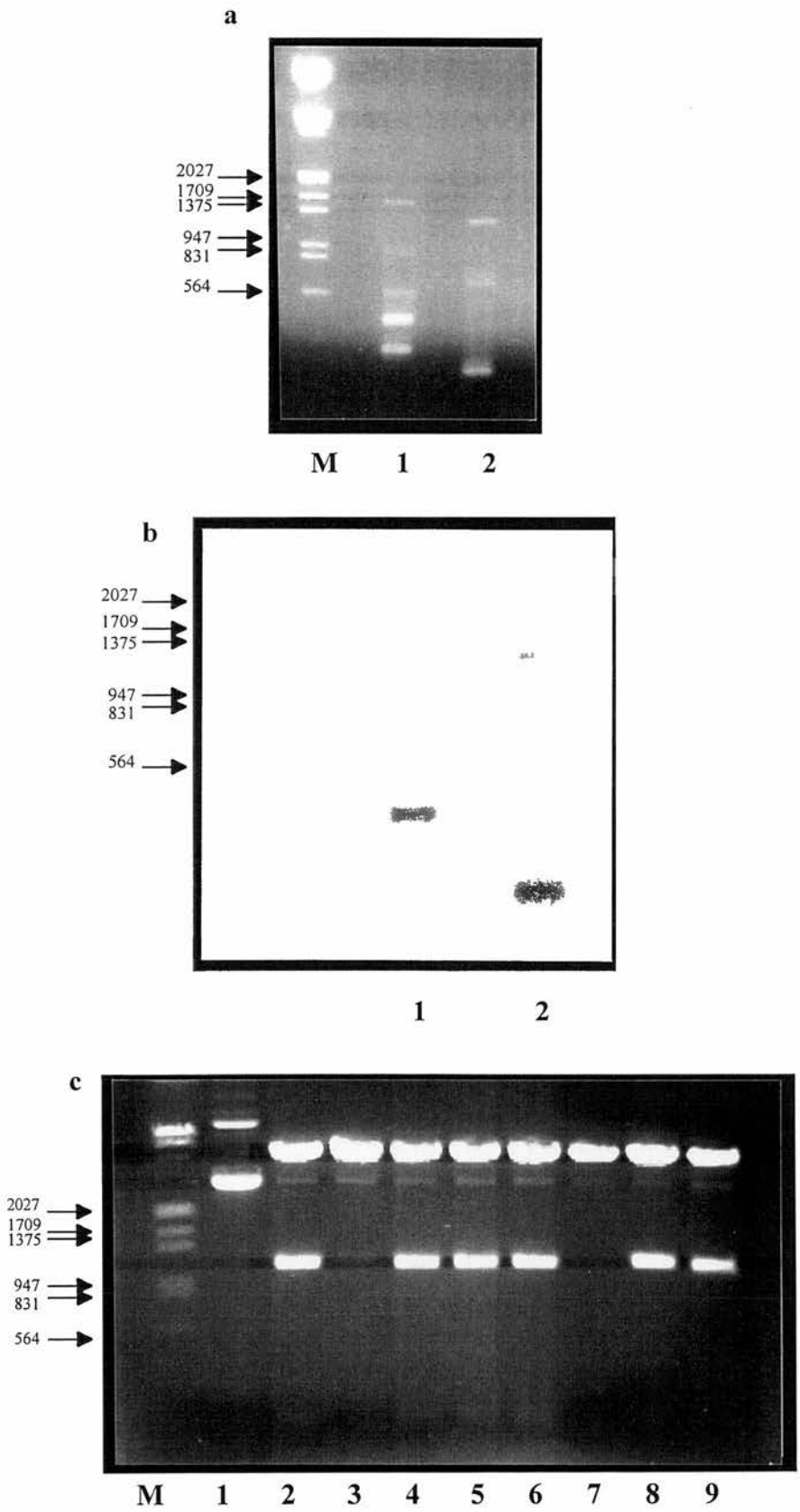
(c) Cloning of the 1.2kb product in Figure 5.9a - lane 2.

M- Marker DNA

Lane 1 - Uncut plasmid.

Lanes 2-9 - EcoRI/HindIII restriction digests of 7 positive clones. Lanes 2, 4, 5, 6, 8 and 9 contain inserts of the correct size and also demonstrate the presence of an EcoRI site within the insert.

Figure 5.9


a
2027
1709
1375
947
831
564
M 1 2
b
2027
1709
1375
947
831
564
1 2
c
2027
1709
1375
947
831
564
M 1 2 3 4 5 6 7 8 9

5.4 Sequence analysis

The SCD1D sequence was compared at the nucleotide and amino acid sequence level to sheep, human, mouse and rat sequences using the GAP programme of UWGCG 8.0. GAP uses the algorithm of Needleman and Wunsch (1970) to find the alignment of two sequences by maximizing the number of matches and minimizing the number of gaps. Default settings for gap weight and gap length (5.0 and 3.0 for nucleotide comparisons, 0.3 and 0.1 for amino acid comparisons respectively) were used.

Table 5.2 shows the percentage identity at both nucleotide and amino acid level between SCD1D and known sheep, human, rat, mouse and rabbit sequences. The sequence has closest homology to human CD1D (76.15% identity at the nucleotide level, 63.8% identity at the amino-acid level) and has greater than 70% identity at the nucleotide level with all the CD1D-like genes (with the exception of rat CD1 which is 69.54% identical). Identity to the group 1 CD1 genes (i.e. CD1A, -B and -C and the sheep CD1 genes) is less than 65% at the nucleotide level whilst identity to HCD1E is 66.67%. This demonstrates that this sheep CD1 gene is most closely related to the CD1D genes.

The PILEUP programme was used to compare SCD1D with the other sheep sequences and with CD1D cDNAs in other species. PILEUP creates a multiple sequence alignment using progressive pairwise alignments. Figure 5.10 shows sequence comparisons between SCD1D and the sheep CD1B-like cDNAs. Numbering is based on the full length sequence of SCD1A25 and SCD1B-42. A similar comparison between this cDNA and cDNAs of the CD1D family (i.e. mouse CD1.1 and CD1.2, ratCD1 cotton-tail rabbit CD1 and human CD1D) are shown in Figure 5.11. It is apparent from Figure 5.11 that identity in the 3' untranslated region appears to be very low with few regions of identity existing across the six genes. Table 5.3 shows the percentage identity between the domains of SCD1D and the domains of the other CD1D-like genes. As expected, the highest degree of homology exists in the $\alpha 3$ domain with the lowest homology in the 3' untranslated region when compared to rodent CD1. It is notable however that SCD1D is 65.45% identical to human CD1D in this region which is apparently higher than that shown in Figure

5.11 when sequences are aligned using PILEUP. Figure 5.12 resolves this contradiction by demonstrating that the GAP programme (which can achieve an optimal alignment of the two genes by introducing gaps into the sequence) shows regions of considerable identity throughout the 3' untranslated region of SCD1D and HCD1D.

The identity in the 3' untranslated regions of the CD1 genes is shown in Table 5.4. This shows that SCD1D is more homologous to HCD1D in this region than any of the rodent CD1D genes. SCD1B-42 and SCD1A25 show a similar percentage identity when compare to HCD1B (63% and 65% respectively).

It is interesting that although mCD1.1 and mCD1.2 are only 54% identical in this region, RtCD1 is 81% identical to mCD1.1. The only other genes which achieve this level of identity are SCD1B-42 and SCD1T10 i.e. genes from one species. This may have implications for the evolutionary relationship of mCD1.1 and RtCD1.

Interspecies comparisons of both groups of CD1 amino acid sequences are shown in Figure 5.13. Potential N-linked glycosylation sites are indicated by an asterisk. Class specific CD1 residues for CD1d-like proteins (as defined by Calabi et al. 1989b) are indicated by boxed residues. SCD1d has three potential N-linked glycosylation sites compared to five in mCD1d and four in human and rabbit CD1d. It can be seen that SCD1d shares the majority of class-specific CD1d residues in the α1 domain (11 out of 12) and in the α2 domain shares 8 out of 13. Since SCD1b proteins do not contain all the CD1a-c class specific residues, it is apparent that as more CD1 genes and proteins are identified in different species, so the original definition of these class specific residues may have to be modified.

The cytoplasmic tail of SCD1d contains a YXXZ motif (where Z is a hydrophobic residue). It has recently been shown that CD1b localizes to an endocytic compartment and that this localization is dependent upon the tyrosine-based motif in the cytoplasmic tail (Sugita et al. 1996). This motif is shared by the all the other CD1 proteins with the exception of HCD1a (and the predicted sequence of HCD1E).

TABLE 5.2

Table comparing percentage identity at nucleotide and amino-acid level between SCD1D and other CD1 sequences. These results were obtained using the GAP programme of UWGCG 8.0.

TABLE 5.2

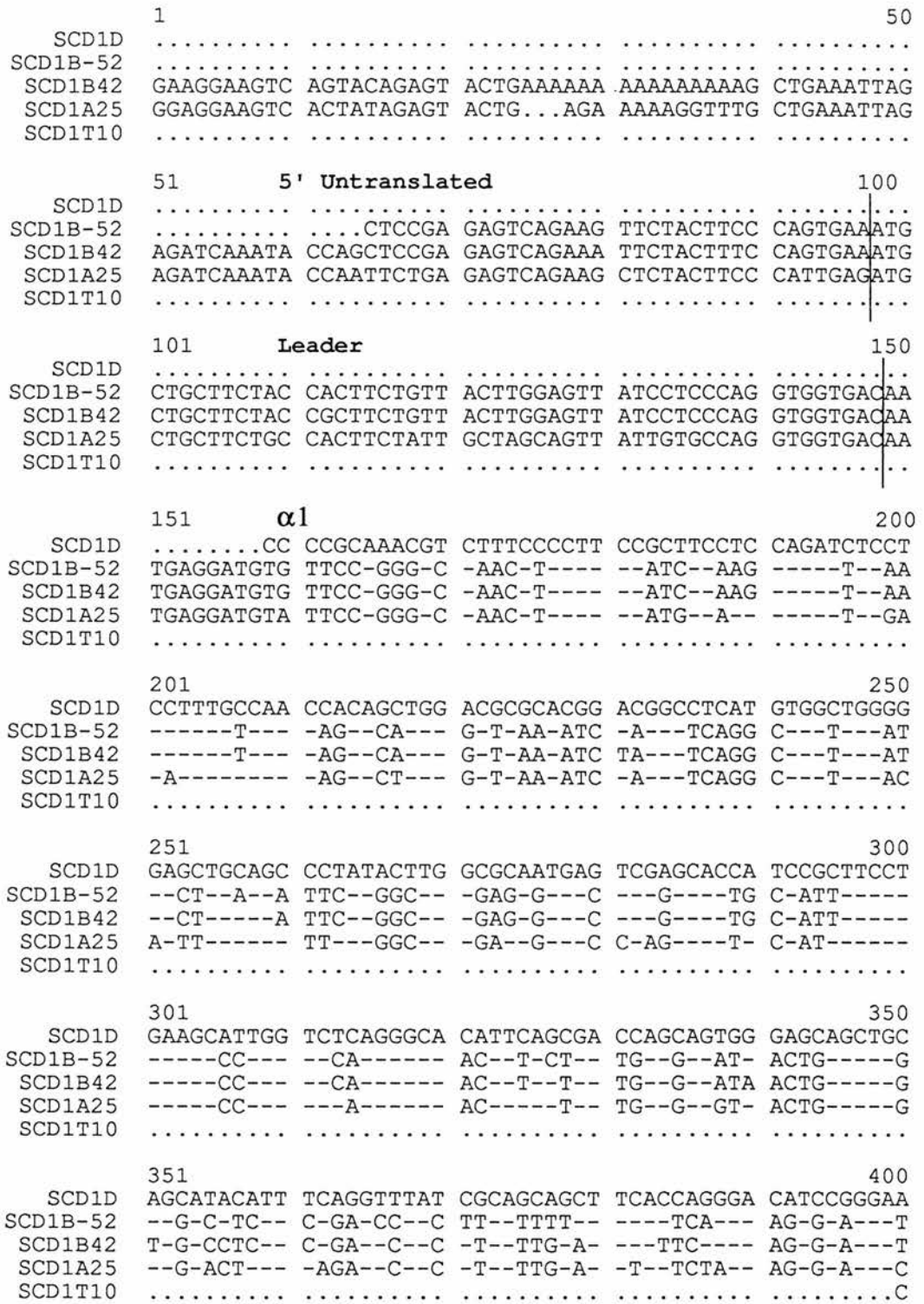
	NUCLEOTIDE	AMINO-ACID
HCD1D	76.15	63.80
CtRabCD1	74.77	64.96
mCD1.1	71.53	60.70
mCD1.2	71.53	59.74
RatCD1	69.54	59.55
HCD1E	66.67	55.30
HCD1A	64.00	52.92
HCD1B	63.31	52.08
SCD1A25	62.76	52.36
SCD1B42	62.66	56.00
SCD1T10	62.28	54.94
HCD1C	62.49	50.96

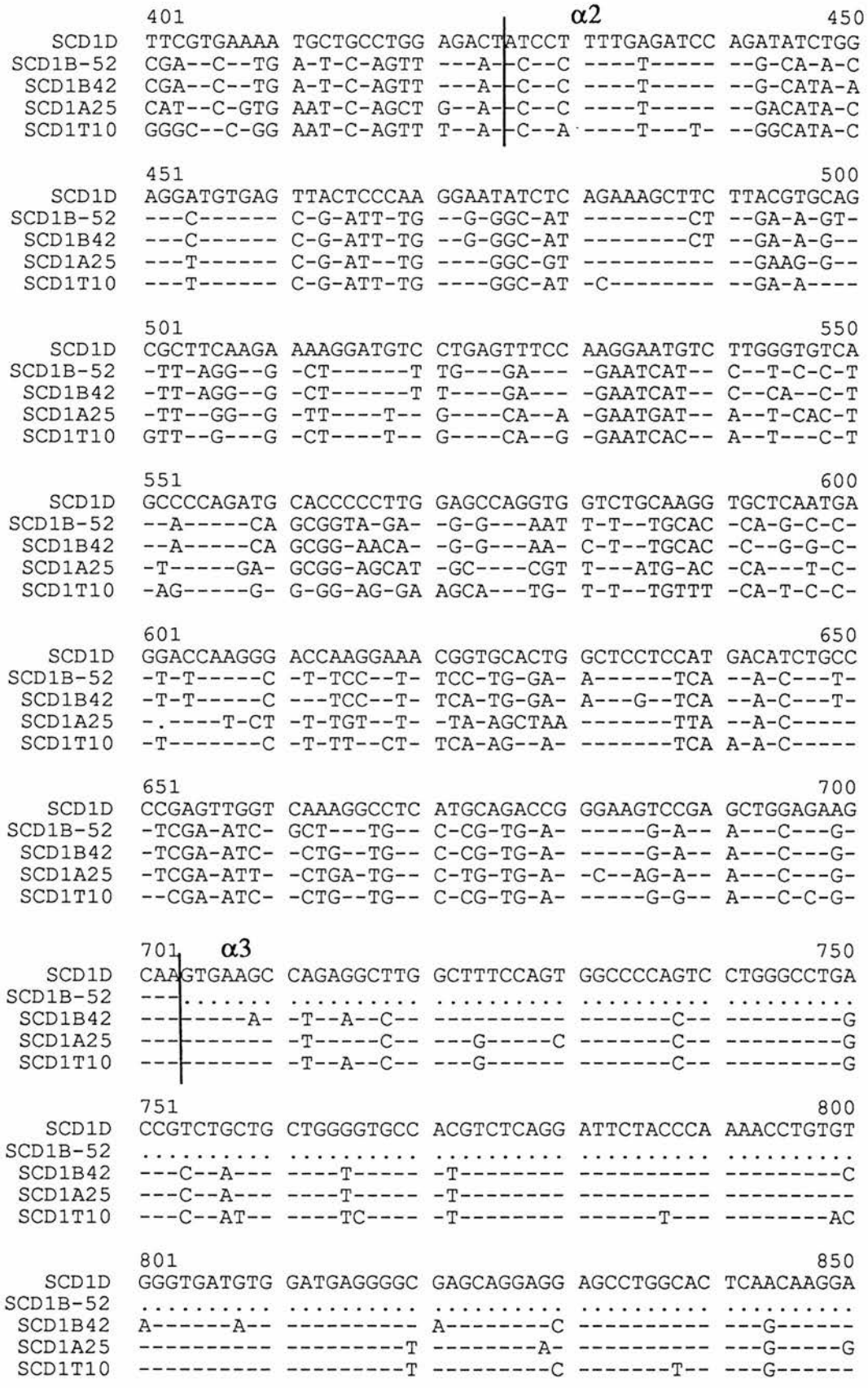
FIGURE 5.10

Alignment of ovine CD1 sequences using the PILEUP programme of UWGCG 8. Domain boundaries were defined by the SCD1B sequences.

Dashes indicate identity to the SCD1D nucleotide sequence. Dots indicate gaps introduced to maintain sequence alignment.

FIGURE 5.10


1
50
SCD1D ....
SCD1B-52 ....
SCD1B42 GAAGGAAGTC AGTACAGAGT ACTGAAAAAA AAAAAAAAAG CTGAAATTAG
SCD1A25 GGAGGAAGTC ACTATAGAGT ACTG...AGA AAAAGGTTTG CTGAAATTAG
SCD1T10 ....
51
5' Untranslated
100
SCD1D ....
SCD1B-52 ....CTCCGA GAGTCAGAAG TTCTACTTCC CAGTGAAATG
SCD1B42 AGATCAAATA CCAGCTCCGA GAGTCAGAAA TTCTACTTTC CAGTGAAATG
SCD1A25 AGATCAAATA CCAATTCTGA GAGTCAGAAG CTCTACTTCC CATTGAGATG
SCD1T10 ....
101
Leader
150
SCD1D ....
SCD1B-52 CTGCTTCTAC CACTTCTGTT ACTTGGAGTT ATCCTCCCAG GTGGTGACAA
SCD1B42 CTGCTTCTAC CGCTTCTGTT ACTTGGAGTT ATCCTCCCAG GTGGTGACAA
SCD1A25 CTGCTTCTGC CACTTCTATT GCTAGCAGTT ATTGTGCCAG GTGGTGACAA
SCD1T10 ....
151
α1
200
SCD1D .....CC CCGCAAACGT CTTTCCCCTT CCGCTTCCTC CAGATCTCCT
SCD1B-52 TGAGGATGTG TTCC-GGG-C -AAC-T---- --ATC--AAG -----T--AA
SCD1B42 TGAGGATGTG TTCC-GGG-C -AAC-T---- --ATC--AAG -----T--AA
SCD1A25 TGAGGATGTA TTCC-GGG-C -AAC-T---- --ATG--A-- -----T--GA
SCD1T10 ....
201
250
SCD1D CCTTTGCCAA CCACAGCTGG ACGCGCACGG ACGGCCTCAT GTGGCTGGGG
SCD1B-52 ------T--- -AG--CA--- G-T-AA-ATC -A---TCAGG C---T---AT
SCD1B42 ------T--- -AG--CA--- G-T-AA-ATC TA---TCAGG C---T---AT
SCD1A25 -A-------- -AG--CT--- G-T-AA-ATC -A---TCAGG C---T---AC
SCD1T10 ....
251
300
SCD1D GAGCTGCAGC CCTATACTTG GCGCAATGAG TCGAGCACCA TCCGCTTCCT
SCD1B-52 --CT--A--A TTC--GGC-- -GAG-G---C ---G----TG C-ATT-----
SCD1B42 --CT-----A TTC--GGC-- -GAG-G---C ---G----TG C-ATT-----
SCD1A25 A-TT------ TT---GGC-- -GA--G---C C-AG----T- C-AT------
SCD1T10 ....
301
350
SCD1D GAAGCATTGG TCTCAGGGCA CATTCAGCGA CCAGCAGTGG GAGCAGCTGC
SCD1B-52 -----CC--- --CA------ AC--T-CT-- TG--G--AT- ACTG-----G
SCD1B42 -----CC--- --CA------ AC--T--T-- TG--G--ATA ACTG-----G
SCD1A25 -----CC--- ---A------ AC-----T-- TG--G--GT- ACTG-----G
SCD1T10 ....
351
400
SCD1D AGCATACATT TCAGGTTTAT CGCAGCAGCT TCACCAGGGA CATCCGGGAA
SCD1B-52 --G-C-TC-- C-GA-CC--C TT--TTTT-- ----TCA--- AG-G-A---T
SCD1B42 T-G-CCTC-- C-GA--C--C -T--TTG-A- ---TTC---- AG-G-A---T
SCD1A25 --G-ACT--- -AGA--C--C -T--TTG-A- -T--TCTA-- AG-G-A---C
SCD1T10 C


α2
α3
401
450
SCD1D TTCGTGAAAA TGCTGCCTGG AGACTATCCT TTTGAGATCC AGATATCTGG
SCD1B-52 CGA--C--TG A-T-C-AGTT ---A--C--C ----T----- --G-CA-A-C
SCD1B42 CGA--C--TG A-T-C-AGTT ---A--C--C ----T----- --G-CATA-A
SCD1A25 CAT--C-GTG AAT-C-AGCT G--A--C--C ----T----- --GACATA-C
SCD1T10 GGGC--C-GG AAT-C-AGTT T--A--C--A ----T---T- --GGCATA-C
451
500
SCD1D AGGATGTGAG TTACTCCCAA GGAATATCTC AGAAAGCTTC TTACGTGCAG
SCD1B-52 ---C------ C-G-ATT-TG --G-GGC-AT --------CT --GA-A-GT-
SCD1B42 ---C------ C-G-ATT-TG --G-GGC-AT --------CT --GA-A-G--
SCD1A25 ---T------ C-G-AT--TG ----GGC-GT ---------- --GAAG-G--
SCD1T10 ---T------ C-G-ATT-TG ----GGC-AT -C-------- --GA-A----
501
550
SCD1D CGCTTCAAGA AAAGGATGTC CTGAGTTTCC AAGGAATGTC TTGGGTGTCA
SCD1B-52 -TT-AGG--G -CT------T TG---GA--- -GAATCAT-- C--T-C-C-T
SCD1B42 -TT-AGG--G -CT------T T----GA--- -GAATCAT-- C--CA--C-T
SCD1A25 -TT--GG--G -TT----T-- G----CA--A -GAATGAT-- A--T-CAC-T
SCD1T10 GTT--G---G -CT----T-- G----CA--G -GAATCAC-- A--T---C-T
551
600
SCD1D GCCCCAGATG CACCCCCTTG GAGCCAGGTG GTCTGCAAGG TGCTCAATGA
SCD1B-52 --A-----CA GCGGTA-GA- -G-G---AAT T-T--TGCAC -CA-G-C-C-
SCD1B42 --A-----CA GCGG-AACA- -G-G---AA- C-T--TGCAC -C--G-G-C-
SCD1A25 -T-----GA- GCGG-AGCAT -GC----CGT T---ATG-AC -CA---T-C-
SCD1T10 -AG-----G- G-GG-AG-GA AGCA---TG- T-T--TGTTT -CA-T-C-C-
601
650
SCD1D GGACCAAGGG ACCAAGGAAA CGGTGCACTG GCTCCTCCAT GACATCTGCC
SCD1B-52 -T-T-----C -T-TCC--T- TCC-TG-GA- A------TCA --A-C---T-
SCD1B42 -T-T-----C ---TCC--T- TCA-TG-GA- A---G--TCA --A-C---T-
SCD1A25 -.----T-CT -T-TGT--T- -TA-AGCTAA -------TTA --A-C-----
SCD1T10 -T-------C -T-TT--CT- TCA-AG--A- -------TCA A-A-C-----
651
700
SCD1D CCGAGTTGGT CAAAGGCCTC ATGCAGACCG GGAAGTCCGA GCTGGAGAAG
SCD1B-52 -TCGA-ATC- GCT---TG-- C-CG-TG-A- -----G-A-- A---C---G-
SCD1B42 -TCGA-ATC- -CTG--TG-- C-CG-TG-A- -----G-A-- A---C---G-
SCD1A25 -TCGA-ATT- -CTGA-TG-- C-TG-TG-A- -C--AG-A-- A---C---G-
SCD1T10 --CGA-ATC- -CTG--TG-- C-CG-TG-A- -----G-G-- A---C-C-G-
701
750
SCD1D CAAGTGAAGC CAGAGGCTTG GCTTTCCAGT GGCCCCAGTC CTGGGCCTGA
SCD1B-52 ---..... ....
SCD1B42 ---.....A- -T--A--C-- ---------- -------C-- ---------G
SCD1A25 ---.....-- -T-----C-- ---G-----C -------C-- ---------G
SCD1T10 ---.....-- -T--A--C-- ---G------ -------C-- ---------G
751
800
SCD1D CCGTCTGCTG CTGGGGTGCC ACGTCTCAGG ATTCTACCCA AAACCTGTGT
SCD1B-52 ....
SCD1B42 ---C--A--- ----T----- -T-------- ---------- ---------C
SCD1A25 ---C--A--- ----T----- -T-------- ---------- ----------
SCD1T10 ---C--AT-- ----TC---- -T-------- ------T--- --------AC
801
850
SCD1D GGGTGATGTG GATGAGGGGC GAGCAGGAGG AGCCTGGCAC TCAACAAGGA
SCD1B-52 ....
SCD1B42 A------A-- ---------- A--------C ---------- ---G------
SCD1A25 ---------- ---------T --------A- ---------- ---G-----G
SCD1T10 ---------- ---------T ---------C -------T-- ---G------

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          851                                                        900
   SCD1D  GACGTCATGC CCAATGCCGA CTCGACTTGG TATCTGCGAG TAACCCTGGA
SCD1B-52  .......... .......... .......... .......... ..........
 SCD1B42  ---A------ -------T-- --G------- -----C---- -------AA-
 SCD1A25  ---A------ -------AA- T-G------- C--------- C------A--
 SCD1T10  A--A----A- T------T-- T-G------- -----C---- ----------

          901                                                        950
   SCD1D  GGTGGCGGCT GGGGAGGCGG CTGGCTTGAG TTGCCGTGTG AAGCACAGCA
SCD1B-52  .......... .......... .......... .......... ..........
 SCD1B42  T-----A--- ---------- -----C---- ------A--- ----------
 SCD1A25  T-----A--- --A------- ----TC---- ------A--- ----------
 SCD1T10  T--------- --------A- -----C---- ---T--A--- ----------

          951                                                 Tm/Cyt 1000
   SCD1D  GTCTAGGAGA CCAGGACATC ATCCTGTACT GGGACGGGAA ACGTGTCTCC
SCD1B-52  .......... .......... .......... .....--AC- C-CCACA---
 SCD1B42  ---------- ---------- -----C---- --...--AC- C-CCACA---
 SCD1A25  ---------- ---------- G--------- --...--AC- C-CCAC----
 SCD1T10  ---------- ------T--- ---------- --...--AC- C-CCA-G-A-

          1001                                                       1050
   SCD1D  AGGGGCTTGA TTGTCGTCCT GGTAATACTG GTGTTCGTCC TTCTGTTTGT
SCD1B-52  -TT------- -AC-T-...- --C----A-A ---CC-TC-T -GA-CC--T-
 SCD1B42  -TT------- -AC-T-...- --C----A-A ---CC-TC-T -GA-CC--TC
 SCD1A25  -CT---C--- -AT-T-...- --C----A-A ----C-TC-- -CA-CC--T-
 SCD1T10  -TT------- -AT-T-...- --C----A-A ---CC-TC-T -GA-CC-CT-

          1051                                                       1100
   SCD1D  TGGAGGCTTA GTCTTCTGGT TTAGGAAGCA CCGCCGCTAT CAAGATATCT
SCD1B-52  GAT-T--C-T -CA--A---- --T---GA-G -T-GTCAC-- -GGA------
 SCD1B42  GAT-T--C-T -CA--A---- --T---GA-G -T-GTCA--- --GA------
 SCD1A25  GATCT-TC-T -CA--A---- --T---G--G -T-GTCA--- -TGAC-----
 SCD1T10  GAT-T-TC-T -CA--A---- --T---G--G -T-GTCA--- --GAC-G-T-

          1101    3' Untranslated                                    1150
   SCD1D  CGTGA..CTC TCCTGCCCAC ACCTATCTGT CTGGACTTCA GGATCTCAGG
SCD1B-52  T----GCACT GA-C-TGTCT C-T-T--CA- T-T-GAA-A- -T---CAG-T
 SCD1B42  T----GC-CT GA-CATGTCT C-T-T--..A T-T-GAA-A- -T---CAG-.
 SCD1A25  T----GC-C. AT-ACTGTCT C-T-T--C.A T-T-GAA-A- -T-C-CAC-A
 SCD1T10  T----TC-CT GA-CATGTCT C-.-T--CCA T-T-GAGCA- TT-C-CAG-A

          1151                                                       1200
   SCD1D  CCTTTAGCCC TCAGAATTTG AGGGTGAAAG AGAGCTACCT TGAAGAAGCA
SCD1B-52  A-CC-GAAA- ---AGT-G-C --CC-AGG-- TC--TCT-A- CAT-TTTCAT
 SCD1B42  AACC-GAAA- -T-AGT-G-C --CC-AGG-- TC-ATCT-A- -AT-TTTCAT
 SCD1A25  A-CCAGAAA- ---AGCGG-C --CCCAGT-- CC-AT-T-A- CAT-TTTCAT
 SCD1T10  G-CCAGAAA- ----GT-GAC --CCCAGG-- TC-AT-T-AC -AT-TTTCAT

          1201                                                       1250
   SCD1D  GAGAAGAGGG AGCTCTTCTC ACACACTTTG AACATTTTAG CTAAGAGGAT
SCD1B-52  C-A-TA-TCA TCACA--TGA TC-A-.CA-T GTTCC-G--- G-TGT-A---
 SCD1B42  C-A-TA-TCA TCACA--TGA TC-A-TCA-T GTTCC-G--- G-TGT-A---
 SCD1A25  C-A-TA-TCA TCTGA-CAAA TC-A...... GTTCC-G--- A-TGC-A---
 SCD1T10  C-A-TA-CCA TCA-A--TGA TC-A-T-AGT GTTC-GA--A GCT.TTA---

          1251                                                       1300
   SCD1D  TTAAGTTTAT TTTTTTCTTT TAGTATACTA CAAGTGTATA TGTCCAAGCA
SCD1B-52  AG-TCA-A-- --A-AC-C-A GCAG-A--A- A--TGAA-AT ---TATTATG
 SCD1B42  AA-TCA-A-- --A-ACTC-A GCAG-A--AT A--TGAA-AT ---TATTATG
 SCD1A25  .......... .......CAG A-AC-G-A-T A---GTGT-C ATATATTATG
 SCD1T10  AA-TCA-A-- --A-A-AC-G GT-G-A-AAT G-..-AA-GT GTA-ATTAT-
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          1301                                                  1350
   SCD1D  GTTCTGTAAA AAAAGAAGTG CAAACTCAGA TACGTCTCAG GGACAAGCAG
SCD1B-52  AGA-CA--TC -CT--C--GA TCC------- -TTCATAG-T -TGATTTGT-
 SCD1B42  AGA-AA--TC -CT--C--GA TCC------- -TTCATAG-T -TGATTTGT-
 SCD1A25  AGA-AA--CT --T-TT--GA TCTG------ -TTC-TGG-T -TGGG-TGC-
 SCD1T10  AGA-AA--TC -CT--T--AA TCC--A-G-- -TTC..AGGT -TGA-GTGT-

          1351                                                  1400
   SCD1D  ATAAAGGTTG CAGTGTAAAA AAAAAAAAAA AAAAACCGGC GCTCGAGCAT
SCD1B-52  -G----AA-A T-CCT-GG-- T---TG---T G-TGTA-ACA AAAAA-AAAA
 SCD1B42  -G----.A-A T-CCT-GG-- T---TG---T G-TGTA-AC. ..........
 SCD1A25  GG----AA-- T-CCT-GT-- T---TG---T G-TGTA-AC. ..........
 SCD1T10  -G----AA-- T-TCTC-G-C T---TG---T G-TGTG-A-G A--AA-TTG-

          1401                                                  1450
   SCD1D  GCATCTAGAG GCCCTATCCT ATAGTGTCAC CTAAATGCTA GAGTAG....
SCD1B-52  AA-AAA-A-A AAAAA-A... .......... .......... ..........
 SCD1B42  .......... .......... .......... .......... ..........
 SCD1A25  .......... .......... .......... .......... ..........
 SCD1T10  -ACAT-CCTT TGG--TCTTA T-TTATAACA T-TTT-T-AC TCC--CTGAA

 SCD1T10  ATATCATTTG TCAAAATGAG CTAATTGTAA TTATGTCAAG ATAATTGTAT

 SCD1T10  TTGCAGAGAT GACCAGCATT TTAACTTAAT TTCATTGTAT CTGCTTGGAT

 SCD1T10  GATTTTCTAT CTTGGAGAAA CTGAGCTCCT TCTTCTTCCA GGGAGCCTTC

 SCD1T10  CTTGGTCCTA CAGTAGAAGT AACCATCCCT TGACTGATGT CATGCAGAGA

 SCD1T10  TCTACTTTCC TCTTTACTCA GAACATTGCA GTTCTTTATG TTTTCTTTTC

 SCD1T10  CTTCTTATGA GTTCTTTGAA AGAAAGTTCT TTGCAAGACT CGGCTCTAAG

 SCD1T10  TGG
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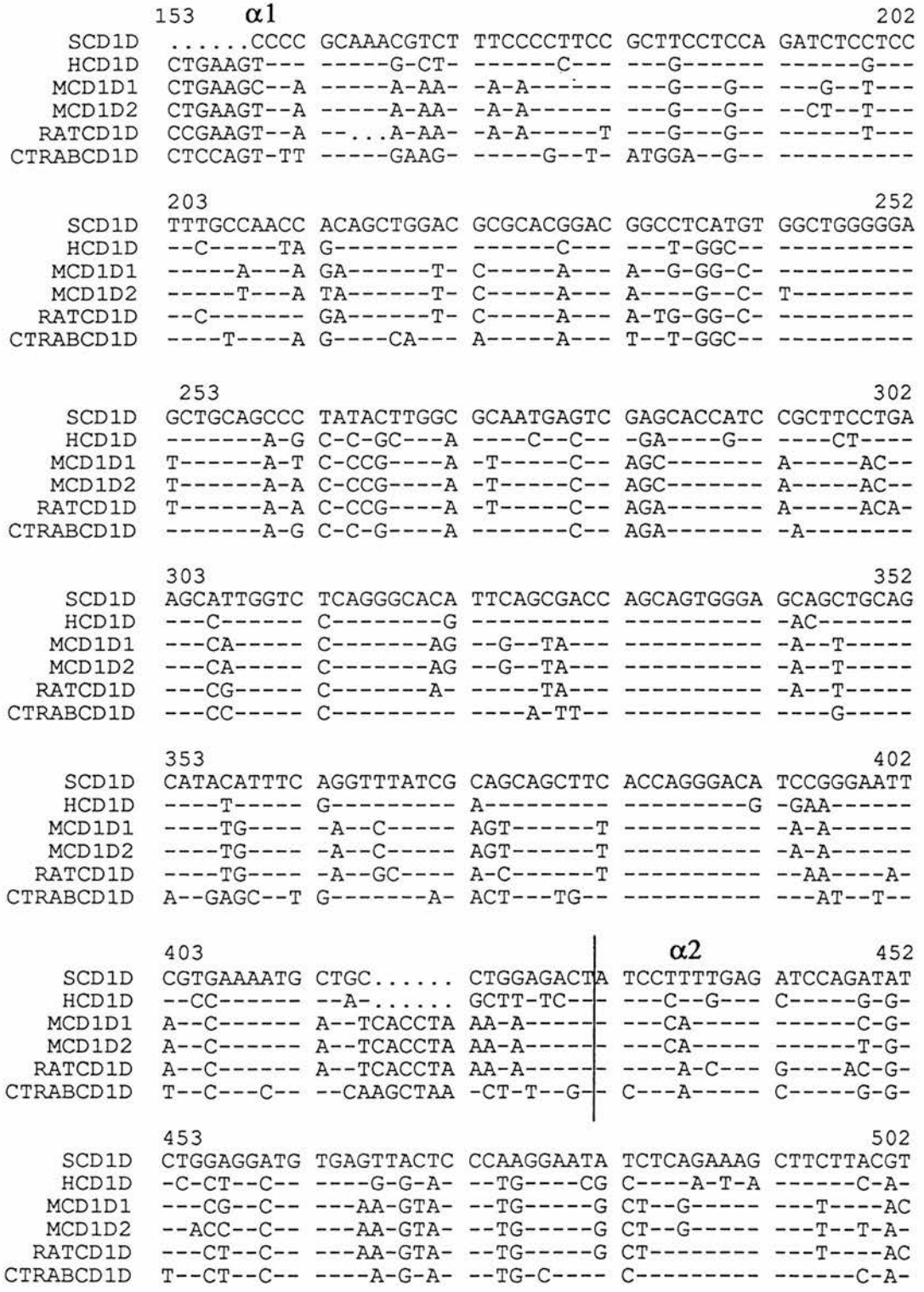
FIGURE 5.11

Alignment of ovine CD1 sequences using the PILEUP programme of UWGCG 8. Dashes indicate identity to the SCD1D nucleotide sequence. Dots indicate gaps introduced to maintain sequence alignment.

FIGURE 5.11


153 α1 202
SCD1D ......CCCC GCAAACGTCT TTCCCCTTCC GCTTCCTCCA GATCTCCTCC
HCD1D CTGAAGT--- -----G-CT- ------C--- ---G------ ------G---
MCD1D1 CTGAAGC--A -----A-AA- -A-A------ ---G---G-- ---G--T---
MCD1D2 CTGAAGT--A -----A-AA- -A-A------ ---G---G-- --CT--T---
RATCD1D CCGAAGT--A --...A-AA- -A-A-----T ---G---G-- ------T---
CTRABCD1D CTCCAGT-TT -----GAAG- -----G--T- ATGGA--G-- ----------
203 252
SCD1D TTTGCCAACC ACAGCTGGAC GCGCACGGAC GGCCTCATGT GGCTGGGGGA
HCD1D --C-----TA G--------- ------C--- ---T-GGC-- ----------
MCD1D1 -----A---A GA------T- C-----A--- A--G-GG-C- ----------
MCD1D2 -----T---A TA------T- C-----A--- A----G--C- T---------
RATCD1D --C------- GA------T- C-----A--- A-TG-GG-C- ----------
CTRABCD1D ----T----A G----CA--- A-----A--- T--T-GGC-- ----------
253 302
SCD1D GCTGCAGCCC TATACTTGGC GCAATGAGTC GAGCACCATC CGCTTCCTGA
HCD1D -------A-G C-C-GC---A ----C--C-- -GA----G-- ----CT----
MCD1D1 T------A-T C-CCG----A -T-----C-- AGC------- A-----AC--
MCD1D2 T------A-A C-CCG----A -T-----C-- AGC------- A-----AC--
RATCD1D T------A-A C-CCG----A -T-----C-- AGA------- A-----ACA-
CTRABCD1D -------A-G C-C-G----A -------C-- AGA------- -A--------
303 352
SCD1D AGCATTGGTC TCAGGGCACA TTCAGCGACC AGCAGTGGGA GCAGCTGCAG
HCD1D ---C------ C--------G ---------- ---------- -AC-------
MCD1D1 ---CA----- C-------AG --G--TA--- ---------- -A--T-----
MCD1D2 ---CA----- C-------AG --G--TA--- ---------- -A--T-----
RATCD1D ---CG----- C-------A- -----TA--- ---------- -A--T-----
CTRABCD1D ---CC----- C--------- ----A-TT-- ---------- ----G-----
353 402
SCD1D CATACATTTC AGGTTTATCG CAGCAGCTTC ACCAGGGACA TCCGGGAATT
HCD1D ----T----- G--------- A--------- ---------G -GAA------
MCD1D1 ----TG---- -A--C----- AGT------T ---------- -A-A------
MCD1D2 ----TG---- -A--C----- AGT------T ---------- -A-A------
RATCD1D ----TG---- -A--GC---- A-C------T ---------- --AA----A-
CTRABCD1D A--GAGC--T G-------A- ACT---TG-- ---------- ---AT--T--
403 α2 452
SCD1D CGTGAAAATG CTGC..... CTGGAGACTA TCCTTTTGAG ATCCAGATAT
HCD1D --CC------ --A-..... GCTT-TC--- ---C--G--- C-----G-G-
MCD1D1 A--C------ A--TCACCTA AA-A------ ---CA----- ------C-G-
MCD1D2 A--C------ A--TCACCTA AA-A------ ---CA----- ------T-G-
RATCD1D A--C------ A--TCACCTA AA-A------ ----A-C--- G----AC-G-
CTRABCD1D T--C---C-- --CAAGCTAA -CT-T--G-- C---A----- C-----G-G-
453 502
SCD1D CTGGAGGATG TGAGTTACTC CCAAGGAATA TCTCAGAAAG CTTCTTACGT
HCD1D -C-CT--C-- ----G-G-A- --TG----CG C----A-T-A ------C-A-
MCD1D1 ---CG--C-- ---AA-GTA- --TG-----G CT--G----- ---T----AC
MCD1D2 --ACC--C-- ---AA-GTA- --TG-----G CT--G----- ---T--T-A-
RATCD1D ---CT--C-- ---AA-GTA- --TG-----G CT-------- ---T----AC
CTRABCD1D T--CT--C-- ----A-G-A- --TG-C---- C--------- ------C-A-

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            503                                                       552
    SCD1D   GCAGCGCTTC AAGAAAAGGA TGTCCTGAGT TTCCAAGGAA TGTCTTGGGT
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   MCD1D1   -T---AT--- ---G---AT- ----G----A ---TGG---- C---C---CA
   MCD1D2   -T---AT--- ---G---AT- --C-G----A ----GG---- CA------CA
  RATCD1D   -T---AT--- ---G-G-AT- ----G----A -----T---- CA--C---CA
CTRABCD1D   -TG--CTA-- ---G--T-C- ---TT----- ----G----- CT-TG----A

            553                                                       602
    SCD1D   GTCAGCCCCA GATGCACCCC CTTGGAGCCA GGTGGTCTGC AAGGTGCTCA
    HCD1D   -C--A---A- --G--C--A- T----GTAA- CT---C-ATT C-A-------
   MCD1D1   -A---T---- -GG--C--AT -----TTAG- CT--CC-AT- --A-------
   MCD1D2   -AGG-T--T- -GG--C--AT -----TTAG- CT--CC-AT- --A-------
  RATCD1D   -AAG-T---- --G--C--AT -----TTAG- TT--CC-AT- --AA------
CTRABCD1D   -A----T--- -GGA-T--G- -G-TTGTAA- -C-T--TGT- --A-A-----

            603                                                       652
    SCD1D   ATGAGGACCA AGGGACCAAG GAAACGGTGC ACTGGCTCCT CCATGACATC
    HCD1D   -CC-----A- GT----G-G- -----A---- -G-------- TA---G--C-
   MCD1D1   -C-CT--T-- ------A-GT -C---C---- -GAT------ GA------C-
   MCD1D2   -C-CT--T-- ------A-GT -C---C---- -GAC------ GA------C-
  RATCD1D   -C-CT--TG- ---A--G-GA -----A---- -GATA----- GA-C----C-
CTRABCD1D   -CCT------ T-----A-GA ----T-A-A- -GGA------ GA--A---C-

            653                                                       702
    SCD1D   TGCCCCGAGT TGGTCAAAGG CCTCATGCAG ACCGGGAAGT CCGAGCTGGA
    HCD1D   ------C-A- -T----GT-- ----C-TG-- T-A------- -G--A---A-
   MCD1D1   ------CTAT -T---CGT-- T---C-AG-- G-A------- -A--C--A--
   MCD1D2   --G---C--- -T-C-CGT-- T---C-AG-- G-A------- -A--C--A--
  RATCD1D   ------C--- -T---CGT-- ----C-AG-A G-A------C -A--C--A--
CTRABCD1D   --T---C--- -T----GT-- T-----TG-- G-G----GA- -A--A-----

          703          α3                                           752
    SCD1D   GAAGCAAGTG AAGCCAGAGG CTTGGCTTTC CAGTGGCCCC AGTCCTGGGC
    HCD1D   --------|-- -----CA--- -C-----G-- -C-------- --------C-
   MCD1D1   --------|A- -------T-- -C---T-G-- -----T---- --CT---CAG
   MCD1D2   --------|A- -------T-- -C---T-G-- -----T---- --CT---CA-
  RATCD1D   A-------|A- -------T-- -C---T-A-- -C-------- -AC----CC-
CTRABCD1D   --------|-- -----C---- -------G-- ---------- --C-------

            753                                                       802
    SCD1D   CTGACCGTCT GCTGCTGGGG TGCCACGTCT CAGGATTCTA CCCAAAACCT
    HCD1D   ---G------ --------T- -----T---- ---------- ------G---
   MCD1D1   A--G--A-AG --A-----T- --T--T---- -T--C----- ----------
   MCD1D2   A--G--A--- --A-----T- --T--T---- -T--C----- ----------
  RATCD1D   A--G--A--- --A-----TA --T--T---- -T--C---C- ------G---
CTRABCD1D   ---G------ A-------TA --C-GT---- -T--C----- ------G---

            803                                                       852
    SCD1D   GTGTGGGTGA TGTGGATGAG GGGCGAGCAG GAGGAGCCTG GCACTCAACA
    HCD1D   --A------- A-------C- ---T------ ---C---AG- -------G-C
   MCD1D1   ---------- ----C---C- ---T--C--- ---C-A-AG- -T-----CAG
   MCD1D2   ---------- --------C- ---T--C--- ---C-A-AG- -T-----CAG
  RATCD1D   ---------- --------C- ---T--C--- ---C-AGGG- -T-----CAG
CTRABCD1D   --ACA----- --------C- ---A--C--- ---C----AC A-----G---

            853                                                       902
    SCD1D   AGGAGACGTC ATGCCCAATG CCGACTCGAC TTGGTATCTG CGAGTAACCC
    HCD1D   ---G---A-- C--------- -T---GA--- A--------C ----C-----
   MCD1D1   ---T--TT-- C----A---- -T--TGA--- A--------T -A--C-----
   MCD1D2   ---T--TT-- C----A---- -T--TGA--- A--------T -A--C-----
  RATCD1D   ---T---A-- C----A---- -G--TGA--- A--------T -A--C-----
CTRABCD1D   ---T---T-- C--------- ----TGG--- G--------C -----T----
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          903                                                        952
   SCD1D  TGGAGGTGGC GGCTGGGGAG GCGGCTGGCT TGAGTTGCCG TGTGAAGCAC
   HCD1D  ----T----T ---------- --A------C --TCC--T-- G---------
  MCD1D1  ----T----A ------A--- -AA------C --GCC---A- G---------
  MCD1D2  ----T----A ------A--- -AA------C --GCC---A- G---------
  RATCD1D ----T----A ------A--C -A-------C --GCC---A- G---------
CTRABCD1D ----T----- ---------T ---------- --TCC--T-- G---------

          953                                                 Tm/Cyt
   SCD1D  AGCAGTCTAG GAGACCAGGA CATC.ATCCT GTACTGGGAC GGGAAACGTG
   HCD1D  ---------- AG-G------ ----.G---- C-------GT ----GCTACA
  MCD1D1  -----C---- ---GA----- T---.----- C--------T -CC-GG-AA-
  MCD1D2  -----C---- ---GA----- T---.----- C--------T -CC-GG-AA-
  RATCD1D ---------- AG-GG----- ----.----- C-------GT --C-G--AA-
CTRABCD1D ---------- ---G------ ----T----- --------GT -A--.-A-GA

          1003                                                1052
   SCD1D  TCTCCAGGGG CTTGATTGTC GTCCTGGTAA TACTGGTGTT CGTCCTTCTG
   HCD1D  C-----T--- --------C- T-GGCA--CC -GGC-TGC-- GC-GT-C--C
  MCD1D1  CAC--GT--- -C----C--- T--A-A---C -GA-CA--C- A--GG-GG--
  MCD1D2  CAC--GT--- -C----C--- T--A-A---C --A-CA--C- A--GG-GG--
  RATCD1D -A---CC--T G-----CT-- T-GA-A---G GCG-AC--G- TC-AG-GG--
CTRABCD1D A--GGG--TC -AG-C-G-AA A-GGGA-... .......... ..........

          1053                                                1102
   SCD1D  TTTGTTGGAG GCTTAGTCTT CTGGTTTAGG AAGCACCGCC GCTATCAAGA
   HCD1D  C-CA---TG- ----TAC--C -C------A- -G---AACTT C------G-G
  MCD1D1  GG--C--T-- T--ACTAT.. .ATC-GG--A -G-AGAA--G CT--------
  MCD1D2  GG--C--T-- T--ACTAT.. .ATC-GG--A -G-AGAA--G CT--------
  RATCD1D -G--C--TG- C--ACTATA- -ATCAGG-AA -G-AGA---T C---------

          1103          3' Untranslated                       1152
   SCD1D  TATCTCGTGA CTCTCCTGCC CACACCTATC TGTCTGGACT TCAGGATCTC
   HCD1D  CG--CT---- ---G---TG- ----T--G-G -C------AC C-----C---
  MCD1D1  C---CG---- ----T-CTTA ---CTGCC-- -.......... C-T-A-AT--
  MCD1D2  C---CG---- ----T-CTTA ---CTGCC-- -.......... C-T-A-AT--
  RATCD1D C---AT---- GAT-T..TTA -C-CTGCC-- -.......... C-T-A-GT--

          1153                                                1202
   SCD1D  AGGCCTT... ......TAGC CCTCAGAATT TGAGGGTGAA AGAGAGCTAC
   HCD1D  T--A-C-CAG GTTCCCA--A -T----TCC- G-TCT-CTC- G--ATTGA-G
  MCD1D1  --A-T--CCA GGCTCTAG-A -T----TCC- G-TCT-CTC- G--TCTGGGG
  MCD1D2  --A-T--CCA GGCTCTAG-A -T----TCC- G-TCT-CTC- G--TCTGGGG
  RATCD1D --A----CCA GGTTCTAG-A -T----TCCC --TCT-CTC- GA-ACTGAGG

          1203                                                1252
   SCD1D  CTTGAAG.AA GCAGAGAAGA GGGAGCTCTT CTCACACACT TTGAACATTT
   HCD1D  A-GT---.G- ATT--AG-T- --AGAGATAC --TGA-A-AG -A--GA-CAG
  MCD1D1  A-GA-G-AG- -G-ATCCT-- A-A--TGAAG AG--GC--G- AC-CT-T---
  MCD1D2  A-GA---AG- -G-ATCCT-- A-A--TGAAG AG--AC--G- A--CT-C---
  RATCD1D A-AA--AAG- -G-ATCCT-- A-A-ATGAAG AG--AC--G- ACACC-T---

          1253                                                1302
   SCD1D  TAGCTAAGAG GATTTAAGTT TATTTTTTTC TTTTAGTATA CTACAAGTGT
   HCD1D  -CATG-G-CA -C---C-TCA C-CCC----A ACA-TTATCT AA-AG-A-T-
  MCD1D1  C-A-ATTA-T T--AAG-AA- --A--A---G AG-.T--T-C G-CAGTT-CC
  MCD1D2  A-AT..... .........- --GGAA-A-T AC--CC--AT TA-ATTAA-A
  RATCD1D .-ATATTAAT T--AAG-AA- C-A--A---A AA-GAT-T-T A--AGCT--G
```

```
          1303                                                      1352
  SCD1D   ATATGTCCAA GCAGTTCTGT AAAAAAAGAA GTGCAAACTC AGATACGTCT
  HCD1D   -A--TCTTTT T--AAAA-TA C-CT-C-AGT T-AT--G-C- -A--GGC---
 MCD1D1   ---GT-TAG- A--AACACAA CTGC---TGT -C-T-CCTG- CAGA-ACAAG
 MCD1D2   TC-AT--A-- TT-CA-G-A- GTGCCGGAGT -A-GTGGGGG T-G....-G-
RATCD1D   G--GT--AG- A--AACACAA CTGC-C-TGT -T-T-CCTG- CAGA-ACAAG

          1353                                                      1402
  SCD1D   CAGGGACAAG CAGATAAAGG TTGCAGTGTA AAAAAAAAAA AAAAAAAACC
  HCD1D   GT-AA-TC-- A--TGC--A- G--TGCAAAC TTGT-TCTG- -G-CCT-C-A
 MCD1D1   -T-TTGGC-- TGTT-T-T-- GATTT-CACT G--CT-G--- GC-T-CTT--
 MCD1D2   -T-T-CTCTT G-ATACT--T GAT-TTAAA- GT-GG-G-GC CTGCGGTTT-
RATCD1D   -T-TG-GC-- TGTC-CGT-- GATTT--ACT G--CT-G-CT GC-CGCTT--

          1403                                                      1452
  SCD1D   GGCGCTCGAG CATGCATCTA GAGGCCCTAT CCTATAGTGT CACCTAAATG
  HCD1D   --GA-AA-CA GG-AAGAGCT --T-TGAGTG TG-G-GA--G G-T--GT-A-
 MCD1D1   T--C-AAACA G-C--TCTG- -GTTAGT-GG -AAG-GTAAA GT--A-C-CC
 MCD1D2   CCT--AGCCG G-GTT--AGC CC-AG-AGGG TGATAT-GAC T-TAGG--CC
RATCD1D   T--C-AAACA G-CA-T-TGG -G-TTGT-GG -AAG-GTAAA GT--AAC-CC

          1453
  SCD1D   CTAGAGTAG. ..........  ..........  ..........  ..........
  HCD1D   GA-CT-G-AC ACACATGTCC TATCCAAAGG AATCAGCTGC AGCTGCTTGT
 MCD1D1   AACCT-GCTG TACTCTGTAT TTTTCAAGGT GACTAGAAAA ATGGAT..T.
 MCD1D2   AACTCTAGTC CTCTGCAAGG CCAGTGTGTG GTCTAAGGGA CTGAATCATC
RATCD1D   AACAC-ACTG TACTCTGTAT TTTTCAAGAT AACGAGAAAA ATGGAT..TT
```

TABLE 5.3

Percentage identity at the nucleotide level between the domains of SCD1D and the other CD1D sequences. These results were obtained using the GAP programme of UWGCG 8.0.

TABLE 5.4

Percentage identity at the nucleotide level when the sequences of all CD1 3' untranslated regions are compared. These results were obtained using the GAP programme of UWGCG 8.0.

Comparisons greater than 50% are highlighted.

TABLE 5.3

	α1	α2	α3	TM/CYT	3'UT
HCD1D	80.90	72.76	84.23	56.30	65.45
MCD1.1	74.16	69.53	77.78	56.90	42.48
RatCD1	71.91	67.38	77.42	52.94	43.36
CtRabCD1	72.66	69.18	85.30	51.43	_

TABLE 5.4

	SCD1A25	SCD1T10	HCD1B	HCD1A	HCD1C	HCD1D	MCD1.1	MCD1.2	RtCD1	SCD1D
SCD1B-42	75.49	81.09	63.93	37.54	41.09	45.71	45.32	35.71	44.04	39.76
SCD1A25		70.04	52.65	41.41	39.32	44.79	46.67	38.61	44.53	39.78
SCD1T10			65.55	41.65	34.50	41.16	42.77	39.00	38.22	39.85
HCD1B				40.32	40.73	44.31	42.80	37.96	43.62	44.35
HCD1A					58.82	38.93	35.98	40.77	39.82	42.25
HCD1C						38.31	55.88	43.51	39.61	36.58
HCD1D							53.87	45.76	55.89	65.46
MCD1.1								54.61	81.62	42.48
MCD1.2									54.16	45.43
RtCD1										43.36

FIGURE 5.12

Pairwise comparison between the 3' untranslated regions of SCD1D and HCD1D. Alignment was achieved using the GAP programme of UWGCG 8.0. Seven gaps have been introduced into the SCD1D sequence to obtain maximum alignment.

FIGURE 5.12

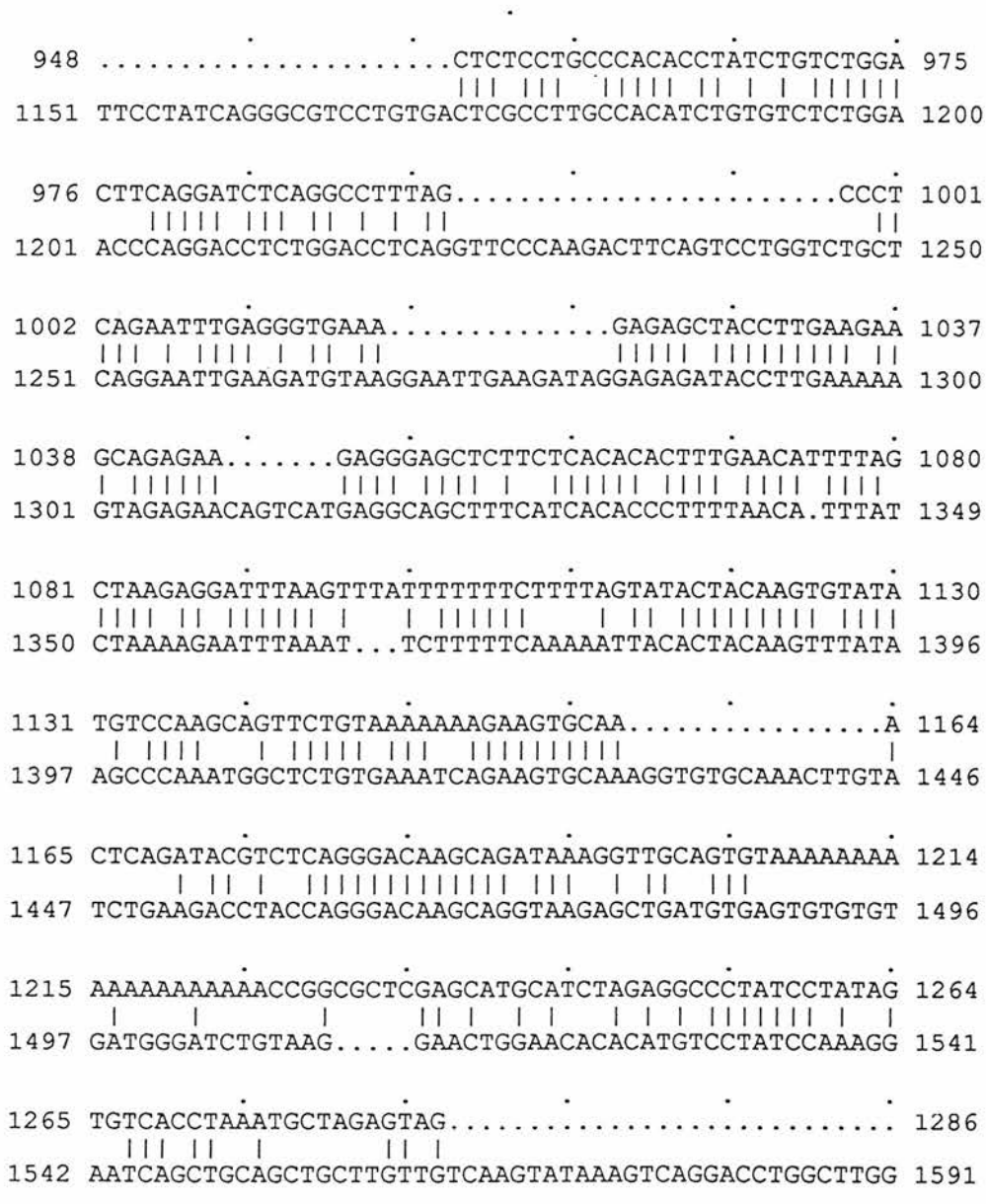

948 .....CTCTCCTGCCCACACCTATCTGTCTGGA 975
1151 TTCCTATCAGGGCGTCCTGTGACTCGCCTTGCCACATCTGTGTCTCTGGA 1200
976 CTTCAGGATCTCAGGCCTTTAG.....CCCT 1001
1201 ACCCAGGACCTCTGGACCTCAGGTTCCCAAGACTTCAGTCCTGGTCTGCT 1250
1002 CAGAATTTGAGGGTGAAA.....GAGAGCTACCTTGAAGAA 1037
1251 CAGGAATTGAAGATGTAAGGAATTGAAGATAGGAGAGATACCTTGAAAAA 1300
1038 GCAGAGAA.....GAGGGAGCTCTTCTCACACACTTTGAACATTTTAG 1080
1301 GTAGAGAACAGTCATGAGGCAGCTTTCATCACACCCTTTTAACA.TTTAT 1349
1081 CTAAGAGGATTTAAGTTTATTTTTTCTTTTAGTATACTACAAGTGTATA 1130
1350 CTAAAAGAATTTAAAT...TCTTTTTCAAAAATTACACTACAAGTTTATA 1396
1131 TGTCCAAGCAGTTCTGTAAAAAAAGAAGTGCAA.....A 1164
1397 AGCCCAAATGGCTCTGTGAAATCAGAAGTGCAAAGGTGTGCAAACTTGTA 1446
1165 CTCAGATACGTCTCAGGGACAAGCAGATAAAGGTTGCAGTGTAAAAAAAA 1214
1447 TCTGAAGACCTACCAGGGACAAGCAGGTAAGAGCTGATGTGAGTGTGTGT 1496
1215 AAAAAAAAAAACCGGCGCTCGAGCATGCATCTAGAGGCCCTATCCTATAG 1264
1497 GATGGGATCTGTAAG.....GAACTGGAACACACATGTCCTATCCAAAGG 1541
1265 TGTCACCTAAATGCTAGAGTAG..... 1286
1542 AATCAGCTGCAGCTGCTTGTTGTCAAGTATAAAGTCAGGACCTGGCTTGG 1591

FIGURE 5.13

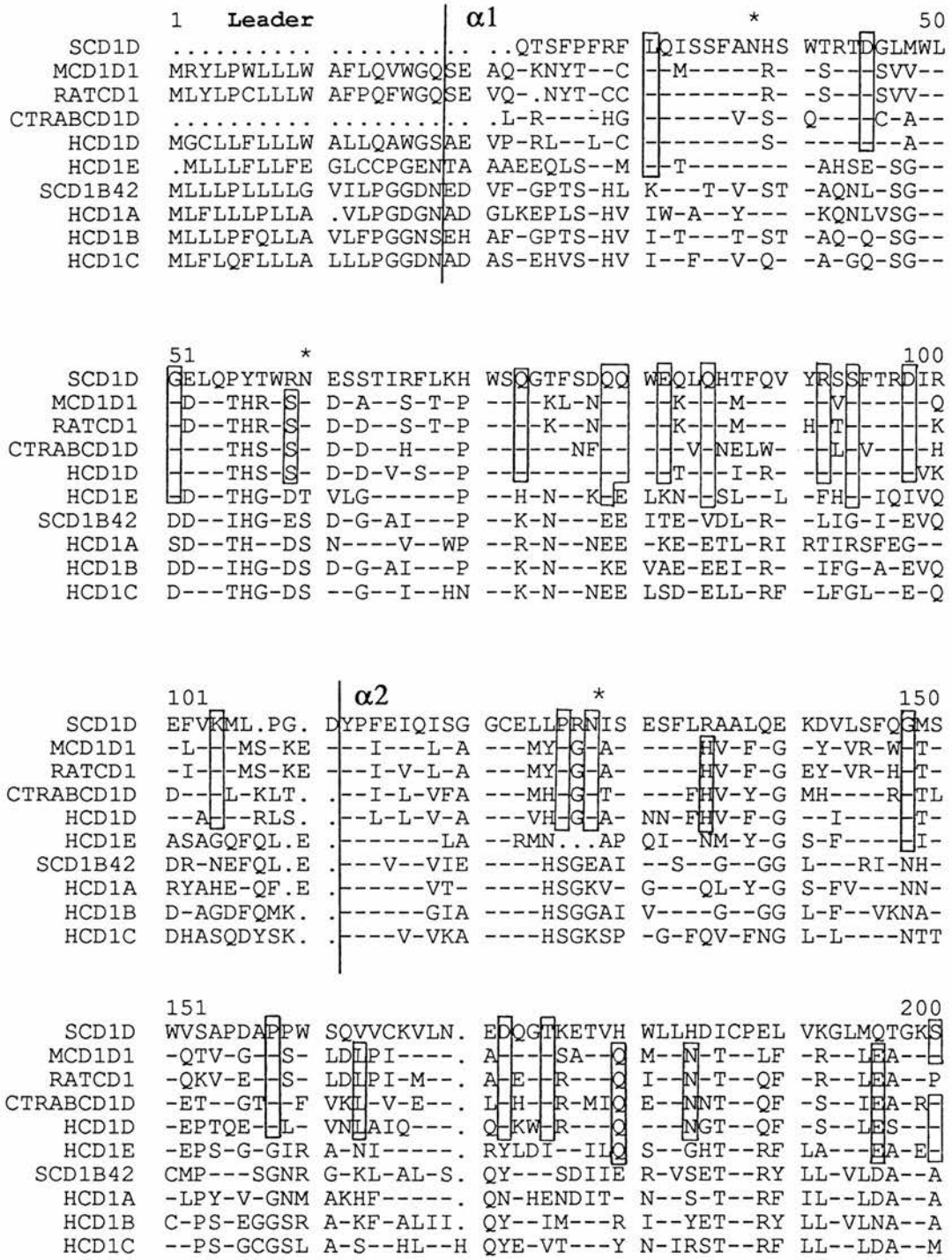
Alignment of deduced amino-acid sequence of SCD1D with the CD1d sequences; Human CD1a, -b and -c; SCD1B-42 and the predicted sequence of HCD1E.

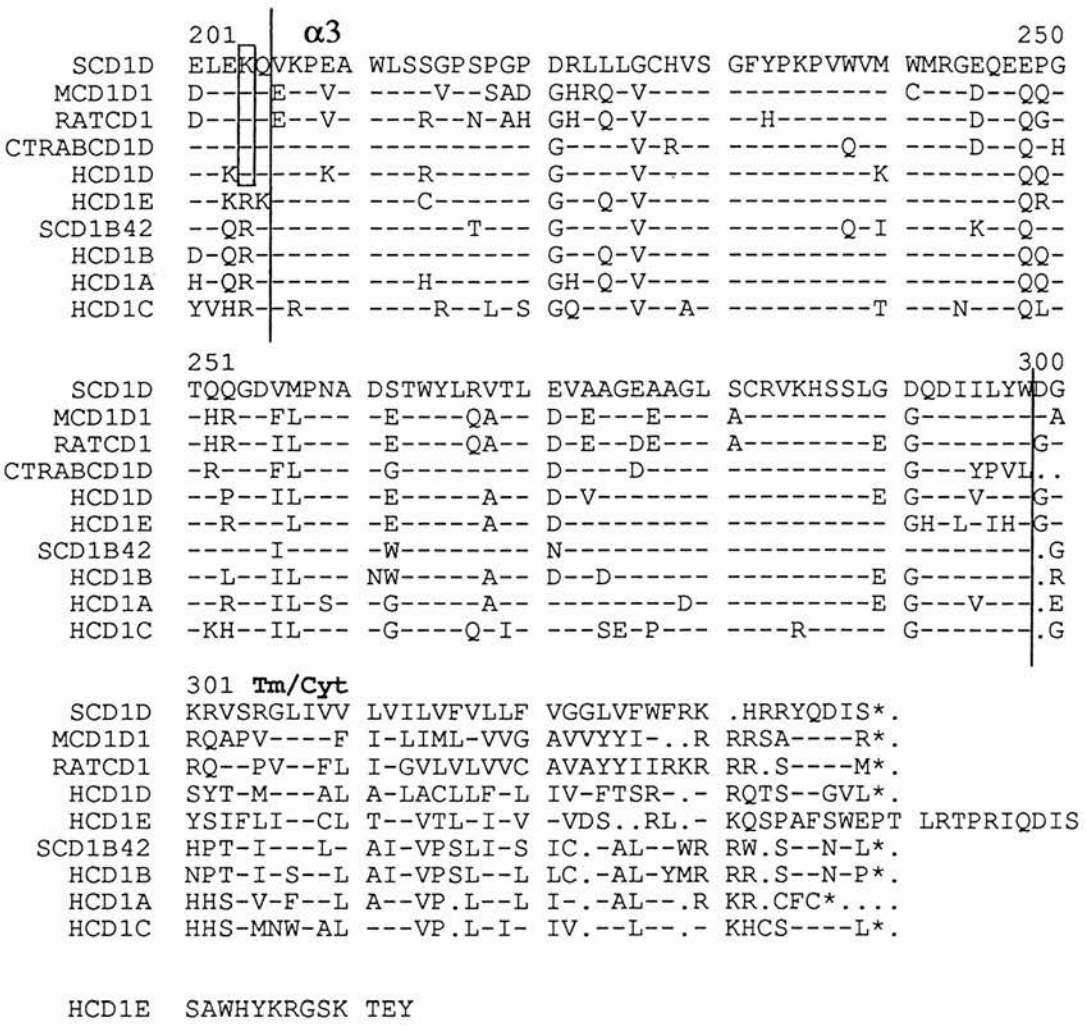
Sequences were aligned using the PILEUP programme of UWGCG 8.0.

Potential N-linked glycosylation sites are indicated by an asterisk.

Class specific residues for CD1d-like proteins (as defined by Calabi et al. 1989b) are indicated by boxed residues.

FIGURE 5.13


1 Leader
α1
*
50
SCD1D ....QTSFPFRF LQISSFANHS WTRTDGLMWL
MCD1D1 MRYLPWLLLW AFLQVWGQSE AQ-KNYT--C --M-----R- -S---SVV--
RATCD1 MLYLPCLLLW AFPQFWGQSE VQ-.NYT-CC ---------R- -S---SVV--
CTRABCD1D .... .L-R----HG ------V-S- Q----C-A--
HCD1D MGCLLFLLLW ALLQAWGSAE VP-RL--L-C ---------S- -----A--
HCD1E .MLLLFLLFE GLCCPGENTA AAEEQLS--M --T-------- -AHSE-SG--
SCD1B42 MLLLPLLLLG VILPGGDNED VF-GPTS-HL K---T-V-ST -AQNL-SG--
HCD1A MLFLLLPLLA .VLPGDGNAD GLKEPLS-HV IW-A--Y--- -KQNLVSG--
HCD1B MLLLPFQLLA VLFPGGNSEH AF-GPTS-HV I-T---T-ST -AQ-Q-SG--
HCD1C MLFLQFLLLA LLLPGGDNAD AS-EHVS-HV I--F--V-Q- -A-GQ-SG--
51
*
100
SCD1D GELQPYTWRN ESSTIRFLKH WSQGTFSDQQ WEQLQHTFQV YRSSFTRDIR
MCD1D1 -D--THR-S- D-A--S-T-P ---KL-N-- --K---M--- --V-----Q
RATCD1 -D--THR-S- D-D--S-T-P ---K--N-- --K---M--- H-T-----K
CTRABCD1D ----THS-S- D-D--H---P ------NF-- ---V-NELW- --L-V----H
HCD1D ----THS-S- D-D-V-S--P ---------- --T---I-R- ---------VK
HCD1E -D--THG-DT VLG------P --H-N--K-E LKN--SL--L -FH--IQIVQ
SCD1B42 DD--IHG-ES D-G-AI---P --K-N---EE ITE-VDL-R- -LIG-I-EVQ
HCD1A SD--TH--DS N----V--WP --R-N--NEE -KE-ETL-RI RTIRSFEG--
HCD1B DD--IHG-DS D-G-AI---P --K-N---KE VAE-EEI-R- -IFG-A-EVQ
HCD1C D---THG-DS --G--I--HN --K-N--NEE LSD-ELL-RF -LFGL--E-Q
101
α2
*
150
SCD1D EFVKML.PG. DYPFEIQISG GCELLPRNIS ESFLRAALQE KDVLSFQGMS
MCD1D1 -L---MS-KE ---I---L-A ---MY-G-A- ----HV-F-G -Y-VR-W-T-
RATCD1 -I---MS-KE ---I-V-L-A ---MY-G-A- ----HV-F-G EY-VR-H-T-
CTRABCD1D D---L-KLT. .--I-L-VFA ---MH-G-T- ---FHV-Y-G MH----R-TL
HCD1D --A---RLS. .--L-L-V-A ---VH-G-A- NN-FHV-F-G --I-----T-
HCD1E ASAGQFQL.E .-------LA --RMN...AP QI--NM-Y-G S-F-----I-
SCD1B42 DR-NEFQL.E .---V--VIE ----HSGEAI --S--G--GG L---RI-NH-
HCD1A RYAHE-QF.E .------VT- ----HSGKV- G---QL-Y-G S-FV---NN-
HCD1B D-AGDFQMK. .------GIA ----HSGGAI V----G--GG L-F--VKNA-
HCD1C DHASQDYSK. .----V-VKA ----HSGKSP -G-FQV-FNG L-L----NTT
151
200
SCD1D WVSAPDAPPW SQVVCKVLN. EDQGTKETVH WLLHDICPEL VKGLMQTGKS
MCD1D1 -QTV-G--S- LDLPI----. A-----SA--Q M--N-T--LF -R--LEA---
RATCD1 -QKV-E--S- LDLPI-M--. A-E--R---Q I--N-T--QF -R--LEA--P
CTRABCD1D -ET--GT--F VKL-V-E--. L-H--R-MIQ E--NNT--QF -S--IEA-R-
HCD1D -EPTQE--L- VNLAIQ---. Q-KW-R---Q ---NGT--QF -S--LES---
HCD1E -EPS-G-GIR A-NI-----. RYLDI--ILQ S--GHT--RF LA---EA-E-
SCD1B42 CMP---SGNR G-KL-AL-S. QY---SDIIE R-VSET--RY LL-VLDA--A
HCD1A -LPY-V-GNM AKHF-----. QN-HENDIT- N--S-T--RF IL--LDA--A
HCD1B C-PS-EGGSR A-KF-ALII. QY--IM---R I--YET--RY LL-VLNA--A
HCD1C --PS-GCGSL A-S--HL--H QYE-VT---Y N-IRST--RF LL--LDA--M


α3
201
250
SCD1D ELEKQVKPEA WLSSGPSPGP DRLLLGCHVS GFYPKPVWVM WMRGEQEEPG
MCD1D1 D----E--V- ----V--SAD GHRQ-V---- ---------- C---D--QQ-
RATCD1 D----E--V- ---R--N-AH GH-Q-V---- --H------- ----D--QG-
CTRABCD1D ---------- ---------- G----V-R-- -------Q-- ----D--Q-H
HCD1D --K-----K- ---R------ G----V---- ---------K -------QQ-
HCD1E --KRK----- ---C------ G--Q-V---- ---------- -------QR-
SCD1B42 --QR----- ------T--- G----V---- -------Q-I ----K--Q--
HCD1B D-QR----- ---------- G--Q-V---- ---------- -------QQ-
HCD1A H-QR----- ---H------ GH-Q-V---- ---------- -------QQ-
HCD1C YVHR--R--- ----R--L-S GQ---V--A- ---------T ---N---QL-
251
300
SCD1D TQQGDVMPNA DSTWYLRVTL EVAAGEAAGL SCRVKHSSLG DQDIILYWDG
MCD1D1 -HR--FL--- -E----QA-- D-E---E--- A--------- G--------A
RATCD1 -HR--IL--- -E----QA-- D-E--DE--- A--------E G-------G-
CTRABCD1D -R---FL--- -G-------- D----D---- ---------- G---YPVL..
HCD1D --P--IL--- -E-----A-- D-V------- ---------E G---V---G-
HCD1E --R---L--- -E-----A-- D--------- ---------- GH-L-IH-G-
SCD1B42 -----I---- -W-------- N--------- ---------- --------.G
HCD1B --L--IL--- NW-----A-- D--D------ ---------E G-------.R
HCD1A --R--IL-S- -G-----A-- --------D- ---------E G---V---.E
HCD1C -KH--IL--- -G----Q-I- ---SE-P--- ----R----- G-------.G
301 Tm/Cyt
SCD1D KRVSRGLIVV LVILVFVLLF VGGLVFWFRK .HRRYQDIS*.
MCD1D1 RQAPV----F I-LIML-VVG AVVYYI-..R RRSA----R*.
RATCD1 RQ--PV--FL I-GVLVLVVC AVAYYIIRKR RR.S----M*.
HCD1D SYT-M---AL A-LACLLF-L IV-FTSR-.- RQTS--GVL*.
HCD1E YSIFLI--CL T--VTL-I-V -VDS..RL.- KQSPAFSWEPT LRTPRIQDIS
SCD1B42 HPT-I---L- AI-VPSLI-S IC.-AL--WR RW.S--N-L*.
HCD1B NPT-I-S--L AI-VPSL--L LC.-AL-YMR RR.S--N-P*.
HCD1A HHS-V-F--L A--VP.L--L I-.-AL--.R KR.CFC*....
HCD1C HHS-MNW-AL ---VP.L-I- IV.--L--.- KHCS----L*.
HCD1E SAWHYKRGSK TEY

5.5 Discussion

The inability of the degenerate 5' primers to detect the group 2 CD1 gene in thymocyte DNA could have arisen for a variety of reasons. It is possible that the cDNA substrate was not full length hence primers designed at the 5' end of the sequence may have no template to bind. This would seem unlikely however as primers based on the 5' end of known ovine CD1 genes amplified their target as expected (data not shown).

It thus appears likely that the problem rests with the inability of the primers to bind to their 'target' under the conditions used in the reactions described in this chapter. Primers were designed based on the amino acid sequence obtained from the purified protein recognised by SBU-T6. As discussed in Chapter 4 however, this sequence may not be 100% accurate and it is obvious that a single amino acid alteration could potentially result in 3 nucleotide errors in the primer sequence. Relevant to this point, it has been stated that the NH_2-terminal sequence can be 'unreliable' (Calabi et al, 1989b) as evidenced by the discrepancy between the reported NH_2 terminal sequence of rabbit CD1 with the subsequent predicted sequence from the gene.

With regard to the amplification of SCD1B-42 cDNA from PBM cDNA, contamination with cloned SCD1B-42 was not possible since this product was generated from cDNA which was adapter ligated and adapter specific primers were used in the PCR reaction. Therefore assuming that this product did arise from PBM cDNA, it raises the question as to why $\alpha 3$ region primers should preferentially detect SCD1B-42 rather than a cDNA predicted to be present at much higher levels. Previous results have shown that the only cell type likely to express the molecule encoded by SCD1B-42 in the PBM compartment is the dendritic cell whereas the molecule recognised by SBU-T6 is present on B cells and monocytes. In retrospect, generation of the adapter ligated cDNA from a population of pure B cells and/or monocytes may have provided a better source of cDNA substrate for this purpose. Regardless of this, it is still surprising to amplify SCD1B-42 from this population albeit that a nested PCR was required to generate the fragment. One explanation for this result is that the group 2 gene(s) possess significant alterations in the sequence

of the α3 domain when compared to other CD1 isotypes or that alternative splicing has deleted the α3 domain altogether. Significant differences within the α3 domain would seem unlikely as all other CD1 genes isolated to date demonstrate marked sequence conservation within this region and in addition, the α3 primers were chosen in the regions of least variability.

This therefore leaves the possibility of alternative splicing resulting in deletion of the α3 domain. As discussed in Chapter 7, the cDNA SCD1B-52 contains a precise deletion of the α3 region. Although this may not necessarily be a feature of other members of the ovine CD1 family, the fact that alternative splicing is known to be a feature of the CD1 genes (Calabi et al. 1989; Bradbury et al. 1990; Woolfson and Milstein, 1994) and that an ovine CD1 cDNA with an α3 deletion does exist would be consistent with this theory. In addition, the fact that immunopurified SBU-T6 antigen contains of a major antigen at 33kd would support, although by no means confirm, this theory.

The identification of a sheep CD1 gene homologous to the CD1D group resulted from a fortuitous binding of the degenerate 5' primer to a CD1D like sequence.

Although this sequence was obtained from lymph node cDNA by utilizing a PCR primer designed on the basis of the NH_2-terminal sequence of the antigen recognised by SBU-T6, it is apparent that the protein encoded by the SCD1D gene does not correspond to the SBU-T6 NH_2-terminal sequence. Figure 5.14a compares the amino-acid sequence of SCD1D with that of SBU-T6 and HCD1D and clearly shows that in addition to greater identity to HCD1D, the SCD1D sequence also shares class specific residues with HCD1D.

Figure 5.14b illustrates the relative homology of the 5' primer (P14365) to the SCD1D, human CD1D and CD1E sequences demonstrating that despite being designed on the basis of the SBU-T6 NH_2-terminal sequence, the primer is a 100% match to the sequence of the SCD1D gene. Thus although the SCD1D sequence has closer overall homology to the CD1D gene, over short regions closer homology may be found to other genes of the CD1 family. Relevant to this point is the possibility that although the amino acid sequence of the SBU-T6 antigen has closest homology

to the HCD1E predicted amino acid sequence, this homology is over a short amino acid stretch which may not reflect homology along the entire length of the sequence. However, as discussed in Chapter 4, the existence of class specific residues within the SBU-T6 sequence which are closest to those of HCD1E, together with the overall homology is highly suggestive that the SBU-T6 antigen is not encoded by the SCD1D gene.

Further evidence for this is the work described in Chapter 8 where specific SCD1D riboprobes do not detect those cells which are stained by the SBU-T6 mAb.

Previous work has shown that four CD1B homologues exist (Ferguson et al. 1996) and the work described in this chapter demonstrates the existence of a CD1D homologue. Combined with the evidence for a CD1e-like protein (Chapter 4), this suggests that the ovine CD1 family is considerably more complex than initial work suggested.

FIGURE 5.14

(a) Amino acid sequence comparison of the SBU-T6 antigen, SCD1D and HCD1D. Class specific residues are boxed.

(b) Comparison of the sequence of primer P14365 with SCD1D, HCD1D and HCD1E.

FIGURE 5.14a

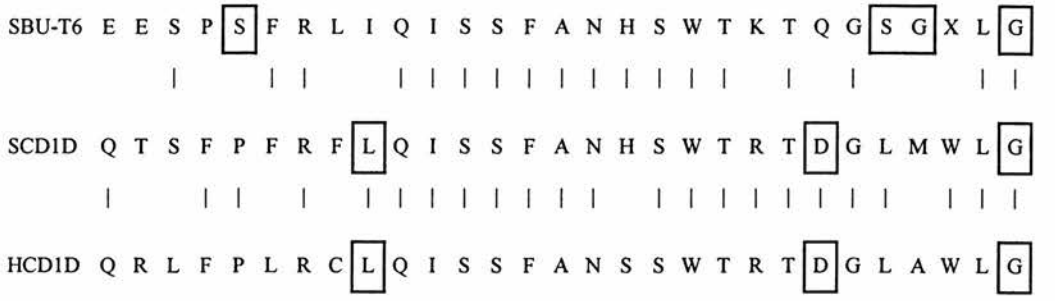


FIGURE 5.14b

P14365	T	C	C	T	T	T	G	C	C	A	A	C	C	A	C	A	G	C	T	G	G	A	C
																	C						
SCD1D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HCD1D	-	-	-	-	-	-	-	-	-	-	-	-	T	A	G	-	-	-	-	-	-	-	-	-	-	-
HCD1E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	

CHAPTER 6

GENERATION AND SCREENING OF A TRANSIENT EXPRESSION LIBRARY

6.1 Introduction

Chapter 5 describes the identification of a new sheep CD1 gene which is shown to have closest homology to the CD1D group of genes. As discussed in Chapter 5, despite the fact that the degenerate primers used were designed on the basis of the NH_2-terminal sequence of the antigen recognised by SBU-T6, the PCR technique failed to identify the sequence of the gene which encodes this CD1 molecule.

At this stage, an alternative approach was therefore adopted to establish the sequence of this novel CD1 gene. The method used was based on the development of a cDNA library within an expression vector. The vector containing the library was then used to transfect COS cells and any cells expressing the molecule of interest were detected using SBU-T6. In this way, the mAb could be used to identify directly the cells containing the gene encoding the molecule of interest i.e. the SBU-T6 antigen.

6.1.1 Transient expression systems

Transient expression systems were initially used extensively to clone cytokine and growth factor genes. These genes are particularly suitable for this system because they are encoded by small mRNAs which are present in high abundance. The original transient expression systems for cytokines involved analysing supernatants from small numbers of transfected COS cells followed by rescreening of positive groups of cells (Simmons, 1993). These techniques are suitable for such molecules which are in high abundance and are likely to have cDNAs well represented in cDNA libraries. However, these systems are less suitable for cell surface molecules encoded by genes which are not abundantly expressed and are generally over 1kb in length. In order to modify the system to improve cloning of cell surface molecules, high efficiency transient expression vector systems have been developed (see below). Utilizing such modified techniques whereby a transient expression system is used followed by antibody mediated selection of the desired cells, it has now been

possible to clone a number of other cell surface molecules including CD2 (Seed and Aruffo, 1987) and CD28 (Aruffo and Seed, 1987) demonstrating the suitability of this system for cloning cell surface molecules. More recently, and relevant to the work carried out in this section, Aruffo and Seed (1989) have utilized this system to isolate and characterise cDNA clones encoding CD1a, CD1b and CD1c.

The method used in this case was based on that of Simmons (1993) with several modifications. The cDNA library was transfected into COS cells by DEAE-dextran facilitated transfection. This efficient method of introduction of DNA into the cells allows the cDNA library to be represented maximally within the cell population (an estimated 10^3-10^4 different cDNA clones can be taken up into the COS cells by this method- Simmons, 1993). Following this initial transfection and subsequent selection using the mAb SBU-T6 to detect positive CD1 expressing cells, subsequent rounds of transfection were carried out using the technique of spheroplast fusion as described in section 2B.12.4. This is a much less efficient technique when compared to DEAE-Dextran transfection and since only a small number of spheroplasts will actually fuse with each COS cell and each spheroplast only contains one cDNA clone, over several rounds, it provides a means whereby enrichment for the clone of interest occurs. This strategy for identifying and enriching for cells expressing the CD1 molecule is illustrated in Figure 6.1.

6.1.2 Vectors

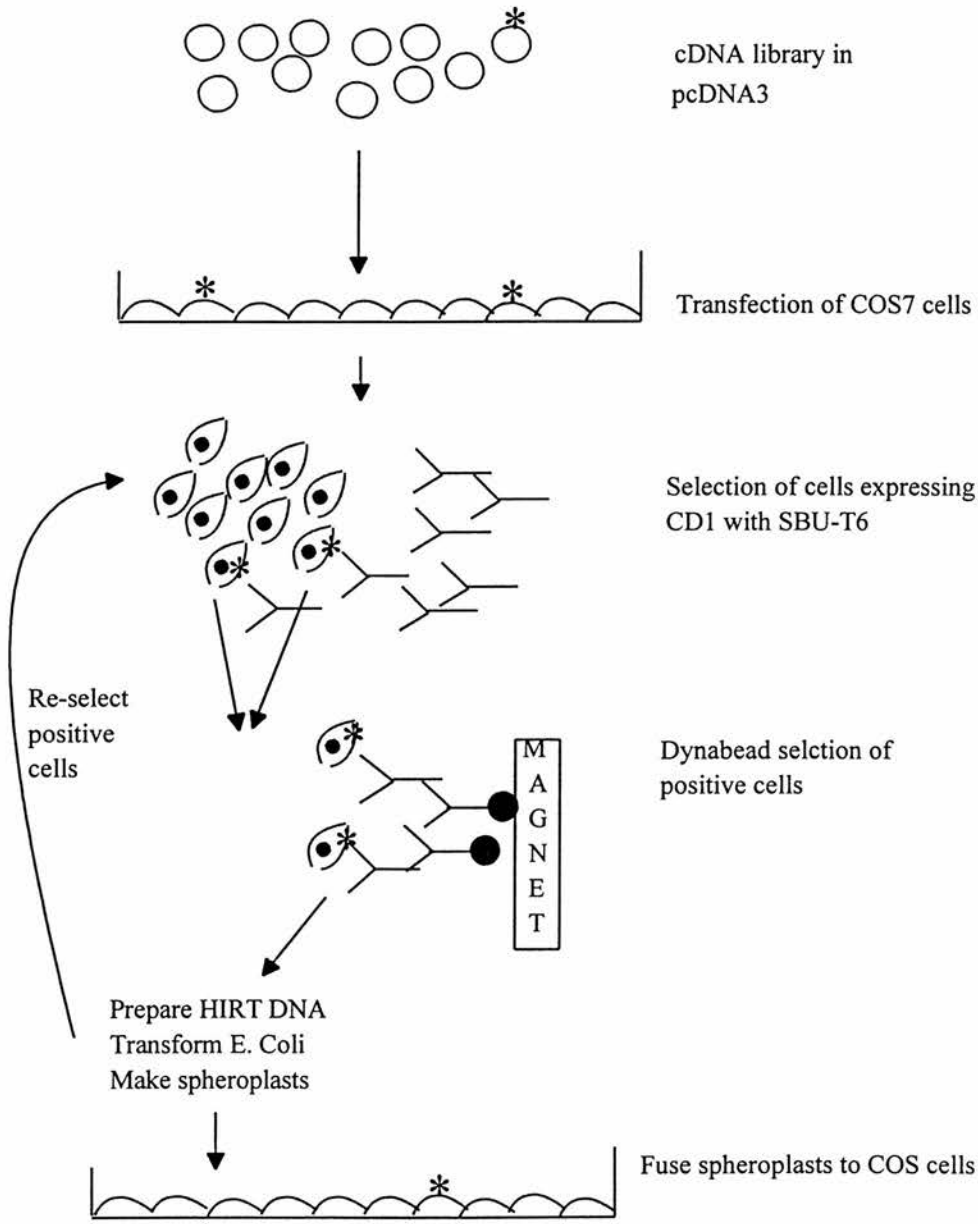
SV40 based plasmids replicate to very high copy numbers per cell (10^3-10^5) and thus result in high expression of specific molecules. The vector pCDM8 (Aruffo and Seed, 1987), has been used to clone the genes encoding several cell surface molecules. The salient features of this vector are illustrated in Figure 6.2a. These features include:

- CMV enhancer and promoter
- Polylinker cloning site flanked by nonpalindromic BstXI sites
- SV40 and polyoma origin of replication

FIGURE 6.1

Diagram illustrating the method for transient expression of a cDNA library in COS cells followed by selection of those cells expressing the molecule of interest using mAbs

FIGURE 6.1


cDNA library in
pcDNA3
Transfection of COS7 cells
Selection of cells expressing
CD1 with SBU-T6
Re-select
positive
cells
M
A
G
N
E
T
Dynabead selction of
positive cells
Prepare HIRT DNA
Transform E. Coli
Make spheroplasts
Fuse spheroplasts to COS cells

- Suppressor tRNA (supF) which suppresses amber stop codons in ampicillin and tetracycline resistance genes which are carried on a stable episome (p3) in the *E.coli* strain MC1061/p3.

The vector chosen to contain the library in this study was pcDNA3. This vector is a modification of the original pCDM8 vector and is illustrated in Figure 6.2b. pcDNA3 shares several features with pCDM8 however, notably it contains an ampicillin resistance gene which avoids the requirement for growth in bacteria containing p3 which is required when using the vector pCDM8.

6.2 Results

6.2.1 cDNA library generation and analysis

cDNA library generation was carried out as described in Chapter 2. RNA was isolated from thymocytes using the RNAzol method. Prior to selection of polyA RNA, total RNA was run on a denaturing formaldehyde gel to confirm that the RNA was not degraded and was suitable for downstream manipulations. Figure 6.3 shows this RNA demonstrating intact 28s and 18s bands with no evidence of RNA degradation. PolyA RNA was therefore isolated from this preparation and cDNA synthesized. Following ligation of adapters to the cDNA and its insertion into the pcDNA3 vector, test transformations were carried out to enable an estimation of the size range of the cDNA population. Twenty-four colonies were selected, grown in liquid culture overnight and plasmid DNA isolated. Restriction digests of purified plasmids were carried out and typical results shown in Figure 6.4. This illustrates the range of insert sizes present and indicates that the cDNAs that are present are not representative of those found in the tissue as they are all less than 1.2kb in length. Repeating the mRNA isolation, cDNA synthesis and transformation gave a similar result.

At this stage, a commercial cDNA library (Clontech) was obtained as a gift from Dr. W. Hein and this library was used in transfection of the COS cells. The library had been made from sheep lymph node.

FIGURE 6.2

(a) Diagram of the vector pCDM8 showing relevant features.

CMV - enhancer-promoter sequences from the immediate early gene of human cytomegalovirus (CMV).

MCS - multiple cloning site.

POLYOMA ori and SV40 ori - polyoma and SV40 origins of replication for episomal replication in cells expressing the SV40 large T antigen or latently infected with polyoma virus.

ColE1 - origin for growth in *E.coli.*

supF - suppresser tRNA for maintenance in *E.coli* strains which carry the p3 episome.

(b) Diagram of the vector pcDNA3 showing relevant features.

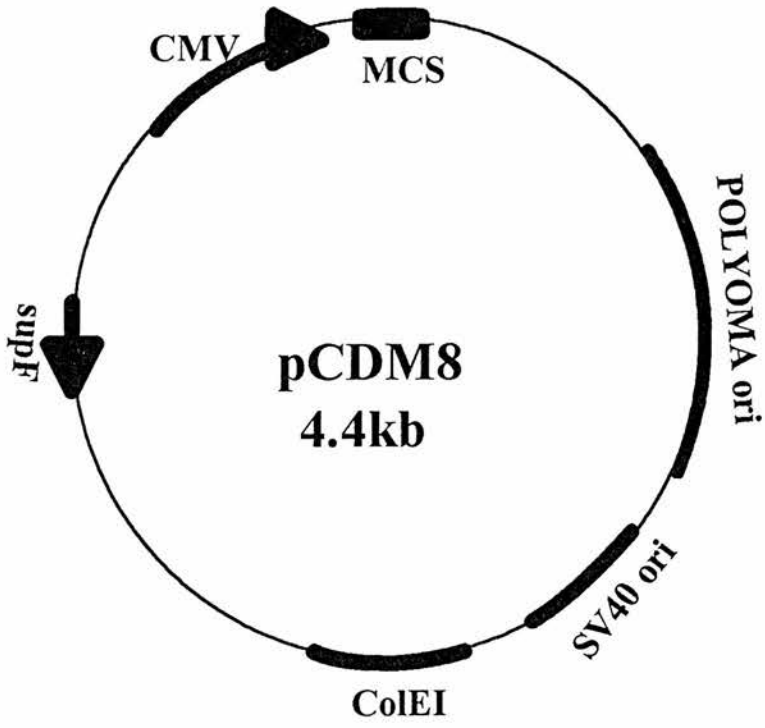
CMV - as above.

MCS - as above.

AMP - ampicillin resistance gene for selection.

FIGURE 6.2

a


CMV
MCS
POLYOMA ori
SV40 ori
ColEI
SupF
pCDM8
4.4kb

b

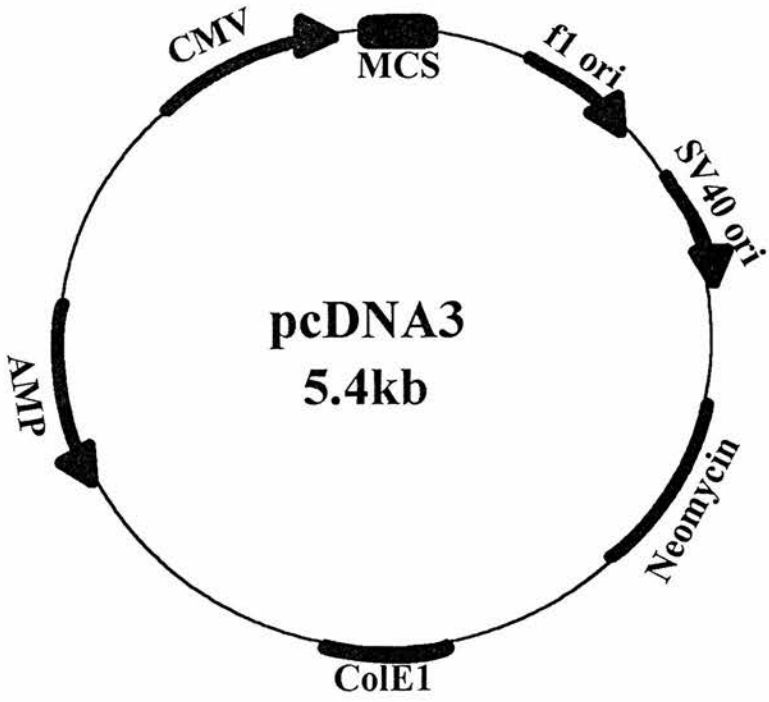

CMV
MCS
f1 ori
SV40 ori
Neomycin
ColE1
AMP
pcDNA3
5.4kb

FIGURE 6.3

Denaturing formaldehyde gel analysis of RNA preparations used for selection of polyA RNA. 18s and 28s bands are marked.

Lane 1 - PBL RNA.

Lane 2 - Thymocyte RNA.

FIGURE 6.4

Agarose gel analysis of inserts obtained following restriction digests of pcDNA3 from 19 clones.

M - marker DNA.

Lane 1 - uncut pcDNA3.

Lanes 2-20 - pcDNA3 digested with XHOI and BamHI.

FIGURE 6.3

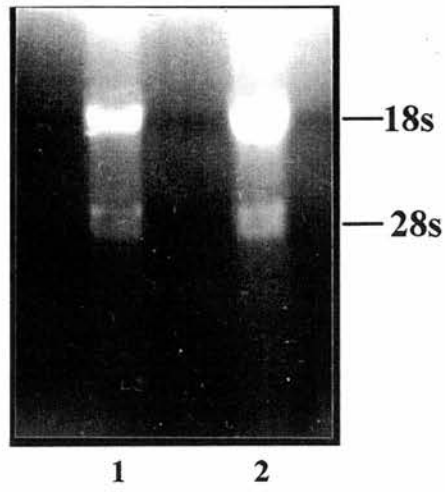
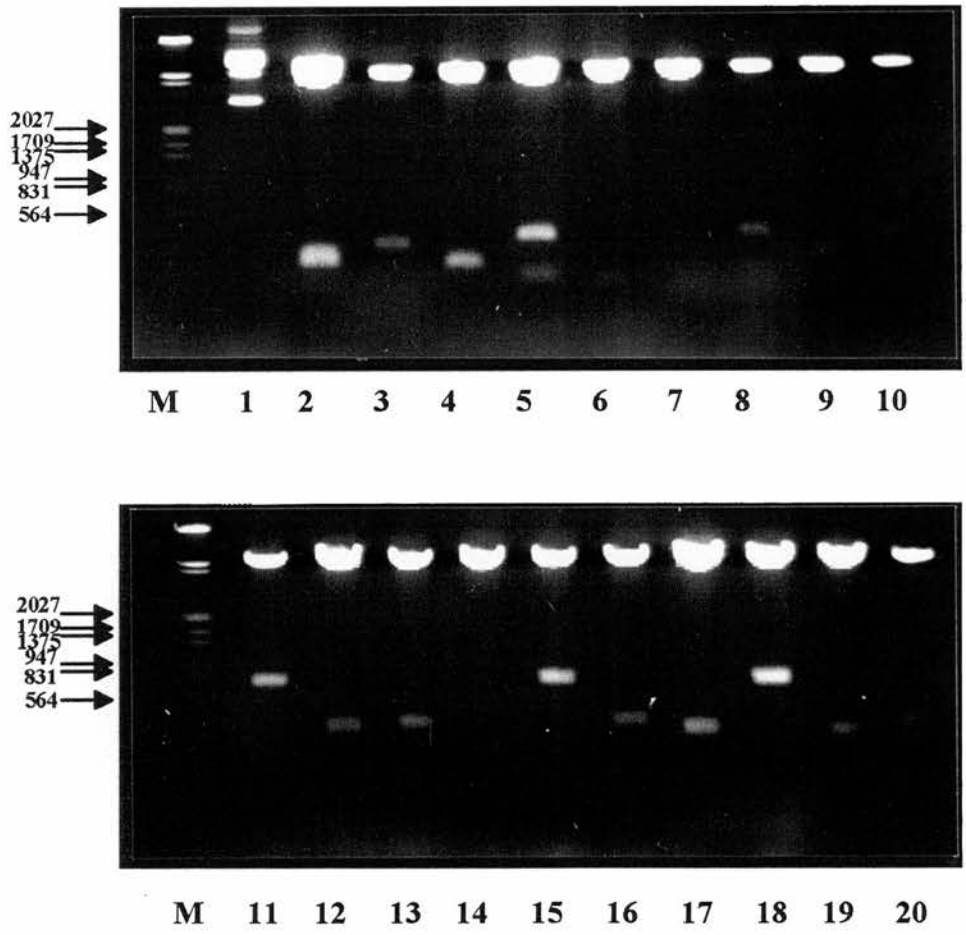


FIGURE 6.4



6.2.2 Screening of the transfected cells

Following transfection of the library into the COS cells, the cells were left for 48 hours before being screened for CD1 expression. Several methods may be used to select cells which are expressing the molecule of interest. Original protocols describe the use of the 'panning' technique whereby Petri dishes are pre-coated with affinity purified goat anti-mouse IgG then cells which have been incubated with the specific mAb are applied to the dishes and allowed to adhere (or 'pan') for 2-3 hours. An alternative technique, and that which was used in this case, was to incubate harvested cells with mAb in the same way but subsequently select the cells using Dynabeads (Dynal) coated with anti-mouse IgG.

This procedure was carried out over 3 cycles of spheroplast fusion and selection however no apparent increase in the number of cells selected was apparent following each successive round. *E.Coli* colonies did grow following transfection with Hirt DNA prepared following each fusion however analysis of these colonies after the three cycles revealed a lack of plasmid. The possible reasons for these results are discussed in Section 6.3.

6.3 Discussion

The reason why colonies were found to exist without apparently containing plasmid is difficult to explain. The logical conclusion is that either the ampicillin on the plates was degraded or not at the correct concentration or that a small number of the bacteria had developed ampicillin resistance.

Despite the reported success of the transient expression cloning system for cloning cDNAs such as CD2 and CD28, there are several major disadvantages of the technique. Multicomponent glycoprotein complexes i.e. those which require expression of other molecules cannot be cloned using this technique unless there is co-transfection of an existing cDNA for other members of the complex. An example of molecules falling into this category are the integrins. CD1 is known to associate non-covalently with β2m although as discussed in Chapter 7, this does not appear to be analogous to the MHC class I/β2m association and β2m may not be an absolute

requirement for CD1 expression. Nevertheless, assuming that β2m is required for CD1 cell surface expression in some situations (suggested by the immunoprecipitation data), it is obviously important to ensure that a source of β2m is available in any experiments involving expression of a CD1 molecule. Human CD1 molecules have been successfully cloned using the technique of transient expression in COS cells (Aruffo and Seed, 1989) however the similarity between monkey β2m and human β2m is likely to be closer than between monkey and sheep β2m. Despite this, however, it has been shown that bovine β2m in tissue culture supernatant can exchange with β2m on the cell surface of the human cell line MOLT-4 (Bernabeu et al. 1984). Hence it is probably safe to assume that monkey β2m can associate with sheep CD1. Thus if the CD1 cDNA is represented in the library, it would be expected that the CD1 molecule could be expressed at the cell surface in association with β2m.

Perhaps the major drawback of this technique is the number of stages to proceed through before being able to analyse colonies for correct sized inserts. If the technique is proceeding according to plan, it is expected that after each round of spheroplast fusion and selection, an increased number of positively selected cells should be apparent. Although positive cells were apparent following the first round of selection (subsequent to the DEAE-dextran transfection), there was no apparent increase in selected cell numbers as each round of spheroplast fusion was carried out. It is therefore likely that the failure of this technique to identify the cDNA encoding the SBU-T6 antigen was the result of a problem at the spheroplast fusion stage. It may also be relevant that in the work described in Chapter 5 in which the SCD1D gene was identified, the SCD1D gene in the pcDNA3 library was found to be truncated at the 5' end. If the gene encoding the molecule recognised by SBU-T6 is similarly truncated in this library then the failure of the technique may have occurred as a result of a lack of full length mRNA within the transfected cells.

Further work will be aimed at optimising this technique such that this cDNA can be isolated as this remains the most rational approach to identify the gene encoding the molecule that this mAb recognises.

CHAPTER 7

ANALYSIS OF THE α3 DELETION MUTANT SCD1B-52

7.1 Introduction

7.1.1 Alternative splicing

Alternative splicing of genes results in the formation of protein isoforms which share extensive regions of identity and vary only in specific domains. The consequences of alternative splicing are many and varied but include alteration in cellular or subcellular protein localization, modification of enzyme activity and, by giving rise to prematurely truncated open reading frames, can result in quantitative regulation of gene expression (Smith et al. 1989).

Alternative splicing is known to occur in several genes encoding human HLA molecules one example of which is HLA-DQ (Briata et al. 1989). Alternative splicing in this gene can result in the production of secretory isoforms of the protein as a result of deletion of the TM exon. Alternative splicing of the gene encoding the human NCCI molecule HLA-G has also been shown both *in vivo* and in HLA-G transfected cell lines (Ishitani et al. 1992; Kirszenbaum et al. 1994). Two splice variants named G2 and G3 lacked the α2 domain and the α2 and α3 domains respectively. The murine NCCI gene Qa-2 also exhibits alternative splicing demonstrated by the expression of soluble Qa-2 molecules resulting from alternative splicing of exon 5 (Tabaczewski et al. 1994). In the mouse, it has been shown that most CD1 mRNA in the thymus is incompletely spliced (Bradbury et al. 1990).

In humans, Northern blots utilizing probes from the 3' UT region have shown that all the CD1 genes (with the exception of CD1D) produce several transcripts ranging from 1.2 - 3kb. In addition, cDNA sequence analysis demonstrates the existence, in CD1B and CD1E cDNA, of cryptic splice sites within the α3 domain which splice to the TM/C exon (Calabi et al. 1989a).

Woolfson and Milstein (1994) provide evidence for the TM/C exon of CD1 genes being under alternative splicing control. Both CD1A and CD1C demonstrate transcripts with a truncated or absent TM domain. Using mouse myeloma cells

transfected with CD1A, the major protein is shown to be a secretory form arising from an unspliced transcript. An additional intracellular form of CD1a also arises as a result of usage of a cryptic splice acceptor site within the TM/C exon. Within the thymus, the CD1C and CD1E genes also possess complex splicing patterns.

Alternative splicing of the CD1D gene resulting in a variety of transcripts, including an α3 deletion, has been cited as unpublished data in Blumberg et al. (1995).

Thus it would appear that the alternative splicing which occurs in both classical and non-classical MHC molecules is also a feature of the CD1 family. It is possible that alternatively spliced transcripts may serve a specific purpose e.g. allowing the molecule to perform its 'function' in a non-cell associated manner.

7.1.2 The Ovine α3 deletion mutant, SCD1B-52

The cDNA clone SCD1B-52 contains a precise α3 deletion (Ferguson et al. 1996). SCD1B-52 was isolated from lamb thymocyte cDNA and sequence analysis reveals 96% identity at the nucleotide level between SCD1B-52 and the equivalent domains of the SCD1B-42 clone implying that SCD1B-52 may be an allele of SCD1B-42 (Ferguson et al. 1996).

As described above, alternative splicing would appear to be a feature of the CD1 genes. Although there is no reported evidence for precise α3 deletions in CD1 genes in other species, it is interesting that the transmembrane protein, endothelial cell protein C receptor (EPCR- a type 1 transmembrane glycoprotein with 28% identity to CD1d), has α1 and α2 domains followed by the TM domain (Fukudome and Esmon, 1994;1995). In addition, a study on the evolution of CD1 genes (Hughes, 1991), suggests that the evolutionary history of the α3 domain of human CD1 genes may differ significantly from that of the α1 and α2 domains, based on the demonstration that the α3 region does not contain isotype specific sequences (as is the case for the α1 and α2 regions - Section 1B.2) but is highly conserved across the CD1 isotypes. In view of this evidence and the existence of the SCD1B-52 clone, it was decided to

undertake studies to investigate whether the protein encoded by the SCD1B-52 gene is expressed, either intracellularly or as a membrane bound form.

7.2 Results

7.2.1 PCR to confirm the presence of SCD1B-52

Due to the precise nature of the α3 deletion, the SCD1B-52 sequence was unlikely to have arisen due to sequencing or cloning error. However, to ensure that this gene was present in a cDNA population derived from thymocyte mRNA, a 3' PCR primer (P10435) was designed which spanned the deletion region (Figure 7.1). This primer was used in conjunction with a 5' primer specific for both SCD1B-42 and SCD1B-52 (P14388) to amplify the sequence of interest from thymocyte cDNA. Although the 3' primer would also be complementary to SCD1B-42 cDNA in the α2 and TM/CYT domains, the annealing temperature used in the PCR reaction (60^0C) would not allow this primer to bind to SCD1B-42.

This PCR produced a product of the expected size from thymocyte cDNA (488bp) - Figure 7.2 (arrowhead). The PCR product was cloned using the TA cloning kit and one of the positive clones sequenced. This confirmed that the product was part of the SCD1B-52 gene. Hence SCD1B-52 is not a cloning artefact but represents mRNA present in more than one sheep.

7.2.2 Insertion of SCD1B-52 into the expression vector pCDNA3

The SCD1B-52 cDNA had been previously cloned into the vector pBluescript (Stratagene). This is not an expression vector thus it was necessary to subclone the gene into an alternative vector to allow investigation of gene expression. The stocks were amplified and the orientation of the gene established by sequencing. The gene was found to be present in the reverse orientation to that required, therefore restriction sites were chosen to allow the insert to be subcloned into pCDNA3 (Invitrogen) such that it was in the correct orientation to be transcribed under the influence of the CMV promoter contained within this vector (Figure 7.3). Figure 7.4 shows the vectors following overnight digestion at 37^0C with the enzymes HindIII

FIGURE 7.1

Diagram of the exon structure of SCD1B-52 showing the location of the primers used to detect the SCD1B-52 gene in thymocyte cDNA.

FIGURE 7.2

Photograph of PCR amplification of SCD1B-52 from thymocyte cDNA.

M - marker DNA

Lane 1 - negative.

Lane 2 - 488bp product generated by primers P10448 and P10435 (arrowhead).

FIGURE 7.1

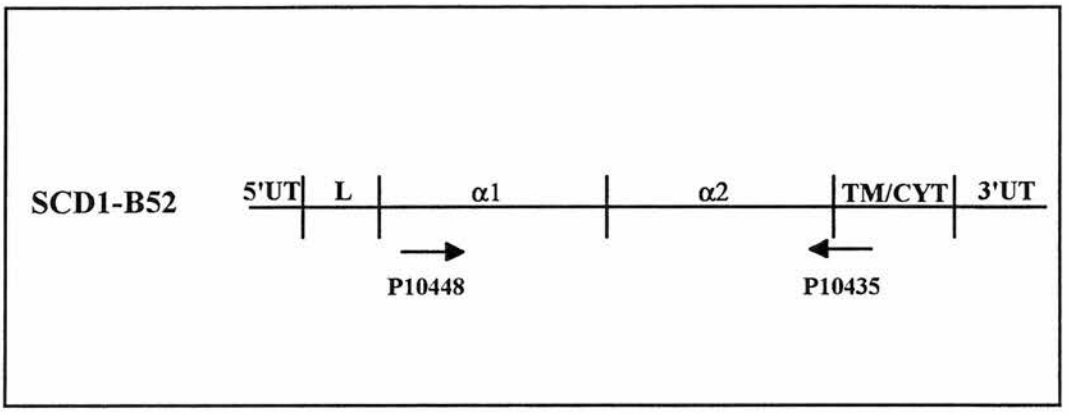


FIGURE 7.2

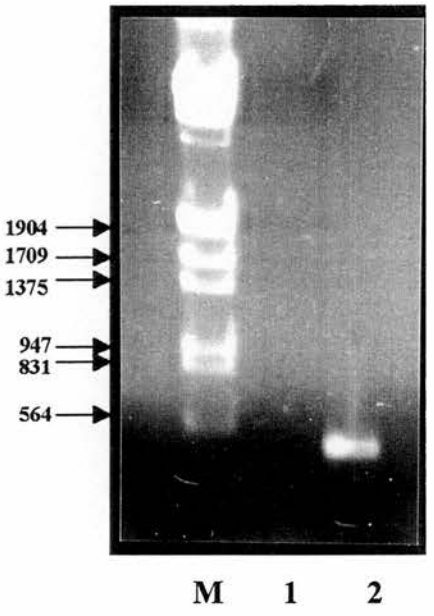
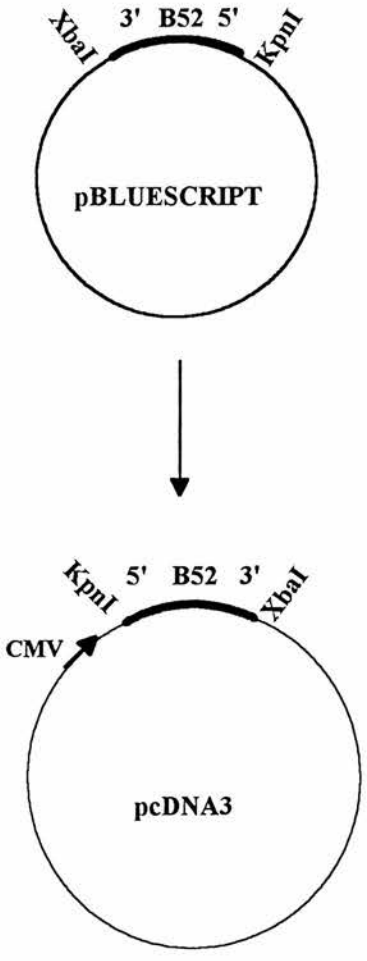


FIGURE 7.3

Diagram illustrating strategy for subcloning SCD1B-52 from pBluescript into pcDNA3.

FIGURE 7.3



and XbaI. The digests were then purified as follows. pCDNA3 was purified using Geneclean and resuspended in 30 µl SDW. The digest containing SCD1B-52 was ethanol precipitated, run on a gel and the SCD1B-52 band excised and purified using a spin-bind column. Subsequently, SCD1B-52 was ligated into pCDNA3 overnight at 12^{0}C. After ligation, 2µl of the ligation reaction was used to transform INVαF' *E.coli.*

Figure 7.5 demonstrates restriction digests of 4 clones resulting from the transfection. This illustrates the successful transfer of SCD1B-52 into PCDNA3.

7.2.3 Transfection of cells and selection of transfectants

C127 cells were transfected using DOTAP transfection reagent as described in Chapter 2. Positive clones were identified as those which appeared as slow growing colonies at the highest dilution when grown in the presence of the selecting agent, the aminoglycoside antibiotic, G418. The identification of positive clones took several days as cells are known to divide once or twice in the presence of lethal doses of G418. Positive clones were isolated then grown up over several weeks until confluent in a T175 flask. G418 selection was maintained at all times.

7.2.4 Analysis of positive clones

Positive clones were analysed for expression of SCD1B-52 at the cell surface and also for mRNA expression.

7.2.4.1 Protein expression

Cells for FACS analysis and cytosmears were harvested by incubating in EDTA at 37^{0}C for 15 minutes. A panel of CD1 mAbs was used to screen transfected cells for CD1 expression using flow cytometry. Four clones were analysed by these methods and the results of one such experiment are shown in Figure 7.6 demonstrating the lack of significant staining by any of the mAbs. Immunostaining of cytospins of transfected cells similarly failed to demonstrate any cell surface or intracellular staining above background.

FIGURE 7.4

Agarose gel analysis of restriction digests of pcDNA3 and pBluescript

M - marker DNA.

Lane 1 - pcDNA3 uncut.

Lane 2 - pcDNA3 following overnight digestion with HindIII and XbaI.

Lane 3 - pBluescript uncut.

Lane 4 - pBluescript following overnight digestion with Hind III and XbaI

FIGURE 7.5

Agarose gel analysis of four clones obtained by transforming InvαF' *E.coli* with pcDNA3 containing SCD1B-52.

M - marker DNA.

Lane 1 - pcDNA3 uncut.

Lanes 2-5 - EcoRI restriction digests of four clones showing transfer of SCD1B-52 into pcDNA3.

FIGURE 7.4

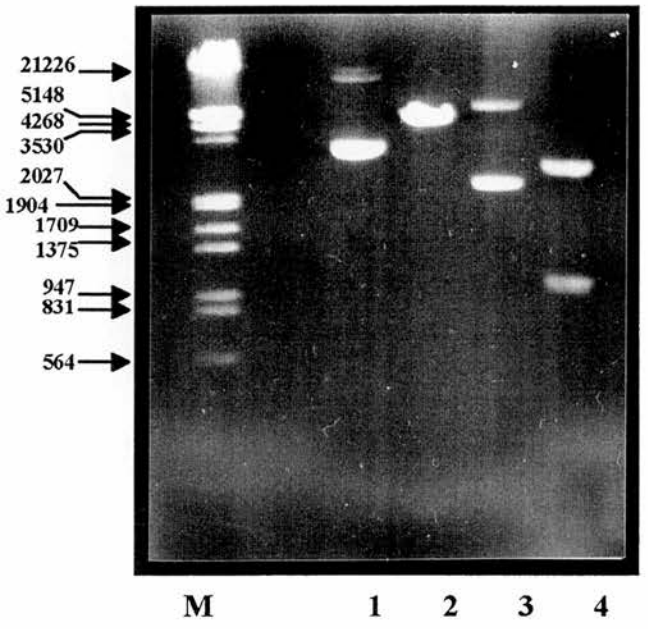


FIGURE 7.5

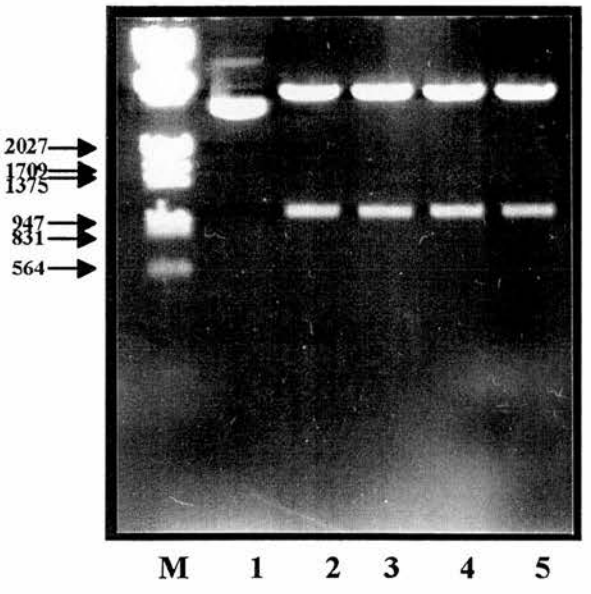


FIGURE 7.6

Flow cytometry analysis of C127 cells.

(a) Forward and side scatter profile of transfected C127 cells showing analytical region.

(b) Frequency histograms of transfected cells stained with negative control (VPM53-top left histogram) and a range of anti CD1 mAbs. The mAb used and percentage of cells stained by each mAb is shown in the top right corner of each histogram.

FIGURE 7.6a

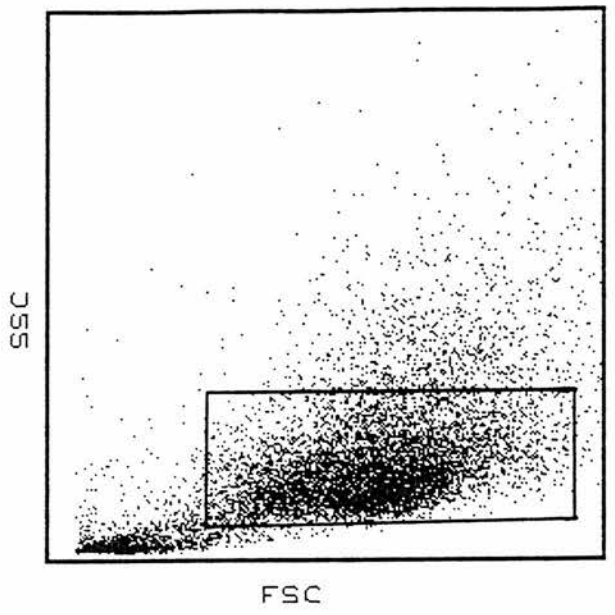
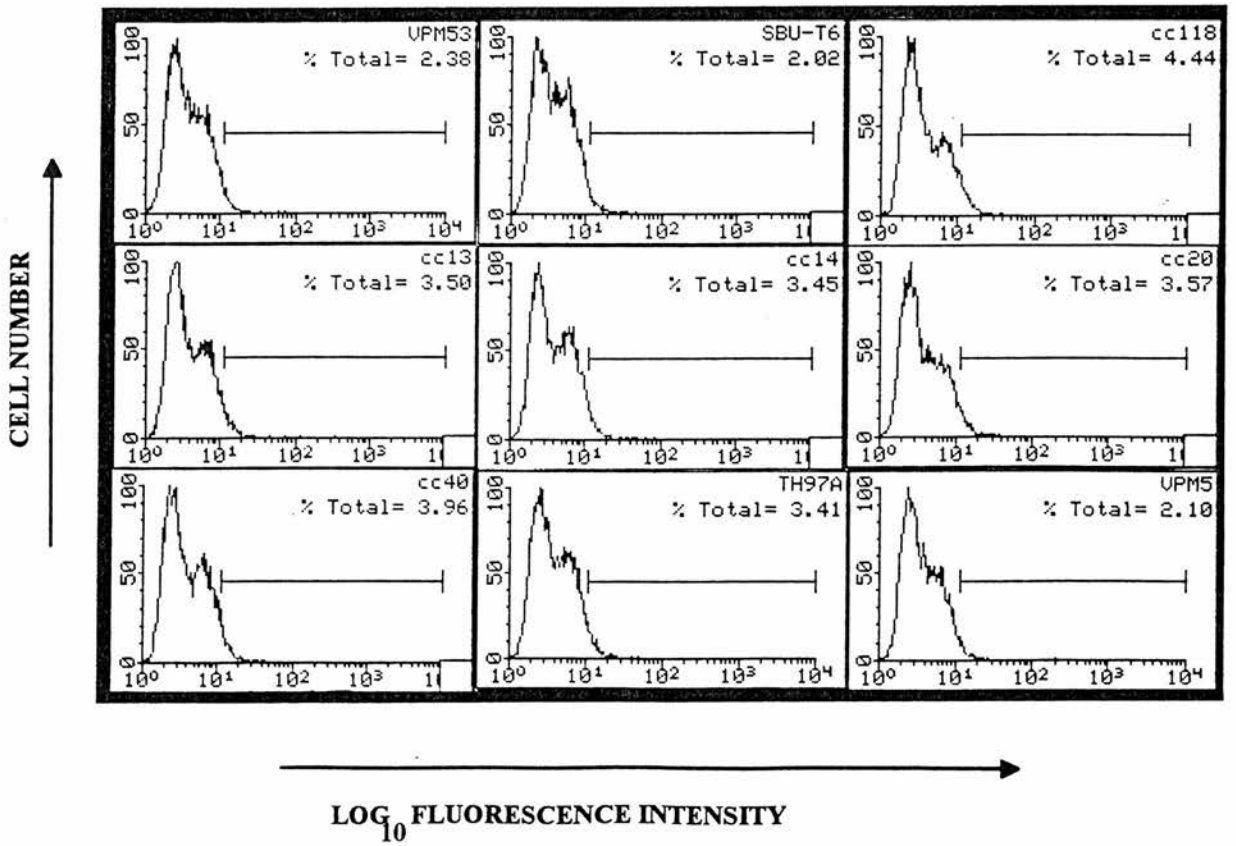


FIGURE 7.6b



Western blotting was carried out by incubating cells in EDTA at 37^{0}C as above followed by TNT lysis, fractionation of proteins by SDS-PAGE and blotting onto nitrocellulose. Non transfected C127 cells were used as a control. Strips of the blot were incubated either in normal rabbit serum or polyclonal rabbit CD1 anti-serum (R-179: this serum had been obtained by immunizing rabbits with SBU-T6 antigen and was shown to recognise CD1 antigen on Western blots). Figure 7.7a shows the results of a blot of lysate from transfected cells incubated with either normal rabbit serum (lane 1) or R179 (lane 2). No positive control was available due to the lack of cells transfected with a full length sheep CD1 clone. Although bands are present at 46kd and 14kd in lane 2 but not in lane 1 (arrowheads), Figure 7.7b shows that this reactivity is also observed using R-179 on untransfected lysate. Deletion of the α3 domain would predict that the size of the α chain in SCD1B-52 would be approximately 30-35kd. No reactivity against a protein of this size was observed using the CD1 antiserum. It is possible that the antiserum is cross reacting with MHC class I and β2m resulting in the bands at 46kd and 14kd which are present in both the transfected and non-transfected cells. Thus there is no evidence for recognition of CD1 antigen in the transfected cells by the polyclonal anti-CD1 rabbit serum.

7.2.4.2 mRNA expression

RNA was also isolated from the cells - in this case, cells were lysed by adding RNAzol (Biogenesis) directly to the flask and gently shaking for 1 minute. The lysate was removed and processed as described in Chapter 2B. RT-PCR was then carried out using this RNA as a substrate and utilizing primers P6087 and P6086 (Appendix II-specific for sequences in the α1 region and 3'UT region of B52 respectively).

Figure 7.8 shows the results of RT-PCR on RNA isolated from the individual clones. Lane 1 is marker DNA. Each PCR was carried out on a sample of RNA which had not received treatment with reverse transcriptase (lanes 2, 4, 6 and 8) in addition to the cDNA from each clone (lanes 3, 5, 7 and 9). This rules out contamination of the RNA sample with original plasmid DNA. Positive and negative controls for the PCR are shown in lanes 10 and 11 respectively. The positive control utilized plasmid

FIGURE 7.7

Western blot analysis of lysate from transfected cells (A) and control nontransfected cells (B).

Lane 1 - normal rabbit serum.

Lane 2 - polyclonal rabbit CD1 antiserum.

The 46kd and 14kd bands are indicated by arrowheads.

FIGURE 7.8

Photograph of RT-PCR analysis from four transfected clones.

M - marker DNA

Lanes 1, 3, 5, 7 - no reverse transcriptase negative control for clones 1-4.

Lanes 2, 4, 6, 8 - RT-PCR utilizing primers P6086 and P6087 on cDNA from transfected clones.

Lane 9 - positive control.

Lane 10 - negative control.

FIGURE 7.7

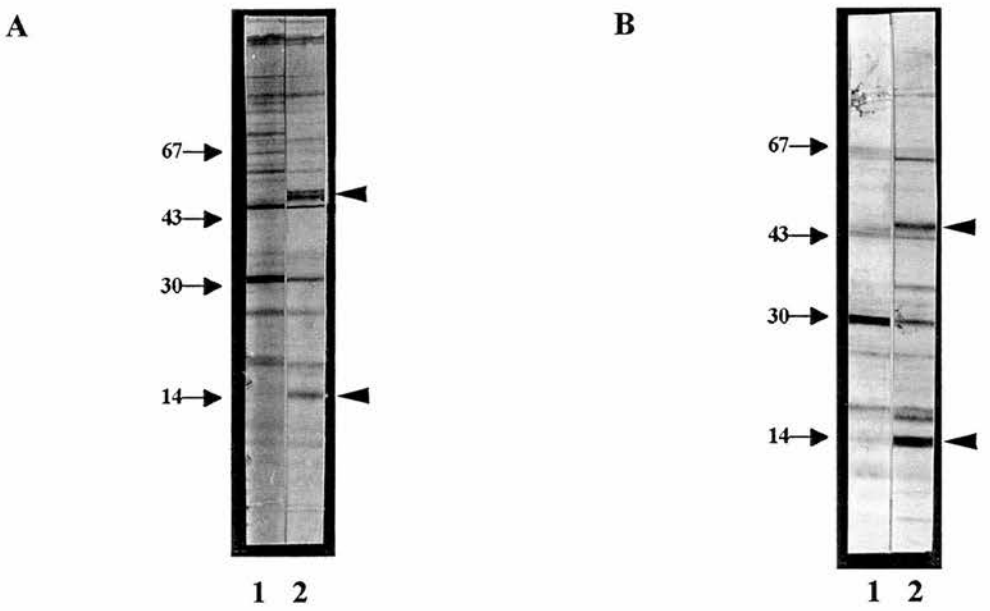
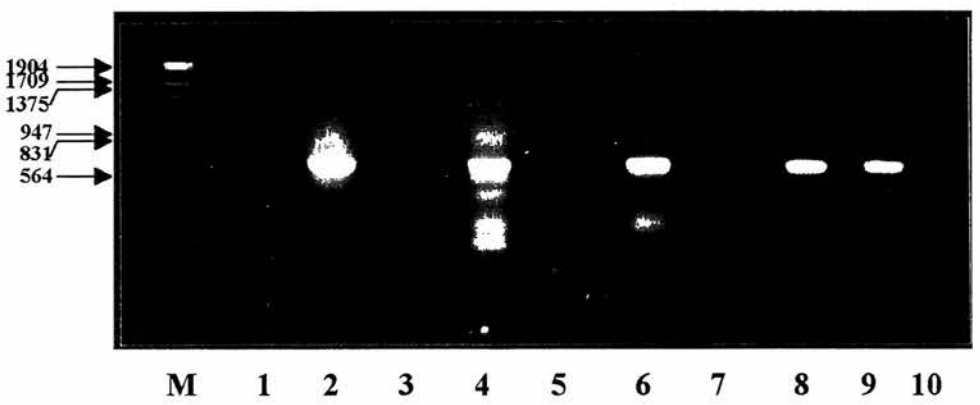


FIGURE 7.8



containing the gene as a substrate and the negative control utilized sterile water as a substrate. A band of the expected size was obtained with the positive control whilst no product was generated in the negative control.

7.3 Discussion

The aim of the work carried out in this chapter was to establish whether the CD1 molecule encoded by the SCD1B-52 gene could be expressed in the absence of the α3 domain. When considering the potential consequences of the lack of an α3 domain, the major feature to consider is the effect of the inability of the molecule to bind β2m.

Studies using β2m knockout mice show little if any surface expression of MHC class I molecules confirming the necessity for a trimolecular MHC/peptide/β2m complex for stable surface expression of MHC class I molecules. Although the α3 region has a critical role in MHC class I molecules in the non-covalent association of the molecule with β2-microglobulin, there is evidence that the requirement for β2m and therefore the α3 domain in CD1 molecules may not be so stringent. Co-capping experiments demonstrated that CD1a and CD1b can exist in isolation from β2m (Kahn-Perles et al. 1985). In addition, it has recently been shown that CD1d can exist on human intestinal epithelial cells in a β2m independent manner (Balk et al. 1994).

Residues within the α3 domain (together with residues in the α2 domain) are involved in the association of the α chain with β2m in MHC class I molecules and similar predictions have been made for CD1 molecules although folding differences in the α1 and α2 domains indicate that the binding may have different characteristics (Tysoe-Calnon et al. 1991). Analysis of potential β2m contact residues in ovine CD1 α chains has been carried out (Ferguson et al. 1996) and demonstrates that of the nine contact residues located within the α3 domain, only two are conserved in the ovine sequences.These data imply that ovine CD1 α chains interact with β2m in a manner different from classical MHC class I/ β2m association and raises the possibility that

β2m association may not have the same significance for CD1 molecules as for MHC class I molecules. If this is the case, then the lack of an α3 region and possible inability of β2m to bind to the α chain may still be consistent with a 'functional' molecule.

Although RT-PCR demonstrated successful transfection of cells with the SCD1B-52 construct, no intracellular or membrane bound protein was detectable using the methods described above. Despite the lack of evidence for either intracellular or expressed protein, the assumption cannot be made that such protein does not exist. It is possible that the mAbs used either recognise a conformational epitope which is lost with the change in conformation of the protein which will obviously occur as a result of the α3 deletion, or that a mAb is not available for the protein encoded by this gene whether complete or lacking the α3 region. The polyclonal antiserum which was used on the Western blots would perhaps have been expected to be more likely to recognise CD1 protein in the clones, however this antiserum was made against purified SBU-T6 antigen at a stage when this mAb was thought to represent a pan-CD1 mAb. The data presented in Chapter 4 argues against this, hence this antiserum may in actual fact be less likely to detect SCD1B-52 protein as it is apparently directed against a novel CD1 isotype. Despite this however, the ELISA data described in Chapter 4 clearly show that SBU-T6 does recognise purified group 1 antigen. In addition, the identity which exists between all CD1 proteins may allow an antiserum generated against one isotype to cross-react with other isotypes.

Although SCD1B-52 was detected by PCR from thymocyte cDNA, there is no evidence of immunoprecipitation of a 30-35kd band by CC14 (a mAb shown to recognise an NH_2-terminal sequence identical to that of SCD1B-52 and SCD1B-42 - Chapter 4). Whilst this may reflect only relative amounts of specific gene products, it would also be consistent with lack of cell surface expression or loss of the epitope recognised by the mAb. As discussed previously, although it is possible that the lack of an α3 domain may result in loss of conformational epitopes recognised by mAbs, it is unlikely that the mAb CC14 specifically recognises an epitope within the α3

domain as α3 domains are highly conserved across the CD1 groups and CC14 is CD1b specific.

In hindsight, the use of a control i.e. transfection of the cells with SCD1B-42 would have been beneficial to prove that ovine CD1 can be expressed in this system. As described in Chapter 4, immunopurified CC14 antigen has an NH_2-terminal sequence identical to that of SCD1B-42. It would thus be expected that CC14 would recognise SCD1B-42 at the cell surface in this system therefore providing a suitable positive control.

In conclusion, it has been shown that the transfected SCD1B-52 gene is transcribed in this system but translation could not be detected. However, this inability to detect protein may be due to a lack of suitable reagents. An alternative approach which could be adopted is to raise antiserum to a peptide which is common to both SCD1B-42 and SCD1B-52. This could then be used as a specific reagent to detect the proteins both *in vivo* and *in vitro*.

CHAPTER 8

ANALYSIS OF OVINE CD1 GENES IN TISSUES

8.1 Introduction

The mAbs which recognise CD1 in sheep form two distinct clusters. The majority of these mAbs recognise a molecule with a similar distribution to human CD1b whilst SBU-T6, CC43 and CC118 recognise a molecule with a much wider tissue distribution. Comparing the pattern of reactivity of these mAbs to the known expression pattern of CD1d in humans and mice suggests that they do not recognise the sheep CD1d molecule. This is supported by the NH_2-terminal sequence of the antigen recognised by SBU-T6 which is clearly more similar to the CD1e sequence than the CD1d sequences (Chapter 4). These data imply that sheep express at least three CD1 isotypes i.e. CD1b, CD1e and CD1d with mAbs only available against the CD1e and CD1b isotypes.

In order to localize the cells and tissues expressing CD1D, it was therefore necessary to employ molecular techniques. RT-PCR and subsequent Southern hybridization using labelled internal oligonucleotides were used to identify the distribution of the CD1 transcripts in various cells and tissues. *In situ* hybridization was also used to identify more precisely those cell types expressing CD1 mRNA.

8.1.1 RT-PCR

PCR reactions were carried out using primers specific for SCD1D and SCD1B-42/ SCD1B-52. Internal primers for SCD1D and SCD1B-42/SCD1B-52 were labelled and used as internal probes to confirm the identity of the PCR product. The primer sequences are listed in Appendix II and conditions for the PCR reactions used to generate these fragments (and the fragments used for riboprobe generation) are shown in Table 8.1.

As described above, PCR for the SCD1D gene allowed identification of those cells and tissues expressing SCD1D mRNA. Performing a similar experiment using primers specific for SCD1B-42/ SCD1B-52 allowed confirmation of results obtained using immunochemistry both in normal cells and tissues and in intestinal

TABLE 8.1

PCR primers and conditions used to detect ATPase, SCD1D, SCD1B-42/SCD1B-52 and SCD1T10 α3 region.

TABLE 8.1

SUBSTRATE					
CDNA CLONE (RIBOPROBES)	**CDNA (RT-PCR)**	**PRIMERS**	**CYCLE NO.**	**CYCLE PARAMETERS**	**EXTENSION**
SCD1T10		G5936 G5935	35	94-0.7', 55-1', 72 1.5'	72-10'
SCD1D		LN01 LN03	35	94-1', 55-1', 72-1'	"
	VARIOUS	ATPase	35	94-1', 55-1', 72-1'	"
	"	10448 6086	35	94-1', 55-1', 72-1.5'	"
	"	LN01 LN03	35	94-1', 55-1', 72-1'	"

tissue infected with *M. paratuberculosis.* The results of immunostaining illustrated in Chapter 3 suggest that up-regulation of CD1b occurs in association with this disease. This may be a result of either a specific role for CD1 in the presentation of mycobacterial mycolic acids and LAM (Beckman et al. 1994, Seiling et al. 1995), or more simply may occur as a result of the elaboration of pro-inflammatory cytokines by activated T cells. This latter hypothesis is suggested by the fact that cytokines (GM-CSF, IL-1, IL-6 and TNFα) have been shown to up-regulate CD1 (Walsh et al. 1986, Ishii et al. 1990, Porcelli et al. 1992, Kasinrerk et al. 1993).

8.1.2 *In situ* hybridization

In situ hybridization has been used in the mouse to investigate the expression of CD1 genes in the intestine. A radiolabelled probe spanning the α3 region of the murine mCD1.1 gene was used in the hybridization experiments and the results showed that, within the small intestine, only Paneth cells at the base of the crypts expressed CD1 mRNA (Lacasse and Martin. 1992). This result was unexpected as immunostaining shows expression of murine CD1 on gastrointestinal epithelium (Bleicher et al. 1990).

Having shown expression of ovine CD1 on intestinal epithelium and also discovered a different pattern of CD1 expression in the intestine with the group 1 and group 2 mAbs, a similar approach was taken to establish the pattern of CD1 mRNA expression in sheep intestine using a riboprobe derived from the conserved α3 region. This riboprobe was also used to examine CD1 mRNA expression in thymus and lymph node.

Subsequently, a riboprobe specific for the SCD1D gene was generated in order to localize SCD1D mRNA specifically. Having established that this gene possesed an α3 domain with 90% identity to the α3 region generated from the SCD1T10 clone (used in generation of α3 riboprobes), it was predicted that cells which are positive for the SCD1D transcript would also be positive for the α3 region. Logically however, it was possible that the α3 probe would generate a positive signal in cells lacking SCD1D mRNA.

8.2 Results

8.2.1 RT-PCR and Southern blotting

RT-PCR was carried out on RNA from thymus, liver, lymph node, PBLs and intestine (normal and infected with *M. paratuberculosis*). Total or mRNA was reverse transcribed into cDNA using random hexanucleotide primers. This cDNA was used as a substrate in the PCR. ATPase was used as a control for the cDNA from each source.

The results of this experiment are shown in Figure 8.1. Figure 8.1a shows the signal generated by the ATPase internal probe, Figure 8.1b by the SCD1D probe and Figure 8.1c by the SCD1B-42/SCD1B-52 probe. In each case, lane 7 represents a negative control in which SDW was used in place of cDNA.

This PCR was not designed to be quantitative and was carried out purely to ascertain whether CD1 mRNA was present in each particular tissue. The signal generated by the ATPase was similar in all samples implying that similar amounts of substrate cDNA were being analysed in each sample. All the tissues examined showed a positive result for the SCD1D gene (Figure 8.1b lanes 1-6). For the SCD1B-42 gene, only lane 1 (thymus), lane 3 (*M.paratuberculosis* infected ileum) and lane 6 (PBL) showed a positive signal.

8.2.2 *In situ* hybridization

In situ hybridization was carried out using riboprobes designed on the conserved α3 region and also on the α1/α2 region of the SCD1D gene. Both sets of riboprobes (sense and antisense for each gene) were approximately 300 base pairs in length. The regions of the CD1 genes represented by these probes are illustrated in Figure 8.2a.

8.2.2.1 Riboprobe generation

Generation of α3 riboprobes

The PCR technique was used to amplify the α3 region from the ovine CD1 clone SCD1T10 using primers G5935 and G5936. The resulting 290bp PCR product was

FIGURE 8.1

Southern hybridization of PCR products generated utilizing primers specific for ATPase (a), SCD1D (b) and SCD1B-42/ SCD1B-52 (c).

The source of the cDNA in each lane was as follows:

Lane 1 - thymus.

Lane 2 - intestine (normal).

Lane 3 - intestine (Johne's disease).

Lane 4 - liver.

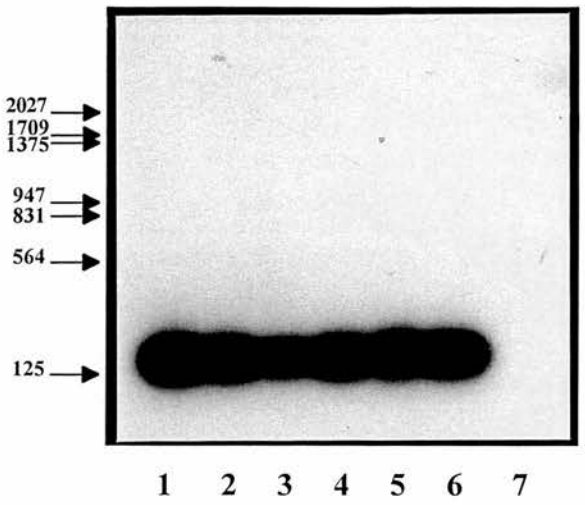
Lane 5 - lymph node.

Lane 6 - PBLs.

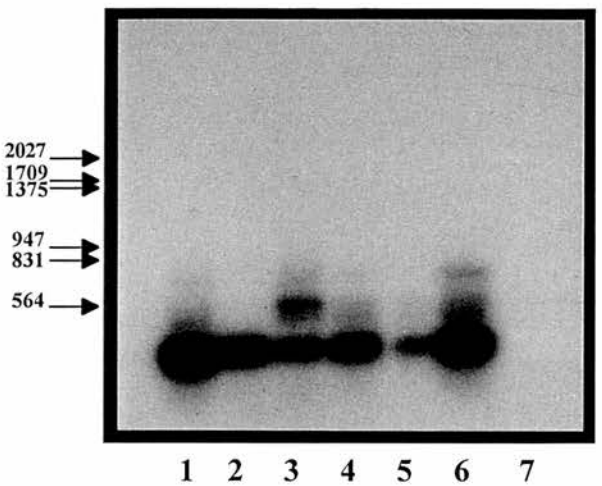
Lane 7 - SDW.

FIGURE 8.1

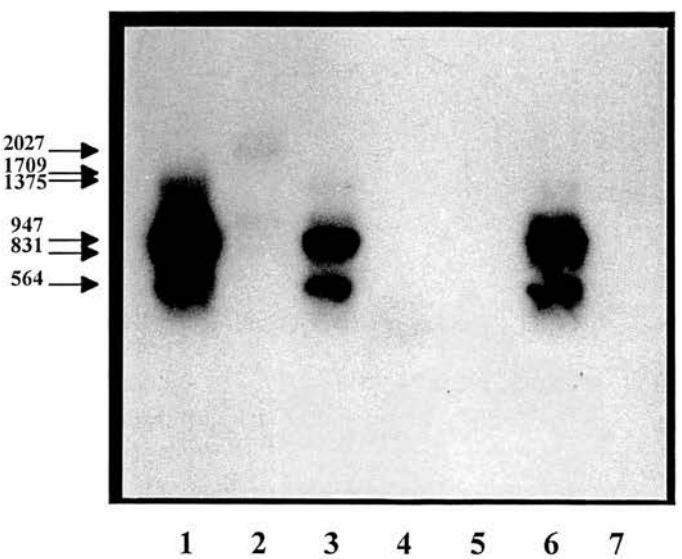
a



b



c



cloned using the TA cloning kit (Invitrogen). The orientation of the insert was established by sequencing on an automatic sequencer.

The sense riboprobe was generated by cleaving the plasmid with HindIII followed by RNA synthesis using T7 polymerase. The antisense riboprobe was generated by cleaving with NotI followed by RNA synthesis using Sp6 polymerase (Illustrated in Figure 8.2b).

Generation of SCD1D riboprobes

PCR was used to generate a 300bp fragment using primers LN01 and LN03. Within this region, sequence homology to the previously described ovine CD1 genes (SCD1A25, SCD1T10, SCD1B-42, SCD1B-52) was less than 50% thus probes generated from this region were considered highly specific with minimal likelihood of cross-reactivity with other CD1 isotypes. The PCR product was cloned as for the α3 region. Sense and antisense probes were generated using T7 and Sp6 RNA polymerase as above.

8.2.2.2 Hybridization

Thymus

Initial experiments were carried out using the α3 riboprobes on sections of thymus in order to validate the probes. Thymus was chosen as a tissue which expresses high levels of CD1 and the results of hybridization on this tissue are illustrated in Figure 8.3. Figure 8.3a clearly demonstrates the detection of CD1 mRNA in the thymic cortex but not in the medulla detected by the antisense riboprobe. This contrasts with results using the sense riboprobe which did not give a significant signal (Figure 8.3b). Using the antisense SCD1D riboprobe in the thymus (Figure 8.3c and 8.3d), a strong signal was also detected in the thymic cortex.

Lymph Node

Hybridization using both the α3 and SCD1D riboprobes revealed a similar pattern of staining within the lymph node. Figure 8.4a and 8.4b show results of hybridization

FIGURE 8.2

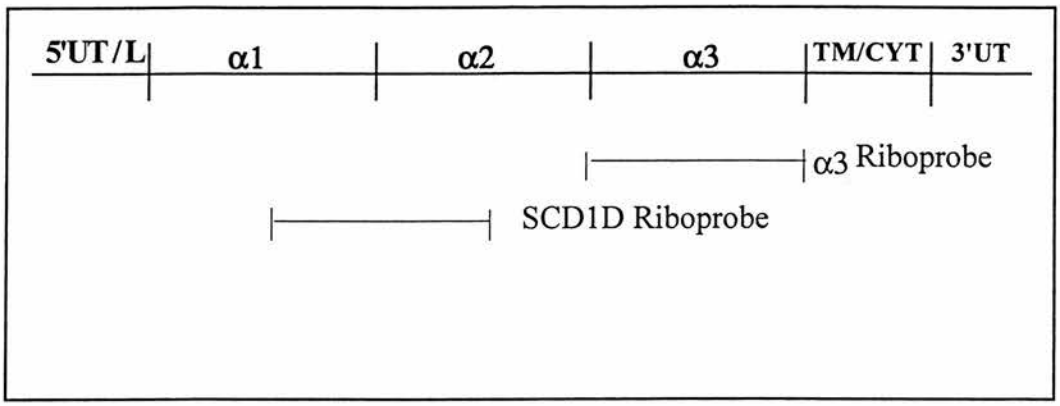
Generation of riboprobes for *in situ* hybridization.

(a) Regions utilized for generation of the α3 and SCD1D specific riboprobes.

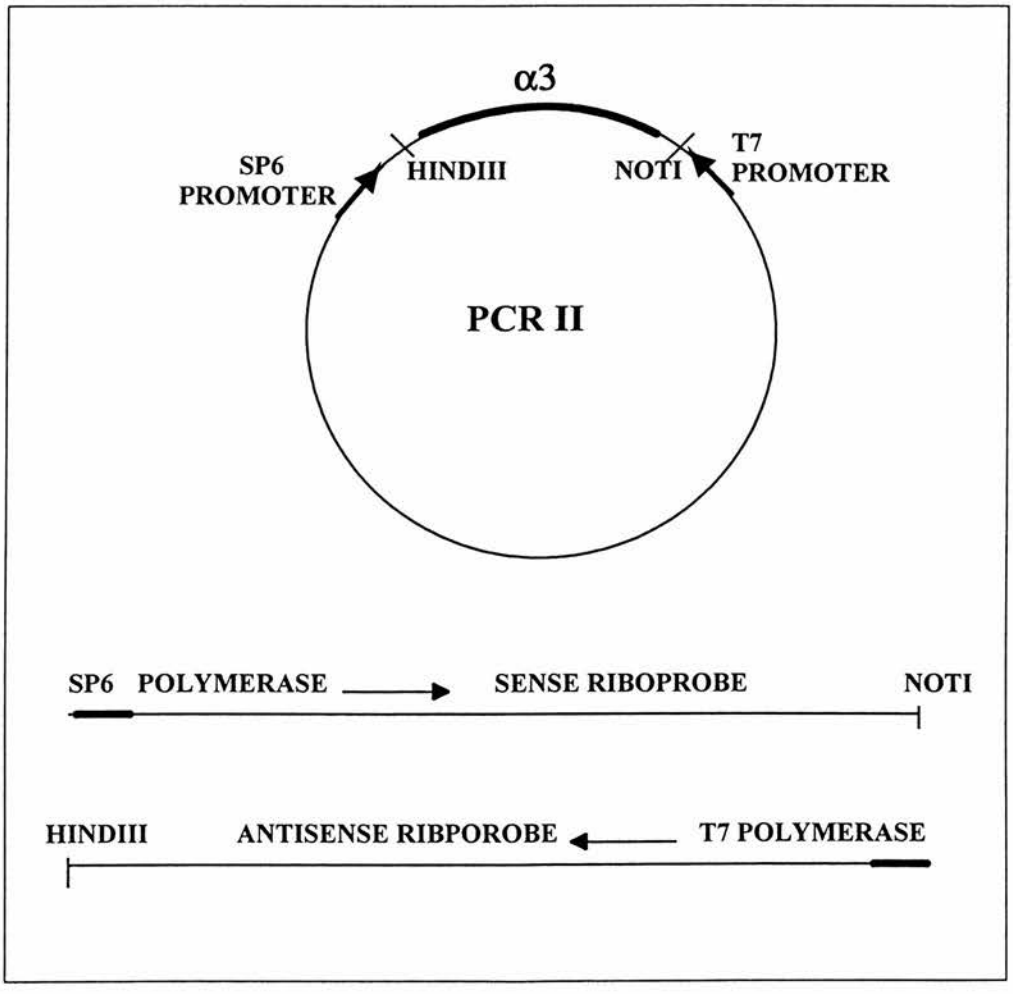
(b) Strategy for generation of the α3 riboprobes.

FIGURE 8.2

a


5'UT/L
α1
α2
α3
TM/CYT
3'UT
α3 Riboprobe
SCD1D Riboprobe

b


α3
SP6 PROMOTER
HINDIII
NOTI
T7 PROMOTER
PCR II
SP6 POLYMERASE
SENSE RIBOPROBE
NOTI
HINDIII
ANTISENSE RIBPOROBE
T7 POLYMERASE

using an α3 probe (no counterstain). Strongly positive cells are evident within the paracortex and in the follicle centre. Figure 8.4c reveals a similar pattern of reactivity with the SCD1D antisense riboprobe. Cells within the centre of the follicle and scattered cells beneath the capsule are stained. Figure 8.4d shows the sense riboprobe negative control.

Intestine

Figure 8.5 shows the results of hybridization on intestinal tissue (ileum). 8.5a shows intense staining which appears superficial on the cells lining the intestinal crypts with an α3 antisense riboprobe. Figure 8.5b and 8.5c show staining of isolated cells within the intestinal epithelium with the SCD1D antisense riboprobe (b) and the α3 antisense riboprobe (c). Figure 8.5d is a sequential section using a SCD1D sense riboprobe.

FIGURE 8.3

In situ hybridization on paraformaldehyde fixed sections of sheep thymus. Development was carried out using an alkaline phosphatase system with no counterstain.

(a) - α3 antisense riboprobe. Signal in thymic cortex.

(b) - α3 sense riboprobe. Background signal only.

(c), (d) - SCD1D antisense riboprobe. Signal in thymic cortex.

Magnification - a, c x250.

Magnification b, d x400.

FIGURE 8.3

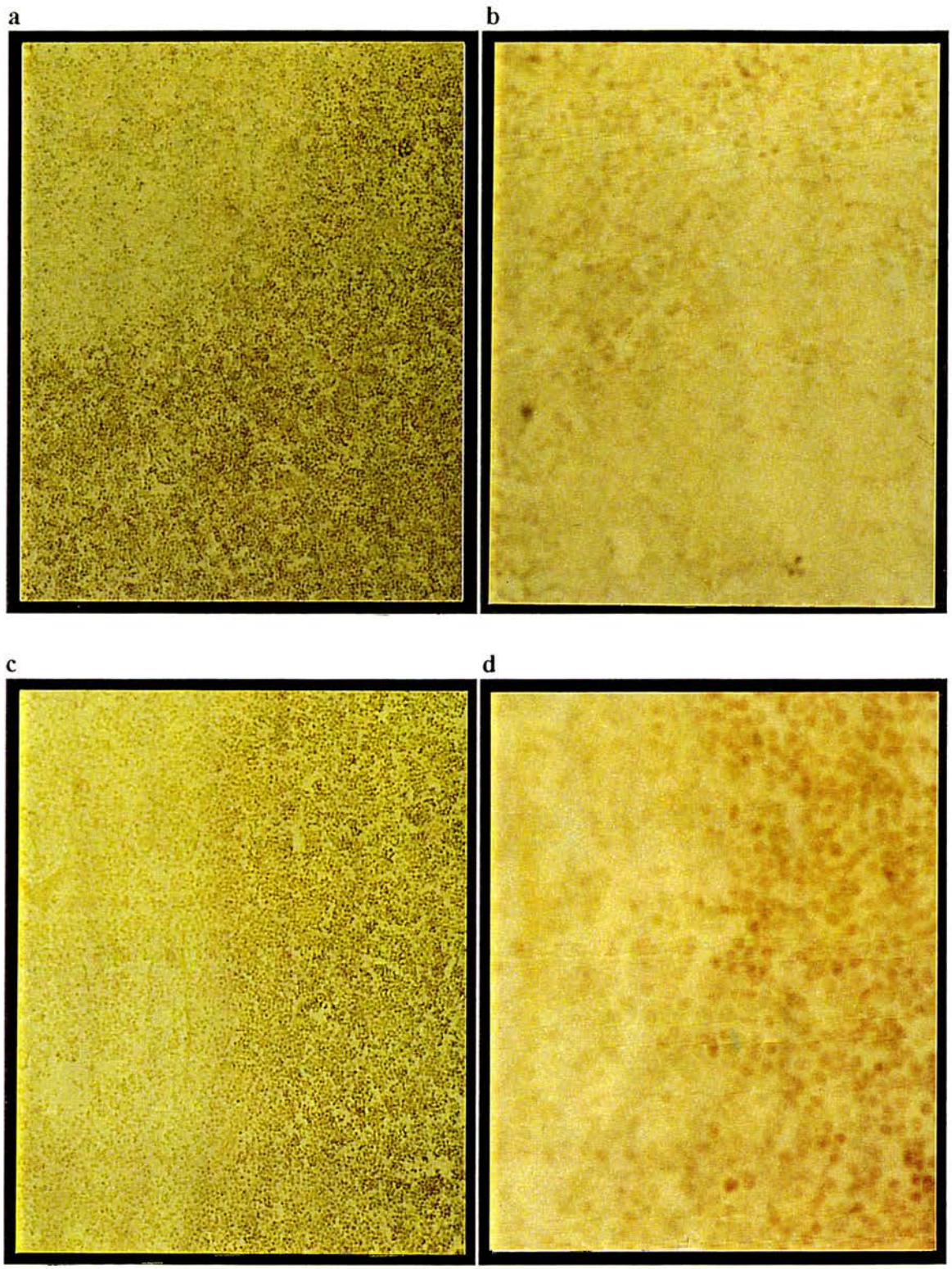


FIGURE 8.4

In situ hybridization on paraformaldehyde fixed sections of sheep lymph node. Development was carried out using an alkaline phosphatase system.

(a) and (b) - α3 antisense riboprobe. Positive paracortical dendritic cells (a) and cells within the follicle centre (b) are shown. No counterstain.

(c) - SCD1D antisense riboprobe. Positive cells within the follicle centre and beneath the capsule. Counterstain - methyl green.

(d) - SCD1D sense riboprobe. No positive cells. Counterstain - methyl green.

Magnification (a), (b) and (c) x250.

Magnification (d) x100.

FIGURE 8.4

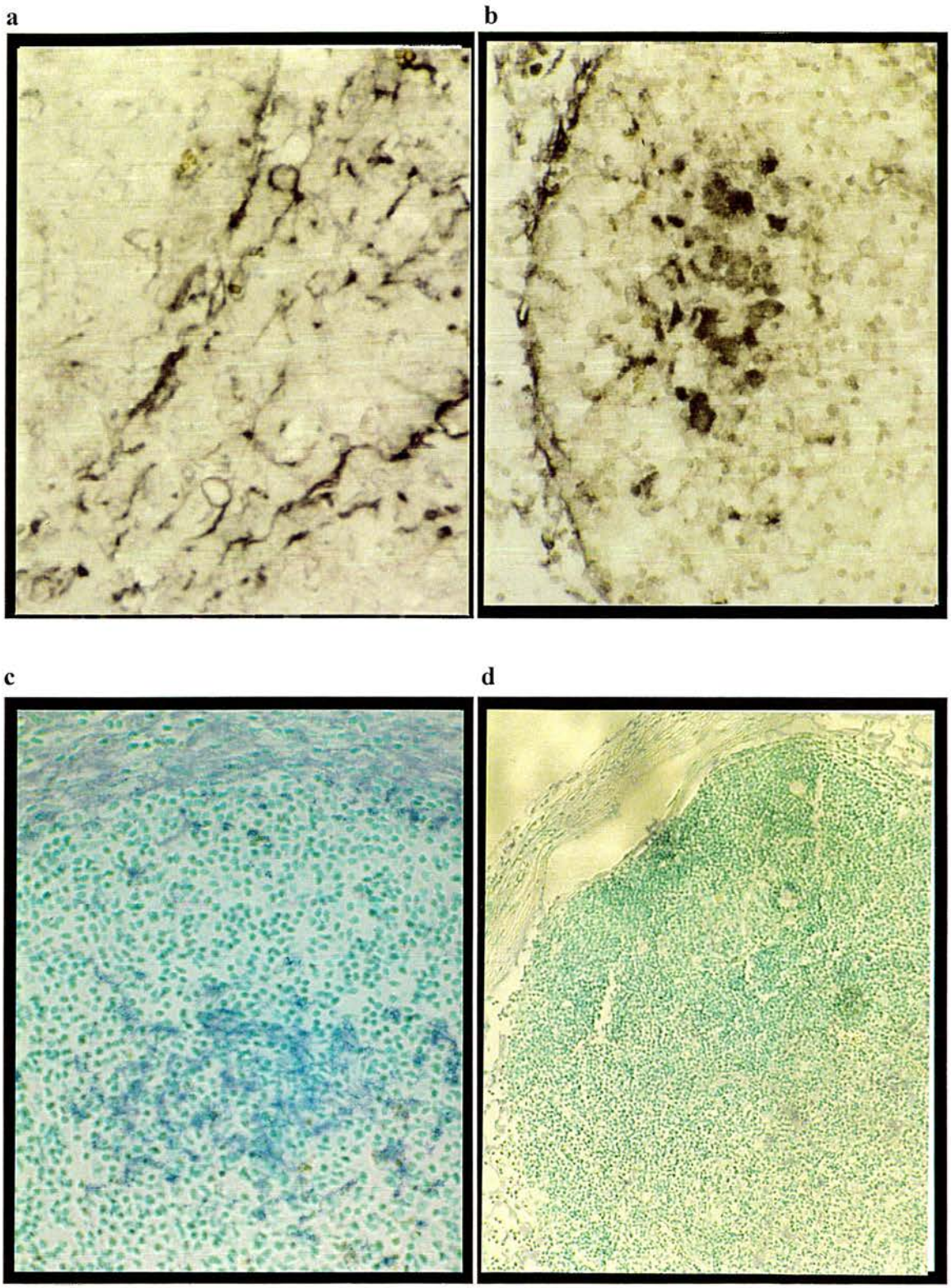


FIGURE 8.5

In situ hybridization on paraformaldehyde fixed sections of sheep intestine. Development was carried out using an alkaline phosphatase system.

(a) - α3 antisense riboprobe. Superficial staining of cells lining the crypts. No counterstain.

(b) - α3 antisense riboprobe. Isolated positive cells within the intestinal epithelium. Counterstain - methyl green.

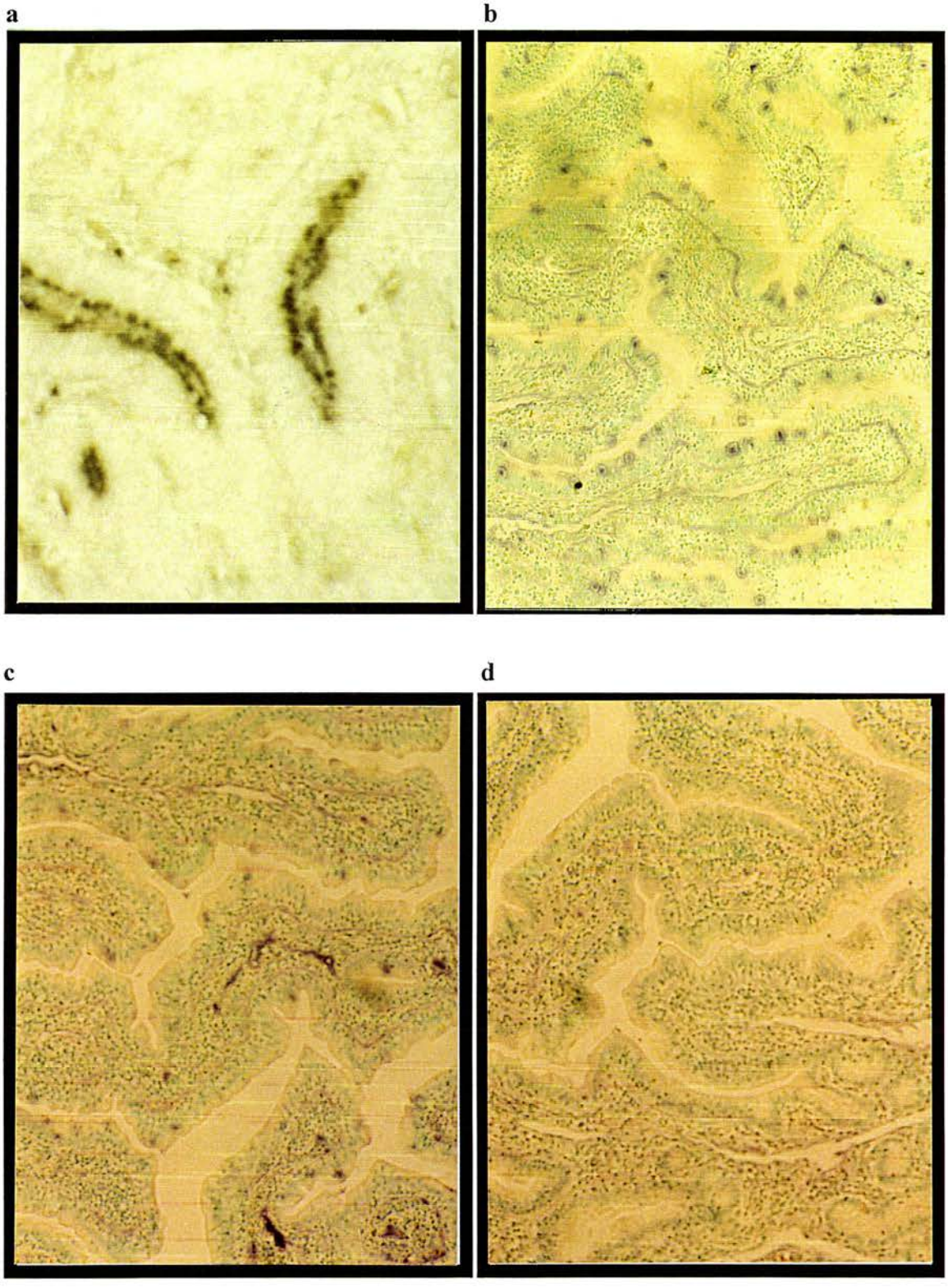
(c) - SCD1D antisense riboprobe. Isolated positive cells within the intestinal epithelium. Counterstain - methyl green.

(d) - SCD1D sense riboprobe. Background staining only. Counterstain - methyl green.

Magnification (a) x400.

Magnification (b), (c), (d) x100.

FIGURE 8.5



8.3 Discussion

8.3.1 RT-PCR

RT-PCR for the SCD1D gene showed that this gene is present in all the tissues tested i.e thymus, intestine, liver, PBLs and lymph node. Table 8.2 compares published data on expression of members of the CD1D group of CD1 genes across the species using molecular techniques. Black boxes indicate that there is no published data for that particular tissue, shaded boxes indicate that the tissue is positive by the technique listed. This table presents published data of studies using molecular techniques only. This demonstrates that although there are individual variations in expression, CD1D genes are characterised by expression in thymus, intestine and liver. One exception is the cotton-tail rabbit CD1 which is not detectable by Northern hybridization in the liver or spleen. It is possible however that a more sensitive technique e.g. RNase protection or RT-PCR may detect RNA in this tissue.

The results shown in Figure 8.1b show that the SCD1D gene is found in the same group of tissues as other members of the CD1D family.

RT-PCR for the SCD1B-42/SCD1B-52 gene is shown in Figure 8.1c. The positive tissues are thymus, PBLs and *M. paratuberculosis* infected intestine. Notable points about this result are the positive signal with PBL RNA consistent with the isolation of the SCD1B-42 gene from the adaptor ligated PBL cDNA (Chapter 5) and the identification of two positive bands consistent with the labelled internal oligonucleotide recognising both SCD1B42 and SCD1B52. Most significantly however, is the demonstration of SCD1B-42/ SCD1B-52 in diseased intestine but not in control intestine confirming induction/up-regulation of this isotype as a consequence of infection. This result is consistent with the immunostaining results using CC14 on *M. paratuberculosis* infected tissue (Chapter 3 - Figures 3.10 and 3.11). This showed induction of CD1b expression in clusters of cells within lymphoid aggregates and also within intestinal epithelial cells. These findings suggest that in this disease, CD1 may have a role in presenting mycobacterial antigens to T cells as has been shown *in vitro* (Beckman et al. 1994, Sieling et al. 1995). This disease may therefore provide a useful *in vivo* model for the study of CD1 - T cell interactions.

TABLE 8.2

Comparison of published data on expression of members of the CD1D group of genes with SCD1D. Black boxes indicate no published data for that particular tissue. Shaded boxes indicate that the tissue is positive by the technique listed according to the appropriate reference. The abbreviations are as follows:

RPA ribonuclease protection assay.

N Northern hybridization.

RT RT-PCR.

ISH *In situ* hybridization.

Table 8.2

	THYMUS	PBL	INTESTINE	LIVER	SPLEEN
mCD1.1	RPA[1]		ISH[2]	RPA[1] ,N[6]	RPA[1]
mCD1.2	RPA[1]		ISH[2]	RPA[1]	RPA[1]
RtCD1	N[3]		RT[3]	N[3, 6]	N[3]
CtRabCD1	N[4]			-ve[4]	-ve[4]
HCD1D	N[5]				
SCD1D	RT, ISH	RT	RT,1SH	RT	

1. Bradbury et al. 1988.
2. Lacasse and Martin 1992.
3. Ichimaya et al. 1994.
4. Calabi et al. 1989b.
5. Balk et al. 1989.
6. Burke et al. 1994.

8.3.2 *In situ* hybridization

In situ hybridization in the thymus demonstrated CD1 mRNA in cortical thymocytes using both the α3 and SCD1D-specific riboprobes (Figure 8.3). CD1D mRNA has been detected in the thymus in other species by various molecular techniques (Table 8.2) and the positive results obtained in this work show that SCD1D mRNA is similarly expressed in the thymus.

Hybridization in lymph node revealed a similar pattern with both α3 and SCD1D riboprobes i.e. signal was detected in paracortical and subcapsular dendritic cells and in cells in the centre of follicles (Figure 8.4). When this pattern of staining is compared to the results of immunostaining using the group 2 mAbs (Chapter 3 - Figure 3.4), it is apparent that the strong staining of mantle zone lymphocytes is not mirrored by a similar pattern of staining using *in situ* hybridization as the strongest signal is generated by cells within the follicle centre.

The results of hybridization in the intestine are somewhat confusing and their interpretation is to a certain extent speculative. The strong signal detected superficially within the intestinal crypts does not appear to be intracellular and may therefore represent an artefact. However, it is notable that the group 1 mAbs recognise cells lining the crypts (Chapter 3 -Figure 3.6) and the pattern of staining by the two methods is similar. In addition, isolated cells within the intestinal epithelium are detected with both the α3 and SCD1D specific probes (Figure 8.5b and 8.5c) and this pattern appears highly specific. This is another surprising finding as it indicates that IELs may contain CD1 mRNA. Both human CD1d and murine CD1 antigens have been detected on intestinal epithelial cells by immunochemical techniques (Bleicher et al. 1990, Blumberg et al. 1991). In contrast to these earlier studies, Sydora and colleagues (1996) demonstrated low levels of mCD1 expression on IELs using 3 different anti-CD1 mAbs (including the mAb 3C11 used by Bleicher et al., 1990) but were unable to to detect expression on epithelial cells. These data support the hybridization results described above in which SCD1D mRNA appears to be expressed in IELs.

Neither the α3 or the SCD1D probe detects those cells within the lamina propria which are strongly stained by the mAbs CC118 and SBU-T6. Whilst not an unexpected result for the SCD1D probe, it is surprising that an α3 probe (which should in theory detect all CD1 isotypes) clearly does not detect cells which express high levels of CD1 as detected by the group 2 mAbs.

The logical conclusion to make from this is that an α3 probe does not detect mRNA which encodes group 2 molecules raising the possibility that the group 2 mAbs recognise a CD1 molecule which lacks an α3 region. This would also explain the discrepancy between the hybridization results in lymph node compared to results obtained using immunostaining.

The SCD1B-52 gene lacks an α3 region and alternative splicing is a recognised feature of the CD1 genes (Calabi et al. 1989, Bradbury et al. 1990). CD1d in humans has been reported to be expressed by intestinal epithelium in a lower molecular weight β2m independent manner (Balk et al. 1994). Although the reduction in size is attributed to altered glycosylation of the molecule rather than any specific alterations in splicing, the fact that CD1d exists in this way independent of β2m would imply that an α3 deleted CD1 molecule with its associated lack of β2m may also exist. Whilst SBU-T6 immunoprecipitates a 46kd protein from the thymus, following affinity purification of the antigen, a 33kd protein in dimeric and monomeric form exist in apparently much greater levels than the 46kd protein (see Chapter 4). Again this would be consistent with an α3 deletion although there is no evidence for this assumption.

The only other report of *in situ* hybridization using CD1 specific riboprobes which was carried out in the mouse. These studies utilized an α3 riboprobe to localize mCD1 gene expression within the intestine (Lacasse and Martin. 1992). Discrepancies between the results of immunohistological analysis of CD1 expression in the intestine and Western blotting on antigen from the same tissue (Bleicher et al. 1990, Mosser et al. 1991) led to the utilization of this technique to attempt to clarify the situation (Lacasse and Martin. 1992). It was shown that only Paneth cells at the

base of the intestinal crypts express CD1 genes - there was no detectable CD1 mRNA in the extrusion zone of the intestinal villi. This study highlighted a discrepancy between results obtained using mAbs to detect CD1d antigen and molecular probes to detect CD1D mRNA. It is clear that a similar discrepancy has also been shown in this study. Resolution of this conflict between immunostaining and *in situ* hybridization results will require isolation of the cDNA encoding the antigens recognised by the group 2 mAbs.

CHAPTER 9
DISCUSSION

The CD1 family is a group of MHC related molecules, members of which have been described in a number of species. Humans possess five different CD1 genes each of which encodes a separate CD1 isotype. In contrast, mice only possess two CD1 genes each encoding a CD1 molecule of the same isotype i.e. CD1d.

Previous molecular studies have identified four ovine CD1 genes all of which have closest homology to CD1B (Ferguson et al. 1996). The results of the work carried out in this thesis now indicate that in addition to CD1B homologues, sheep also possess a CD1D-like gene and are also likely to possess a CD1E like gene although the sequence of this gene has yet to be ascertained. This information renders the sheep an intriguing model for study of the CD1 family in that multiple isotypes exist (as in humans and rabbits), multiple genes encoding molecules belonging to the same isotype exist (as in mouse) and a novel CD1 protein has been demonstrated with closest homology to the predicted amino-acid sequence of human CD1E.

9.1 Groups of CD1 genes and proteins

The original grouping of CD1 molecules by Calabi and colleagues (1991b) allowed division of CD1 molecules into 'classic' CD1 i.e. CD1a-c and the CD1d group on the basis of conserved sequences within the leader, α1 and α2 domains. These two classes are now referred to as group 1 (CD1a-c) and group 2 (CD1d) (Porcelli, 1995). The human CD1E gene remains 'unclassified' and in an intermediate position as a protein product has yet to be identified.

The studies described in Chapter 3 investigating expression of ovine CD1 using mAbs revealed 2 distinct patterns of expression. The mAbs which revealed a pattern of staining consistent with recognition of a CD1b like molecule were classed as group 1. The mAbs that recognise a molecule with a wider distribution i.e. SBU-T6, CC43 and CC118 were classed as group 2. It is apparent that a distinction must be made between the group 1 and group 2 'classes' as defined above and this grouping

of mAbs according to staining pattern. As a result of the NH_2-terminal sequencing data presented in Chapter 4, it appears that at least one member of the group 2 mAbs (SBU-T6) recognises the product of an intermediate/unclassified CD1E-like gene. Thus it is proposed that, as illustrated in Table 9.1, any CD1E gene products will form group 3. Since SBU-T6, CC43 and CC118 cluster together (Chapter 3), these three mAbs will be considered to recognise a group 3 molecule to make the important distinction between mAbs which may recognise a CD1e homologue and group 2 CD1 i.e. CD1d. This nomenclature will be adopted throughout the remainder of this discussion.

9.2 Interspecies isotype comparison

9.2.1 CD1 evolution

Two alternative theories have been proposed to explain the origin of the CD1 family and its relationship to the MHC. Since CD1 is approximately equally related to both MHC class I as MHC class II, this implies that CD1 diverged from the MHC at the same time as MHC class I diverged from MHC class II. An alternative theory is that MHC class I was the first to diverge from the common ancestor followed by divergence of MHC class II and CD1 at a later time (Calabi et al. 1991).

Regardless of the origin of the CD1 family, it is apparent that throughout the process of evolution, different species have retained or acquired different CD1 isotypes. Table 9.2 shows the variation in numbers and classes of CD1 genes between species. It is tempting to assume that the patterns of CD1 expression observed in different species together with retention of members of the different CD1 classes in some but not all species has some functional significance for, or has conferred some selective advantage on that particular species. It is generally accepted that the two groups of CD1 genes diverged prior to the radiation of the placental orders (Calabi et al. 1989c). The lack of group 1 CD1 genes in rodents implies partial deletion of the CD1 gene cluster during rodent speciation (Calabi et al. 1989b).

A number of studies have been carried out on the evolution of the CD1 family. Phylogenetic trees have been constructed based either on the number of

TABLE 9.1

Table demonstrating division of the CD1 genes into three groups. The sheep CD1 genes are highlighted by shading.

TABLE 9.2

Interspecies comparison of CD1 genes.

TABLE 9.1

GROUP 1	GROUP 2	GROUP 3
HCD1A	HCD1D	HCD1E
HCD1B	MCD1.1	?SCD1E
HCD1C	MCD1.2	
SCD1B42/ SCD1B-52	RtCD1	
SCD1T10	CtRabCD1	
SCD1A25	SCD1D	
DoRabCD1		

TABLE 9.2

SPECIES	NUMBER OF CD1 GENES	KNOWN GENES	OTHER GENES
HUMAN	5	CD1A-E	No
MOUSE	2	MCD1.1, MCD1.2	No
RAT	1	RtCD1	No
RABBIT	Up to 8	CtRabCD1, DoRabCD1	Yes
SHEEP	Up to 7	SCD1A25, SCD1B42/ SCD1B-52, SCD1T10, SCD1D	SCD1E Possibly other(s)

non-synonymous (i.e.amino acid altering) nucleotide substitutions (d_N) in the α1 and α2 domains (Hughes, 1991) or on alignments of full-length CD1 protein sequences (Porcelli, 1995). Using the former method, Hughes (1990) showed that the mouse and cotton-tail rabbit CD1 genes, HCD1D and HCD1E cluster together and are separated significantly from the other CD1 genes. In addition, a close relationship of HCD1A and HCD1C is shown and HCD1B is shown to represent a separate lineage that diverged prior to HCD1A and HCD1C. This separation of HCD1B is not however shown in the phylogenetic trees (based on amino-acid sequences) constructed by Porcelli (1995). Phylogeny based on the full length sequence or on the α2 domain sequence clearly shows the two groups of CD1 antigens (i.e. CD1d and CD1a-c) with CD1e being significantly separated. Phylogeny based on the α1 domain amino acid sequences however shows CD1a to be the most divergent. It is evident therefore that no consistent evolutionary relationships are demonstrated by these various methods.

Functional divisions within the CD1 family may also relate to the evolutionary history. CD1b is known to present mycolic acids and LAM to T cells (Beckman et al. 1994, Sieling et al. 1995) and whilst not necessarily being significant *in vivo*, mouse CD1 has been shown to bind peptides. This represents the first functional division i.e. on the basis of antigen binding.

A second functional division is suggested following analysis of the CD1 cytoplasmic tails. CD1b, -c and -d all share an endosomal targeting motif in the cytoplasmic tail. CD1a differs in that it has a shorter cytoplasmic tail which may be involved in interactions with tyrosine kinases (Porcelli, 1995). The recent demonstration by Sugita and colleagues (1996) that localization of CD1b to an endocytic compartment is dependant on the tyrosine-based motif in the cytoplasmic tail further supports the functional division between CD1a and CD1b-d.

The absence of members of the CD1a-c group in rodents is considered to result from a deletion occurring after the separation between rodent and the rabbit/human lineage (Calabi et al. 1989b). It has been suggested that the TL antigens in mice perform the

function of the missing group 1 CD1 antigens in this species (Bradbury et al.1988) and whilst this is a possibility, it may also be that rodents do not require the 'function' of the group 1 CD1 antigens and thus they have not been retained.

Another interesting feature of the CD1 family when comparisons are made across the species is the observation that in the mouse and sheep, two or more genes encode molecules of the same isotype. This may also be the case in the rabbit as up to eight genes have been shown to exist by Southern blot (Calabi et al. 1989b). Of these potential eight genes, however, only one group 2 (CD1D) gene was shown to exist as demonstrated by hybridization with an α1 probe from the CtRabCD1 gene. Hence if multiple genes do exist encoding CD1 antigens of the same isotype in the rabbit, they are likely to be group 1 CD1 as in sheep. The fact that this is a feature of the CD1 family in at least two (i.e. sheep and mice) and probably three (i.e. rabbit) of the species in which the CD1 family has been studied would suggest that this feature is of some functional significance to the animal. What could be the benefit to a species of retaining different genes that apparently encode highly similar molecules with no (or limited) allelic variants? There is now compelling evidence that group 1 CD1 molecules act as restriction elements for non-protein antigens and two of these reports have specifically shown that CD1b restricts the response of T cells to mycolic acids and LAM (Beckman et al. 1994, Sieling et al. 1995). Hence the existence in the sheep of multiple CD1b-like isotypes may imply a greater need or reliance upon this type of antigen presentation. It is important to note that this presentation of 'alternative' non-protein antigen has thus far only been shown to be a feature of CD1b. With the lack of polymorphism which is a feature of the CD1 molecules, a larger number of genes of one particular isotype capable of performing a similar function but with differences, e.g. in the particular range of lipid that may be presented, could conceivably enhance the ability of this species to deal with antigenic challenge of this nature by increasing the range of antigens which may be recognised and presented.

9.2.2 CD1D - an isotype common across the species

Previous studies on CD1 have shown that all species studied to date possess at least one member of the CD1d group of CD1 molecules i.e. man, mouse, rat, rabbit. The identification of an ovine CD1D gene (Chapter 5) shows that, in addition to the multiple CD1b-like isotypes, a member of the CD1d group has also been retained in this species. CD1d is the only isotype which appears in all species in which the CD1 family has been studied. This suggests that functionally, this isotype is required in all these species as there would appear to have been evolutionary pressure to retain it.

At the functional level, as discussed in Chapter 1, there is evidence that members of the group 1 family may differ from group 2 i.e. CD1d molecules. The demonstration of peptide binding by mouse CD1 in a manner which differs from, yet has similarities to, both MHC class I and MHC class II peptide binding (Castano et al. 1995) suggests that group 2 CD1 may present antigen in a more 'conventional' sense than group 1 CD1 i.e. presentation of short peptides. Although it is by no means certain, if it is assumed that group 2 CD1 molecules do bind and present peptides *in vivo* then the key to the specific role and requirement for a molecule of this nature may lie in its unusual tissue distribution. Although there are species variations, a general feature of the group 2 CD1 molecules is high levels of expression on gastrointestinal epithelium and hepatocytes. It is therefore tempting to speculate that there may be a role for group 2 CD1 molecules in mucosal immunity within the intestine. Although not reported in any other species, also relevant to this discussion is the apparent induction of ovine CD1b on intestinal epithelial cells in *M.paratuberculosis* infection and also in neonatal lambs. It is intriguing that the rat neonatal receptor for Ig (FcRn), in addition to being expressed in the intestine is also expressed outside the neonatal period on the canalicular surface of adult hepatocytes (Blumberg et al. 1995). Whilst there is no suggestion that FcRn and CD1 have any precise functional similarities, a more general role in immunosurveillance of epithelial surfaces may be a shared feature of the two classes of molecule.

9.2.3 Group 3 CD1/ CD1E

Although the phylogenetic trees constructed by Porcelli (1995) indicate that the predicted protein product of the HCD1E gene is significantly separated from the group 1 and group 2 CD1 proteins, sequence analysis of the 5'UT, leader, α1 and α2 domains show that in terms of sequence, CD1E is in an intermediate position between the group 1 and group 2 genes (Calabi et al. 1989c). Notably, in the 5' region, there are specific blocks of sequence conservation (including two heptamers) which are conserved between all the human CD1 genes with the exception of HCD1D (Calabi et al. 1989b).

Man is the only species to date in which a CD1E gene has been described. Despite indications that the gene is transcriptionally active (), no protein product has yet been described. Thus the identification of a NH_2-terminal sequence of a protein which has closest homology to the predicted product of this gene represents a significant finding as this is the first description in any species of a CD1e-like protein. The fact that SBU-T6 apparently recognises a protein encoded by a novel CD1 gene is perhaps not surprising considering the pattern of expression observed using this mAb (Chapter 3) which clearly does not resemble that of any recognised CD1 isotype. The mAbs CC43 and CC118 exhibit a similar but not identical pattern of reactivity when compared to SBU-T6. The data presented in Chapter 3 showed that CC43 and CC118 do not stain epidermal Langerhans' cells (which are stained by SBU-T6) and they stain thymocytes and DCs with lower intensity. In cattle, CC43 and CC118 exhibit similar reactivity to SBU-T6 in *Bos taurus* with the exception of weak staining of small intraepithelial cells (Howard et al. 1993a). These minor differences in reactivity between the group 2a (SBU-T6) and group 2b (CC43 and CC118) mAbs in both cattle and sheep suggest that more than one CD1 antigen of the group 3 class is being recognised.

With the recent interest in the functional properties of members of the CD1 family and the apparent dichotomy which exists between group 1 and group 2 CD1 molecules in terms of antigens which they are capable of presenting, a CD1 molecule

which is, at least in terms of sequence, in an intermediate position between the two groups would be an intriguing molecule to study in terms of function.

Once the sequence of the gene encoding this molecule is identified, it may also be of interest to ascertain if this isotype is present in other related species (e.g. cattle) as is suggested by mAb studies (Howard et al. 1993a, 1993b). Assuming that both cattle and sheep do possess a group 3 gene(s) it is possible that the retention of this isotype represents a functional requirement for a CD1 molecule in ruminants which does not exist in other species.

9.3 Summary and conclusions

The work described in this thesis has demonstrated that the ovine CD1 family appears to be one of the most complex studied to date. In conjunction with the previously described sheep CD1B genes (Ferguson et al. 1996), the demonstration of a sheep CD1D gene and identification of an antigen which is likely to be encoded by a CD1E like gene renders the sheep an intriguing model for the study of CD1. Although no specific functional questions were asked in this work, the identification of CD1 expression in cells and tissues in which CD1 molecules have not been described in other species may have some functional relevance. In particular, the identification of constitutive CD1 expression (group 3) by tissue macrophages, all B cells, monocytes and microglial cells in the CNS demonstrates that sheep possess isotypes with a wider tissue distribution than those in man and mice. Notably, this is the first description of CD1 expression within the CNS.

Expression of CD1 as recognised by the group 1 i.e. CD1b-like mAbs was shown in normal tissue to resemble expression of CD1b in man with the exception of the expression on intestinal epithelial cells in neonates and following infection with *M. paratuberculosis*. This *in vivo* up-regulation of CD1 in association with mycobacterial disease is consistent with a role for CD1 in presentation of mycobacterial antigens to T cells.

9.4 Future work

Future work will focus on the isolation and subsequent characterisation of the gene encoding the molecule recognised by SBU-T6. This will then allow detailed comparison at the molecular level of genes belonging to each of the three CD1 classes.

Further studies on the SCD1D gene product would be facilitated by the generation of antipeptide antiserum specific for the SCD1d molecule.

Further investigation of the mRNA splicing complexity of the ovine CD1 family would also be interesting in view of the presence of the SCD1B-52 gene which contains a precise α3 deletion and also the indirect evidence that the antigen recognised by the group 3 mAbs may lack an α3 region. If this is a general feature of ovine CD1 (as has been shown for human CD1 - Woolfson and Milstein, 1994), then it may have functional relevance as a result of the generation of alternative CD1 isoforms.

In view of the current interest in the role of CD1b as a molecule capable of presenting lipid antigen to T cells, the demonstration of CD1 up-regulation in a naturally occurring mycobacterial disease of sheep may provide a useful model to study presentation of lipid antigen by members of the CD1 family.

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APPENDIX I

Phosphate buffered saline (PBS)
137mM NaCl
27mM KCl
8mM Na_2HPO_4
15mM KH_2PO_4
pH 7.2

Borate buffered saline (BBS)
0.1M Boric acid
25mM $Na_2B_4O_7$
75mM $NaCl_2$

TNT lysis buffer
20mM Tris
140mM NaCl
0.5% Triton X 100
pH8

LB Broth
1%(w/v) tryptone
0.5%(w/v) yeast extract
0.17M NaCl
pH7

TYM media
2%(w/v) tryptone
0.5%(w/v) yeast extract
0.1M NaCl
10mM $MgSO_4$

TE buffer (Tris-EDTA)
10mM TrisCl pH8
10mM EDTA pH8

TAE buffer (Tris-acetate-EDTA)
40mM Tris
0.114% glacial acetic acid
1mM EDTA

20x SSPE
3M NaCl
0.2M NaH_2PO_4
25mM EDTA
pH7.4

20x SSC
3M NaCl
300mM Tris sodium citrate pH7

Dig buffer 1
0.1M Tris
0.15M NaCl
pH 7.5

Dig buffer 3
0.1M Tris
0.1M NaCl
0.05M MgCl
pH9.5

APPENDIX II

NAME	SEQUENCE	SPECIFICITY	
G5936	TGAAGCCTGGCTGTCCAGT	α3	5'
G5935	CCAGTACAGGATGATATCC	α3	3'
G4252	TACCAGGCTGCTCCTGCTCA	α3	3'
P10763	GCCAACCACASCTGGACWAARAC	Degen.	5'
P14365	TCCTTTGCCAACCACASCTGGAC	Degen.	5'
P6087	TCCTGAAGCCCTGGTCC	B52 α1	5'
P6086	TGACTCCTAGGCTGACAAC	B52 3'UT	3'
P10448	GTCAACAGCACATGGGCTC	B52 α1	5'
P10435	TGTGGGGTGTCCTTGCCTCTG	B52 TM/CYT	3'
LN01	CAGGGCACRTTCAGCGACC	LN1 α1	5'
LN02	GTCCCTGGTGAAGCTGCTKCG	LN1 α2	3'
LN03	CATGGAGGAGCCAGTGCACCG	LN1 α2	3'
	GCTGACTTGGTCATCTGC	ATPase	5'
	CAGGTAGGTTTGAGGGGATAC	ATPase	3'
H3660	CACTGCTTAACTGGCTTATC	PCDNA3	5'
H3661	CTAGCATTTAGGTGACACT	PCDNA3	3'
PCD1	GAGAACCCACTGCTTAACTGGC	PCDNA3	5'
PCD2	GGCACAGTCGAGGCTGATCAGC	PCDNA3	3'
PCD3	CTGGCTAACTAGAGAACCCACTGC	PCDNA3	5'

CODE	REDUNDANCY
M	AC
W	AT
Y	CT
R	AG
S	GC
K	GT

APPENDIX III

Amersham International	Slough, UK.
Applied Biosystems	Foster City, California, USA.
BDH	Poole, Dorset, UK.
Becton Dickinson	Oxford, UK
Biocell	Cardiff, UK.
Biogenesis	Bournmouth, UK.
Bio 101 Inc.	La Jolla, USA.
Biorad	Hemel Hempstead, Herts., UK.
Boehringer Mannheim	Lewes, Sussex, UK.
Clontech	Palo Alto, California, USA.
Cruachem Ltd.	Glasgow, UK.
Dynal	Oslow, Norway.
FMC Bioproducts	Rockland, USA.
GibcoBRL	Paisley, UK.
Invitrogen	San Diego, California, USA.
Melford Laboratories	Ipswich, UK.
NBL	Cramlington, UK.
Nycomed	Birmingham, UK.
Oxoid	Basingstoke, UK.
Perkin-Elmer	Warrington, Cheshire, UK.
Pharmacia	St. Albans, UK.
Promega	Southampton, UK.
Qiagen Ltd.	Dorking, Surrey, UK.
SAPU	Carluke, Lanarkshire, UK.
Schwarz/Mann Biotech	Cleveland, Ohio, USA.
Shandon	Pittsburgh, USA.
Sigma	Poole, Dorset, UK.
Tissue Tek	Miles, USA.
The Binding Site	Birmingham, UK.
United States Biochemical Corporation	Cleveland, Ohio, USA.
Vector Laboratories	Peterborough, UK.