SEROLOGICAL DEFINITION OF
THE BOVINE MAJOR HISTOCOMPATIBILITY COMPLEX

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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The immunoprecipitation reported in Chapter 5 was done in collaboration with Dr M.L. Bath (Department of Immunology, John Curtin School of Medical Research). The experiments reported in Chapter 8 were done in collaboration with Dr M.J. Stear (Department of Immunology, John Curtin School of Medical Research). All other experiments reported in this thesis were done by myself. The text of the thesis is my own original work.

[Signature]
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ABSTRACT

Some of the MHC polymorphisms in cattle have been identified by serological analysis of the bovine lymphocyte antigen system (BoIA), using a microlymphocytotoxicity assay.

A panning technique was developed to prepare panels of test cells for the detection of class II antigens. The cell panels consisted of 63-90% B lymphocytes.

Anti-class II reagents were prepared by absorbing alloantisera with platelets to remove anti-class I antibodies. Skin implant immunizations proved to be a more efficient method of raising alloantisera than leucocyte immunizations. The anti-class II activity of skin implant alloantisera compared favourably with that of antisera raised by leucocyte immunizations.

After absorption with platelets, 56% of alloantisera had preferential anti-B cell activity. Partial absorption of sera rather than complete absorption required less platelets and could be done more quickly yielding better class II typing reagents. Typing reagents were produced by platelet absorptions followed by leucocyte absorptions (to reduce the complexity of antisera), and these were screened against 117 individual cattle. Five class II antigens were defined by 13 antisera, using cluster analysis.

The defining antisera displayed preferential anti-B cell activity over a number of doubling dilutions. The polymorphic nature of these antigens and their restricted cellular distribution suggested that they belong to the class II antigen system of the MHC of cattle.
The definition of these antigens is however very provisional. Further immunizations will need to be done, and all antisera will need to be tested against a more heterogeneous group of animals and subjected to further absorption analysis before these antigens are adequately defined.

Family data suggested that these antigens are inherited as Mendelian co-dominant traits and belong to the one genetic system, which is linked to the BoLA class I system. Formal linkage analysis demonstrated close linkage between class I antigen CA30 and class II antigen B, with one observed recombination.

Seven putative anti-bovine class II monoclonal antibodies appeared to detect monomorphic determinants, in that they reacted with B lymphocytes from all cattle against which they were tested. Immunoprecipitation studies confirmed the anti-class II activity of antibody TH81A5. Two anti-human class II monoclonal antibodies cross-reacted with bovine B lymphocytes but appeared to detect monomorphic or broadly polymorphic determinants.

The definition of the BoLA class I antigen CA21 was improved by directing immunizations against the apparent antigen and performing absorptions of these alloantisera. Two cases of joint inheritance of BoLA class I antigens W6 and W11 suggested that these 2 antigens do not always behave as if coded for by alleles at a single locus. They may be coded for by 2 linked loci.
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CHAPTER 1

INTRODUCTION
1.1 DEFINITIONS

The major histocompatibility complex (MHC) is a cluster of loci coding for polymorphic cell-surface antigens which appear to control several functions of the immune system, the most notable of which is allograft rejection (Klein, 1981). In the mouse, the MHC is located on chromosome 17 (Klein, 1981) and in man, on chromosome 6 (van Someren et al., 1974).

The following definitions which are used in this thesis in relation to the MHC are based on definitions used by Klein (1977; 1981):

- **Complex** denotes the cluster of H2 loci; the word "system" is also used in the same sense.

- **Region** is a chromosomal portion of the complex occupied by at least one (and possibly more than one) marker locus and separated from similar portions by at least one recombination on each side. The term recombination is used to describe a reciprocal crossing-over event occurring in a pair of homologous chromosomes during meiosis, whereby two genes on the same chromosome are not transmitted together (Cavalli-Sforza & Bodmer, 1971a).

- **Subregion** is a term used in situations where a chromosomal portion, originally defined as a single region, has subsequently been divided by recombination into shorter units which are functionally related.

- **Locus** is a chromosomal segment coding for single polypeptide product and separable from other similar segments by recombination. The strict definition of "locus" has sometimes fallen by the wayside, and "locus" has also been used to describe subregions.
Gene is a specific form of genetic material occupying a locus ("locus" refers only to a position on the genetic map, but the terms "gene" and "locus" are often used interchangeably). A pseudogene is a DNA sequence that displays significant homology with a functional gene, but which has mutations that prevent its expression (Jacq et al., 1977; Proudfoot & Maniatis, 1980).

Alleles are alternative forms of genetic material at a given locus (the terms "gene" and "allele" are also often used interchangeably).

Haplotype is a particular combination of alleles at individual loci inherited on a single chromosome.

Class is a term used to group MHC loci according to their apparent functional and biochemical homology. The four classes of MHC genes are class I, class II, class III, and class IV genes. Conventionally, the class I genes consist of a number of regions, while the class II genes belong to the "class II region" which consists of a number of subregions.

Serologists have traditionally designated the antibody-reactive entities on MHC molecules as "antigens". However, the immunochemical concept of an "antigen" is the whole molecule, and so the alternative terms "determinant", "epitope" and "specificity" are used by some serologists (Klein, 1981). In practice, the four terms are generally used interchangeably. Serologically defined determinants are often divided into two groups: private determinants which are unique to an allele, and public determinants which are shared by two or more alleles in the same region or in different regions (Klein, 1981).

This chapter will deal with the current state of the definition of the MHC of two species, mouse and man, and where relevant will
describe some of the steps leading to this definition. These are the two species in which the MHC has been most extensively studied, mice being studied mainly as members of an inbred population, and human beings as members of an outbred population. Emphasis will be placed on MHC class I and class II genes and their products, and in particular the class II genes in man, as the majority of the experimental work described in this thesis is concerned with the definition of class II gene products in an outbred species. Class IV genes will be discussed in conjunction with class I genes. Class III genes, which code for some complement components, and other MHC-associated loci will not be dealt with; some authors prefer to classify them as being separate to the MHC (Klein et al., 1981). Some consideration will also be given to the biological function of the MHC. Finally, the current state of definition of the MHC of cattle, which has not been as extensively studied as the MHC of the mouse or man, will be reviewed.

1.2 MHC - EARLY HISTORICAL DEVELOPMENT

MHC molecules came to be recognized for two reasons: they were involved in the rejection of grafted tissue, and one could make antisera against them and study them serologically. The mouse MHC was the first to be proposed, following studies on tumour immunology and tumour transplantation by Gorer (1938). The term "histocompatibility" was introduced to describe the genes determining allograft rejection (Snell, 1948). The subsequent definition of this genetic system came about largely because of the development of inbred strains of mice
which differed from each other only at the MHC haplotype (MHC congeneric strains) (Snell et al., 1976).

MHC antigens were defined using histogenic approaches (Snell, 1951), and with alloantisera in both haemagglutination (Gorer & Mikulska, 1954) and cytotoxicity assays (Gorer & O’Gorman, 1956). H-2 was established as the major histocompatibility locus in the mouse (Counce et al., 1956), being the most important barrier to allograft survival and being responsible for rapid skin allograft rejection (average 8.5 days). Minor histocompatibility loci also exist, scattered throughout the mouse genome (Graff, 1978). Skin graft rejection by mice which differ at minor histocompatibility loci takes 24 days or more, and individually the contribution of these loci to graft rejection is small (Counce et al., 1956; Klein, 1975; Graff, 1978). The red cell antigen systems in the mouse do not appear to affect significantly skin allograft survival, with the possible exception of the Ea-2 locus (Klein, 1975). In man, in addition to the MHC, the red cell ABO antigen system also has a significant effect on allograft survival (Ceppellini et al., 1965).

Individual genetic regions within the MHC, and multiple loci within each region and their position, were identified by observing recombination within haplotypes (Snell et al., 1971; Klein, 1975).

1.3 MOUSE MHC - CLASS I LOCI

1.3.1 Serology and biochemistry

The class I loci are considered to consist of two groups: those coding for the classical transplantation antigens, and the Qa/Tla
genes which code for the so-called "lymphocyte differentiation antigens".

Two class I regions coding for classical transplantation antigens are recognized: K and D. Serologically defined antigens could initially be arranged into two mutually exclusive series, controlled apparently by alleles at two genetic loci (H-2K and H-2D), and this was supported by recombination data (Snell et al., 1971; Klein, 1975). The gene products are expressed co-dominantly in F1 hybrid animals.

The K region appears to consist of a single K locus while there is evidence that the D region consists of the D locus and possibly three additional loci (L, M, and R). However, little information exists on these additional loci (Demant & Neauport-Sautes, 1978; Ivanyi et al., 1979; Ivanyi & Demant, 1979; Hansen et al., 1981). A genetic map of the mouse MHC is shown in Figure 1.1 (Klein et al., 1983; Vaiman et al., 1986). The term "class IV genes" has been used to describe the loci on the telomeric side of H-2D, including H-2L, H-2M and the Qa/Tla loci (Klein, 1981). However, more often these genes are described as class I genes, due to their molecular relationship with the K and D loci (Steinmetz & Hood, 1983).

The Qa and Tla genes control expression of the Qa and TL antigens respectively. The TL antigens are a system of alloantigens expressed on some murine leukaemic cells and on the thymocytes of certain mouse strains (Old & Stockert, 1977). They may even be expressed at a very low level on thymocytes of all mouse strains (Michaelson et al., 1986). The Qa loci, which map between H-2D and Tla, control the expression of alloantigens on some lymphoid and myeloid cells and each has a characteristic strain and tissue
A genetic map of the H-2 complex. The centromere is located to the left. Based on maps described by Klein et al. (1983) and Vaiman et al. (1986).
CHROMOSOME 17 OF THE MOUSE

Diagram showing the loci Q, K, I-A, I-E, CLASS III LOCi, D (L,M,R), Qa, and Tla arranged in sequence.
distribution (Stanton & Boyse, 1976; Flaherty et al., 1978; Kincade et al., 1980). It has been suggested that Qa molecules might be involved in cell differentiation (Kincade et al., 1980).

Over 100 class I serological determinants have been defined in the mouse (Klein, 1981). In inbred mice, 33 K alleles and 14 D alleles have been identified (Klein & Figueroa, 1981).

Mouse class I molecules have been shown by immunoprecipitation studies to be transmembrane glycoproteins of molecular weight 40,000-45,000 daltons (Schwartz et al., 1973; Nathenson & Cullen, 1974; Rask et al., 1974). They are bound non-covalently to β2-microglobulin, which is a glycoprotein with a molecular weight of around 12,000 daltons (Natori et al., 1974; Rask et al., 1974; Silver & Hood, 1974; Vitetta et al., 1975). The β2-microglobulin chain gene is not located with the rest of the MHC on chromosome 17 (Klein, 1981).

A single class I molecule has been shown biochemically to carry both private and public specificities (Cullen et al., 1972; Nathenson & Cullen, 1974; Hauptfield & Klein, 1975). The antigenic specificity of the class I molecule appears to be due to its amino acid sequence rather than to carbohydrate moieties (Shimada & Nathenson, 1971; Pancake & Nathenson, 1973).

Each class I molecule consists of five domains, based on the three-dimensional structure created by intra-chain disulphide bonding: an intracellular domain, a transmembrane portion and three extracellular domains (α1, α2, and α3) (Coligan et al., 1981; Steinmetz & Hood, 1983). Most of the variation between antigens, as determined by protein and DNA sequence analyses, occurs in the α1 and α2 domains (Steinmetz & Hood, 1983). Class I antigens generally differ from each other by multiple amino acid substitutions, many of
which are clustered in areas of the molecule distal to the cell surface.

The products of the Qa and Tla loci have a similar molecular weight to the products of the K and D regions, and are also associated with β2-microglobulin (Oetberg et al., 1975; Vitetta et al., 1975; Michaelson et al., 1981; 1986).

1.3.2 Tissue distribution

Class I antigens are expressed on most cells of the body, although the amount expressed per cell varies widely (Klein, 1981). Lymphocytes and macrophages express class I antigens strongly while expression on some other cell types is either very weak or absent. These cells include spermatozoa, trophoblast cells, a number of epithelial cell types (such as the lining cells of the vas deferens, parietal and chief cells of the stomach, and ciliated tracheal cells) and hepatocytes (Klein, 1981).

1.4 MOUSE MHC - CLASS II LOCI

1.4.1 Serology and biochemistry

The identification of the class II region developed from the observation that the antibody and delayed-type hypersensitivity response of inbred strains of mice to a number of simple polypeptides was controlled by genes linked to the H-2 complex (Benacerraf & McDevitt, 1972). These so-called immune response (Ir) genes were mapped between the K and D regions (McDevitt et al., 1972), and this region was called the I region (Klein et al., 1974).
Cross-immunization between strains differing only at the I region resulted in the definition of an alloantigen system which was present mainly on B lymphocytes (Sachs & Cone, 1973; Hammerling et al., 1974). The determinants were termed Ia (I region-associated) antigens (Shreffler et al., 1974), and later, class II antigens (Klein, 1977). Alloantisera raised between strains carrying intra-MHC recombinations enabled the identification of four class II subregions (A, C, E, and J) (Shreffler et al., 1977). A fifth subregion, I-B, was defined not by serology but by a split in Ir gene responsiveness (Lieberman & Humphrey 1972).

Class II molecules are transmembrane cell surface glycoproteins, consisting of two non-covalently linked polypeptide chains; an α chain of molecular weight 30,000-35,000 daltons, and a β chain of 25,000-30,000 daltons (Cullen et al., 1976b; Silver et al., 1977; Cook et al., 1978). The molecular weight variations may be due to differences between subregions and haplotype differences between inbred strains (Cook et al., 1978). A non-polymorphic chain (the "invariant chain") with a molecular weight of around 31,000 daltons is associated with the class II molecule prior to its appearance at the cell surface (Jones et al., 1979), and is encoded by a gene on chromosome 18 (Yamamoto et al., 1985).

I-B has not been serologically defined, and appears to be a functional artefact (Baxevanis et al., 1981). Some I-C specificities have been moved into the I-E subregion (David & Cullen, 1978) and no separate I-C molecules have been detected biochemically. I-J codes for determinants found on subsets of T cells, on a number of suppressor factors described in in vitro systems, and on some macrophages (Murphy et al., 1976; Tada et al., 1976; Niederhuber et
I-J determinants have been shown to function in *in vitro* suppressor cell systems (Dorf & Benacerraf, 1985) but there has been no biochemical identification of I-J gene products. In addition, DNA and RNA studies have failed to detect a candidate I-J sequence in the position within the MHC to which I-J has been mapped by classical genetic techniques (Steinmetz *et al*., 1982; Kronenberg *et al*., 1983). These discrepancies involving I-J have not yet been resolved. Therefore, it has been suggested that the class II region be considered to consist of two subregions, I-A and I-E(C), until better evidence is found that additional loci exist (Klein *et al*., 1981). This simplified model is in keeping with DNA analysis (Klein & Nagy, 1982a; Steinmetz *et al*., 1982). The positions of the class II subregions in the genetic map of the mouse MHC are shown in Figure 1.1.

Biochemical evidence suggested that the I-A subregion codes for the α and β chains of the I-A molecule and also for Eβ, while the I-E subregion codes for Ea only (Jones *et al*., 1978; Silver & Russell, 1979; Uhr *et al*., 1979). Some inbred strains and some wild mice do not express a cell surface I-E molecule, due to mutations affecting the Ea gene or to defective Ea mRNA (Ozato *et al*., 1980; Mathis *et al*., 1983; Dembic *et al*., 1985).

A single class II molecule has been shown biochemically to carry both private and public antigens (Cullen *et al*., 1976a). Some 70 class II determinants have been serologically identified in inbred mice; most are public determinants and most are in the I-A subregion (Klein *et al*., 1983). Extensive allelic variation has been demonstrated between β chains, as shown by amino acid and DNA sequence analyses, while the α chains show limited polymorphism (Cook *et al*.,
The numbers of class II alleles defined in inbred mice are: 12 Aβ, one Aα, 11 Eβ, and 5 Ea (Klein & Figueroa, 1981).

The antigenic specificity of the class II molecule appears to be due to its amino acid sequence rather than to carbohydrate moieties (Cullen et al., 1975; 1976b). However, a family of low molecular weight, carbohydrate-defined class II antigens has been described in the mouse (Parish et al., 1978). The function of these molecules has not been established.

Each class II α and β chain consists of four structural domains created by intra-chain disulphide bonding; the intracellular domain, the transmembrane portion and two extracellular domains, of which the N-terminal domain is the most distal to the cell surface. There are extensive differences between alleles of the polymorphic loci as determined by both protein and DNA sequence analyses. The differences are largely in the membrane-distal domain and specific areas of hyper-variability occur (Steinmetz & Hood, 1983; Kaufman et al., 1984; Bell et al., 1985).

1.4.2 Tissue distribution

Class II antigens are normally expressed on B lymphocytes and on a subpopulation of macrophages/monocytes (Unanue et al., 1974; Hammerling, 1976; Beller et al., 1980; Unanue, 1984). The percentages of macrophages/monocytes expressing class II antigens in vivo increases after infection or immunization (Beller et al., 1980; Nussenzweig et al., 1981).

The amount of class II antigen expressed on other cells is generally quite small compared with the amount expressed on B
lymphocytes. Some cell types do not appear to express class II antigens. Differences in cellular expression may be quantitative rather than qualitative, and variations between reports may reflect differences in the sensitivity of assays.

Class II antigen expression has been reported on thymocytes (David et al., 1973; Schwartz et al., 1977) and on some T lymphocytes (Gotze, 1975; Hammerling, 1976; Murphy et al., 1976). Class II antigen expression has also been reported on epidermal Langerhans cells (Klareskog et al., 1977; Rowden et al., 1978), and on some epithelial cells including in the thymus, the intestine and the mammary gland during pregnancy and lactation (Klareskog et al., 1980; Klein 1981).

Class II antigens do not appear to be expressed on red cells or platelets (Shreffler & David, 1975; Colombani et al., 1976; Staines et al., 1976).

1.4.3 Mixed lymphocyte reaction (MLR)

The genetic control of the mixed lymphocyte reaction (MLR) maps predominantly to the H-2 complex (Dutton, 1966), and the strongest stimulation is associated with differences in the class II region (Bach et al., 1972; Meo et al., 1973). Evidence that class II determinants are the major MLR determinants includes the fact that the reaction can be inhibited by antisera against the class II determinants on stimulator cells (Meo et al., 1975), and the fact that MHC mutations have been found to simultaneously affect both class II determinants and MLR determinants (McKenzie et al., 1979). Some non-MHC MLR determinants have also been identified (Festenstein et al., 1972).
1.5 HUMAN MHC - CLASS I LOCi

1.5.1 Serology and biochemistry

MHC antigens were originally defined in humans using multiparous alloantisera and a haemagglutination assay (Dausset, 1958, cited by Albert & Gotze, 1977; Payne & Rolfs, 1958; van Rood et al., 1958). This assay was subsequently replaced by the microlymphocytotoxicity test (Terasaki & McClelland, 1964), which allowed antisera to be screened on a large scale. The human MHC antigen system is known as the HLA system (WHO-IUIS, 1975).

Because of their significant effect on skin graft survival times, HLA antigens are considered to be the human equivalent of the mouse H-2 antigens (Dausset et al., 1970). HLA definition has been greatly assisted by nine International Histocompatibility Workshops, the most recent of which was held in Munich in 1984 (Albert et al., 1984). Seventy serologically defined HLA class I antigens are currently recognized (23 HLA-A, 49 HLA-B, and 8 HLA-C antigens) (Bodmer, W. et al., 1984). A genetic map of the human MHC is shown in Figure 1.2 (Giles & Capra, 1985; Vaiman et al., 1986).

An interesting feature of HLA antigens at a population level is linkage disequilibrium, which refers to the preferential (non-random) population association between two alleles coded for by separate linked loci (Bodmer & Bodmer, 1978). Such sharing of parts of haplotypes has also been described between wild mice and strains of inbred mice (Klein & Figueroa, 1981). This non-random association may be due to some form of natural selection advantage, non-random mating, or insufficient evolutionary time for the population to reach equilibrium (Bodmer & Bodmer, 1978).
Figure 1.2

A genetic map of the HLA complex. The centromere is located to the left. Based on maps described by Giles & Capra (1985) and Vaiman et al. (1986).
HIA class I molecules are transmembrane glycoproteins of molecular weight 43,000-44,000 daltons, non-covalently bonded to a 12,000 dalton molecule, β2-microglobulin (Springer et al., 1974; Barnstable et al., 1978). The class I molecules are encoded by genes on chromosome 6 (van Someren et al., 1974), and β2-microglobulin is encoded by a gene on chromosome 15 (Goodfellow et al., 1975). The antigenic specificity of class I molecules appears to be due to their amino acid sequence (Parham et al., 1977). Private/public antigen relationships have been described biochemically (Schwartz et al., 1980).

HIA class I molecules consist of five structural domains, as in the mouse (Ploegh et al., 1981). Both the α1 and α2 domains appear to carry most of the polymorphisms and much of the biochemical variation between alleles can be localized to a few discrete areas within these domains (Orr et al., 1979).

1.5.2 Tissue distribution

HIA class I antigens appear to be expressed on most cells of the body (Daar et al., 1984a). Cells and tissues reported to express little or no class I antigen include red cells, spermatozoa, seminiferous tubules, cerebral cortex, cerebellum, sympathetic ganglia, hypophysis, parathyroid gland, thyroid gland, exocrine pancreas, hepatocytes, skeletal muscle, smooth muscle, corneal endothelium and duodenal glands of Brunner (Law & Bodmer, 1978; Daar et al., 1984a; Harris & Gill, 1986; Kuhlman et al., 1986).
1.6 HUMAN MHC - CLASS II LOCI

1.6.1 Mixed lymphocyte reaction (MLR)

An association was found between HLA incompatibility and the strength of the MLR (Bach et al., 1967; Ivanyi et al., 1967). Subsequent mapping studies showed that the determinants controlling strong mixed lymphocyte reactivity were encoded by a locus which was separate to the class I loci (Yunis & Amos, 1971).

The definition of MLR determinants was simplified by the use of cell panels which were homozygous for HLA antigens (Dupont et al., 1973; Jorgensen et al., 1973; Mempel et al., 1973; van der Tweel et al., 1973). The region coding for MLR determinants (which are known as HLA-Dw determinants) was termed the HIA-D region, and 19 HLA-Dw determinants are currently recognized (Bodmer, W. et al., 1984). Some non-HLA-D genes control weak mixed lymphocyte reactivity (Suciu-Foca & Dausset, 1975; Fainboim & Festenstein, 1979).

1.6.2 Serology and biochemistry

The existence of class II antigens was deduced from two phenomena: (a) the ability of some alloantisera to block the MIR (Ceppellini et al., 1971; Hattler et al., 1971; Revillard et al., 1973); and (b) the "extra reactions" observed with some anti-class I typing antisera when tested against cultured lymphoid and leukaemic cell lines (Dick & Steel, 1971; Pegrum et al., 1971; Walford et al., 1971). The MIR-blocking activity appeared to be due to antibodies against MIR determinants expressed on B cells, indicating that it might be possible to define MIR types serologically (van Leeuwen et al., 1973). Gosset et al. (1975) suggested that the "extra
reactivity" of anti-class I typing antisera was predominantly directed against B lymphocytes.

The class II antigen series was subsequently defined and these antigens were named HLA-DR antigens (Bodmer, J., 1978a). This antigen series shows close association with HLA-Dw types (Winchester et al., 1975a; Bodmer, J., 1978a) and is linked with the HLA class I antigen series (Winchester et al., 1975a; Pickbourne et al., 1978a). At the Ninth International Histocompatibility Workshop in 1984, 14 "conventional" allelic DR antigens and 2 "supertypic" DR antigens (DRw52 and DRw53) (see below) were recognized (Bodmer, W. et al., 1984). Some of the conventional DR antigens (e.g. DRw4, DRw6) display serological heterogeneity and are associated with several different MIR types (Pazderka et al., 1980; Reinsmoen & Bach, 1982; Termijtelen et al., 1982; Schreuder et al., 1984).

HLA class II molecules are transmembrane cell surface glycoproteins, consisting of two non-covalently linked polypeptide chains of molecular weights 33,000-34,000 daltons (the α chain), and 28,000-29,000 daltons (the β chain) (Snary et al., 1977a; Springer et al., 1977; Klareskog et al., 1978). An invariant chain, similar to that found in the mouse, has been identified (Charron & McDevitt, 1980) and is encoded by a gene on chromosome 5 (Claesson-Welsh et al., 1984). The antigenic specificity of HLA class II molecules appears to be due to the amino acid sequence (Snary et al., 1977b; Charron & McDevitt, 1980; Shackleford et al., 1982; Shackleford & Strominger, 1983).

Private/public antigen relationships have been described biochemically (Hurley et al., 1984). Antisera which reacted with particular groups of DR specificities led to the definition of some
new series of class II antigens or "supertypic specificities" (Tosi et al., 1978; Duquesnoy et al., 1979; Park et al., 1980). Tosi et al. (1978) and Duquesnoy et al. (1979) suggested that the supertypic specificities were carried by separate molecules, encoded by a separate subregion which was linked to the DR subregion, and this was verified by further biochemical studies (Katigari et al., 1979; Mann et al., 1979; Makert & Cresswell, 1980; Corte et al., 1981; Shackleford et al., 1982; 1983; Tanigaki et al., 1983).

The HIA-DQ subregion and 3 DQ specificities were formally recognized at the Ninth International Workshop (Bodmer, W. et al., 1984). These specificities occur at a relatively high frequency in the population and appear to be supertypic, as there is serological and biochemical evidence that they will be subdivided (Giles & Capra, 1985).

Evidence for a third class II subregion, HLA-DP, came from the results of primed lymphocyte typing (Shaw et al., 1980), and was confirmed by observing recombination between this subregion and HIA-DR (Shaw et al., 1981), by the use of deletion mutant cell lines (Kavathas et al., 1981) and by biochemical studies (Giles & Capra, 1985). Alloantisera defining the products of this subregion are rare (van Leeuwen et al., 1982), but some anti-DP monoclonal antibodies exist (Shaw et al., 1982). At the Ninth International Workshop, 6 DP determinants were recognized (Bodmer, W. et al., 1984).

Some members of the "Ml" supertypic series (Park et al., 1980), namely DRw52 and DRw53, have been shown by biochemical studies to be carried on DRβ chains distinct from those carrying the conventional DR antigens, and may also be carried on the β chains carrying the conventional DR antigens (Hurley et al., 1984).
The positions of the class II subregions in the genetic map of
the human MHC are shown in Figure 1.2. The DR and DQ subregions are
tightly linked, which has led to characteristic population
associations between DR and DQ specificities (Bodmer, W. et al.,
1984). Unusual population associations have also been described
(Termijtelen et al., 1980; Oudshoorn et al., 1984). The DP subregion
has been mapped adjacent to DQ, but frequent recombination appears to
occur between them (Bodmer, 1984).

As in the mouse, each class II α and β chain consists of four
structural domains (Kaufman et al., 1984). Amino acid sequence and
DNA analyses have suggested that most DR polymorphisms are carried by
the N-terminal domain of the β chains (Shackleford et al., 1982;
Kaufman et al., 1984; Trowsdale et al., 1985). In contrast, both DQα
and DQβ chains and their genes are quite polymorphic, and both DPa and
DPβ chain genes have been reported to be polymorphic (Giles & Capra,
1985; Trowsdale et al., 1985; Ando et al., 1986). The exact molecular
localizations of the defined DQ and DP specificities is unknown; they
could reside on α chains or on β chains or on both.

For all class II chains, most of the polymorphisms are carried
in the N-terminal domains and distinct hyper-variable regions exist.
Extensive variation exists between alleles (Schenning et al., 1984;
Korman et al., 1985; Rask et al., 1985).

1.6.3 Relationship between serological and MIR determinants

Although there is evidence that HLA-DR antigens are the HLA-Dw
determinants (Albrechtsen et al., 1978; Bodmer, J., 1978a;
Termijtelen et al., 1982), the population associations between Dw and
DR determinants are not absolute and apparent recombinations have also
been reported (Troup et al., 1978; Sachs et al., 1981; Jaraquemada et al., 1984).

Some instances of MLR incompatibility between DR-identical cells have been explained by DR differences detectable only by monoclonal antibodies or biochemical analysis (Groner et al., 1983; Nepom et al., 1983), and it is possible that DQ differences explain some of the other reactions (Cohen et al., 1986; Moller et al., 1986). The HIA-D type, as determined by the MLR, might not represent separate determinants, but rather the added stimulatory effect of a mosaic of determinants coded for by DR and possibly DQ, DP and other as yet unknown subregions (Schreuder & Degos, 1984; Bach, 1985; Cohen et al., 1986; Moller et al., 1986). On the other hand, some investigators still hold the view that most MLR stimulation is due to determinants which are encoded by genes within the DR subregion but which are independent of the serologically defined specificities (Jaraquemada et al., 1986).

1.6.4 Tissue distribution

HIA class II antigens are normally expressed on B lymphocytes, monocytes, and some early myeloid and erythroid progenitors (Winchester & Kunkel, 1979; Sieff et al., 1982; Nunez et al., 1984). As in the mouse, class II expression on some other cell types is contentious, but where these antigens have been identified on other cells, their expression is weak compared with the expression on B cells.

Class II antigens have been reported on a minor subpopulation of peripheral blood T lymphocytes in normal individuals. The percentage is increased in vivo during some disease states or during an immune
response (Winchester & Kunkel, 1979), and after in vitro stimulation (Winchester & Kunkel, 1979; Charron et al., 1980; Indiveri et al., 1980). Class II antigens have also been identified on some vascular endothelial cells (Hirschberg et al., 1979; Natali et al., 1981; Daar et al., 1984b), on epidermal Langerhans cells, alveolar macrophages, Kupffer cells (Klareskog et al., 1977; Rowden et al., 1977; Natali et al., 1981), and on epithelial cells of a number of organs including the thymic cortex, parts of the gastro-intestinal tract, parts of the respiratory tract, the tubules of the kidney, and the lactating mammary gland (Newman et al., 1980; Natali et al., 1981; Daar et al., 1984b). Class II antigen expression has also been reported on some thymocytes (Williams et al., 1980).

Class II antigens do not appear to be expressed on red cells, platelets or spermatozoa (van Leeuwen et al., 1973; Law & Bodmer, 1978; Williams et al., 1980; Kuhlman et al., 1986).

1.7 COMPARISON BETWEEN MOUSE AND HUMAN MHC

1.7.1 Molecular genetics

More than 30 class I-like gene sequences have been described in mice, depending on the strain being studied. Most are located in the Qa/Tla region and some of these are probably pseudogenes (Steinmetz & Hood, 1983; Winoto et al., 1983; Flavell, et al., 1985). The K and D region loci are very polymorphic but there is relatively little polymorphism in the genes of the Qa/Tla region (Steinmetz & Hood, 1983; Tewarson et al., 1983; Flavell et al., 1985). The number of class I-like gene sequences in man has been estimated at 20-40, many of which correspond to pseudogenes (Steinmetz & Hood, 1983; Cohen et
al., 1985; Jordan et al., 1985). Some of these sequences are probably the equivalent of the mouse Qa/Tla loci (Orr & De Mars, 1983). The variation in gene number observed between mouse strains and between different human haplotypes suggests that there is an expansion and contraction of gene number occurring by mechanisms which could include duplication, translocation or unequal crossing-over (Winoto et al., 1983; Cohen et al., 1985).

The typical class I gene in both mouse and man consists of 8 exons, with each structural domain of the molecule being encoded by a single exon (Steinmetz & Hood, 1983; Kaufman et al., 1984; Jordan et al., 1985).

Only 4 functional class II loci have been described in the mouse (Aα, Aβ, Eα, and Eβ); additional loci have been revealed by gene cloning, but their expression and functional significance are not yet clear (Steinmetz & Hood, 1983; Kaufman et al., 1984; Figueroa & Klein, 1986).

In man, the DR subregion appears to consist of one α chain gene, and one to 4 β chain genes (depending on the haplotype), not all of which are expressed (Bodmer, 1984; Bohme et al., 1985; Rask et al., 1985; Moller et al., 1986). All haplotypes appear to contain 2 DQα chain genes and 2 DQβ chain genes which might both be expressed (Bohme et al., 1985; Okada et al., 1985a; Rask et al., 1985; Moller et al., 1986). The DP subregion consists of 2 α chain genes and 2 β chain genes, of which one of each is probably a pseudogene (Kappes et al., 1984; Servenius et al., 1984; Trowsdale et al., 1984; Okada et al., 1985b). The existence of additional HLA class II genes has been suggested (Inoko et al., 1985; Tonnelle et al., 1985; Trowsdale et al., 1985).
A typical class II α chain gene in both mouse and man consists of 5 exons, while a β chain gene consists of 6 exons; each domain of the molecule is encoded by a single exon (Steinmetz & Hood, 1983; Kaufman et al., 1984; Korman et al., 1985; Rask et al., 1985).

1.7.2 General

Amino acid sequence homology between class I molecules of mouse and man is about 70% (Coligan et al., 1981), and extensive sequence homology exists with other species including the rat, the guinea pig, and the chicken (Cook et al., 1978). Human class II DR molecules show homology with mouse I-E molecules and DQ with I-A, as determined by amino acid and DNA sequence studies (Shackleford et al., 1982; Kaufman et al., 1984; Giles & Capra, 1985).

The most striking difference between the two species is that in the mouse, the class II region is situated between class I loci, whereas in man it is situated outside the class I loci (Barnstable et al., 1977). A further difference is that there are fewer class II α chain genes in the mouse, and fewer expressed class II α and β chain genes. No murine equivalent to the DP subregion has been described, although there is some evidence of multiple I-E-like molecules in certain mouse haplotypes (Lafuse et al., 1982; Ozato & Sachs, 1982). It is possible that additional sequences remain to be identified in both species.

1.8 MHC OF OTHER SPECIES

The definition of the MHC in other species has generally been based on the methods used to define it in either the mouse or man,
depending on whether the population under study has been inbred or outbred. The existence of an MHC has been proposed in a number of species including the chicken (Schieman & Nordskog, 1961), cattle (Caldwell et al., 1977), the chimpanzee (Balner et al., 1974; Seigler et al., 1974), the dog (Vriesendorp et al., 1971), the goat (van Dam et al., 1979), the guinea pig (de Weck et al., 1971), the horse (Bailey et al., 1979), the pig (Vainan et al., 1970) the rabbit (Black, 1967), the rat (Palm, 1964), the rhesus monkey (Balner et al., 1971), the sheep (Millot, 1978) and the Syrian hamster (Duncan & Streilein, 1978).

In the Syrian hamster, no class I polymorphisms have been identified serologically or biochemically, even in strains derived from wild hamsters (Duncan & Streilein, 1977; Streilein & Duncan, 1983). However, class II differences exist, as evidence by class II alloantisera, allograft rejection, graft-versus-host reactivity, and mixed lymphocyte reactivity (Duncan & Streilein, 1978; Streilein et al., 1981). At the DNA level, limited polymorphism of class I genes has been demonstrated (McGuire et al., 1985).

1.9 BIOLOGICAL FUNCTION OF THE MHC

1.9.1 Immune response (Ir) genes

Although most of the initial interest in MHC polymorphism was related to organ transplantation, further interest developed from the discovery of MHC-linked immune response (Ir) genes.

The first specific Ir genes were described in guinea pigs immunized with synthetic polymers, where the ability of an individual to mount an antibody response or a delayed-type hypersensitivity
dermal response to the polymer was inherited as a simple Mendelian dominant trait (Levine & Benacerraf, 1965; Benacerraf et al., 1967). Similar genes were also described in mice (McDevitt & Sela, 1965). In both species, the genes were mapped to within or near the MHC (Benacerraf & McDevitt, 1972; McDevitt et al., 1972), and this so-called I region was found also to control expression of the class II antigens (Shreffler et al., 1974). In vitro models of Ir gene effects were developed, using antigen-induced proliferation of sensitized T cells (Shevach & Rosenthal, 1973).

Ir genes are of high antigenic specificity (Mozes et al., 1974; Hill & Sercarz, 1975; Keck, 1975). They have been shown to exist not only for simple synthetic antigen of restricted heterogeneity, but also for cell-surface alloantigens and for limiting doses of multi-determinant antigens such as albumin, myeloma proteins, insulin, and cytochrome c (Benacerraf & McDevitt, 1972; Stimpfling & Durham, 1972; Green, 1974; Keck, 1975; Solinger et al., 1979). The suggestion is that one can only detect "all or none" control of an immune response when a single antigenic determinant, or a very small number of antigenic determinants, is recognized.

Analysis of recombinant mouse strains has localized Ir genes to the I-A subregion or to both the I-A and the I-E subregions (Nagy et al., 1981; Paul, 1984; Mengle-Gaw & McDevitt, 1985). Ir gene control has also been described for in vitro cytotoxic T lymphocyte responses to virally infected cells in mice. The genes are virus-specific and map to either the K or D class I region (Doherty et al., 1978; Zinkernagel, 1979). Ir gene control has also been described for cytotoxic T cell responses to TNP-modified syngeneic cells (Levy & Shearer, 1980).
The antibody response to some antigens is characterized by Ir gene complementation (Benacerraf & Germain, 1978). This can be explained at a molecular level by the fact that I-E molecules are coded for by genes in both the I-A and I-E subregions, and by the fact that hybrid class II molecules can be formed by the α chain of one haplotype and the β chain of the same class II subregion of the other haplotype (Jones et al., 1978; Uhr et al., 1979; Schwartz, 1986). Hybrid class II molecules have been described in the mouse and in man (Jones et al., 1978; Cook et al., 1980; Lafuse et al., 1980; Charron et al., 1984).

It is difficult to define Ir genes unequivocally in outbred species because of the extensive genetic heterogeneity which tends to obscure such single gene effects. The existence of MHC-linked Ir genes has been described or at least suggested in a number of other species, including the chicken (Balcarova et al., 1975; Benedict et al., 1975; Pevzner et al., 1978), the pig (Vaiman et al., 1978), the rat (Gunther & Stark, 1977), and the rhesus monkey (Dorf et al., 1975). In man, there is evidence suggesting the existence of HLA-linked Ir genes (or immune suppression genes in some cases). Associations have been described between HLA haplotypes or antigens and in vivo antibody responsiveness to the malarial agent Plasmodium falciparum (Osoba et al., 1979) and pollen allergens (Marsh et al., 1982). HLA associations have also been described with in vitro cellular responsiveness to a variety of antigens (Sasazuki et al., 1978; 1980; 1983; Greenberg et al., 1980; Hsu et al., 1981; Hensen & Elferink, 1984). The IgE antibody response to cedar pollen antigen in vitro and in vivo has also been linked with HLA haplotypes (Sasazuki et al., 1983). In addition, some human diseases have been
associated with HLA polymorphisms and such associations could be due to Ir gene effects (Svejgaard et al., 1983) (see Section 1.10).

1.9.2 MHC restriction

The first demonstration of a role for MHC molecules in cell-cell interactions was the observation that co-operation between T and B lymphocytes for antibody production in vivo, in both the mouse and guinea pig, required that the co-operating lymphocytes be from MHC-compatible donors (Kindred & Shreffler, 1972; Katz et al., 1973). The compatibility required was mapped to the class II region (Katz et al., 1975). Similar MHC compatibility was required for the antigen-specific proliferation of sensitized guinea pig and mouse helper T cells when stimulated by antigen-pulsed macrophages in vitro (Rosenthal & Shevach, 1973; Erb & Feldmann, 1975; Pierce et al., 1976). The compatibility required was mapped to the class II region in both species (Erb & Feldmann, 1975; Shevach, 1976). The need for macrophages to be present in addition to antigen suggested that T cells recognized antigen only on cell surfaces (Waldron et al., 1973). MHC compatibility was also required for the transfer of delayed-type hypersensitivity between mice by sensitized T cells (Miller, 1978).

In 1974, the experiments of Zinkernagel & Doherty showed that efficient in vitro lysis of virally infected target cells required cytotoxic T cells specific for the virus and compatible with the target cells at either the K or D class I region of the MHC. This phenomenon was described as MHC restriction (Zinkernagel & Doherty, 1974a), and led to the hypothesis that T cells did not recognize foreign antigen alone but recognized it in the context of self MHC. Cytotoxic T cells appeared to recognize foreign antigen in the context
of class I molecules, and helper T cells and T cells mediating delayed-type hypersensitivity reactions appeared to recognize it in the context of class II molecules (Doherty & Zinkernagel, 1975; Miller, 1978). MHC restriction did not appear to be an absolute phenomenon, but rather appeared to control the efficiency with which immune cells interacted.

Experiments with F1 animals and competitive inhibition studies suggested that cytotoxic T cells do not recognize the whole MHC class I type of an animal, but that sub-populations of T cells exist, each with specificity for foreign antigen and individual H-2K or H-2D molecules, coded for by each parental haplotype (Zinkernagel & Doherty, 1974b; 1975). Similarly, the existence of separate sub-populations of helper T cells in F1 animals, specific for foreign antigen in association with class II molecules from each parent, was demonstrated both in vitro (Paul et al., 1977a) and in vivo (Sprent, 1978).

Class I restriction of cytotoxic T cell activity in the in vitro target cell lysis assay, and class II restriction of T cell proliferation in vitro have been demonstrated in other species including man (Dickmeiss et al., 1977; Goulmy et al., 1977; McMichael et al., 1977; Bergholz & Thorsby, 1978; Hansen et al., 1978; Rodey et al., 1979).

There are several lines of evidence which suggest that MHC class I and class II molecules are the actual restricting elements. MHC-restricted responses in vitro can be blocked by antibody (Schwartz et al., 1976; Zinkernagel & Doherty, 1979; Baxevanis et al., 1980; Lerner et al., 1980). Mutations affecting the structure of MHC molecules have also been shown to affect in vitro T cell responses in
MHC-restricted systems (Doherty et al., 1976; Lin et al., 1981; Nathenson et al., 1986). In the case of class II molecules, both I-E molecules and I-E-associated restriction elements appear to be controlled by genes in both the I-A and I-E subregions (Paul, 1984). Further evidence assigning restrictive function to MHC molecules has come from the results of transfection experiments (see Section 1.9.6 below), and from studies with MHC transgenic mice (Yoshioka et al., 1987).

There is similar evidence that class II molecules are the products of Ir genes. Ir gene effects can be blocked by anti-class II antibody in vitro (Schwartz et al., 1976; Baxevanis et al., 1980; Lerner et al., 1980), and class II mutations have been shown to affect Ir gene expression (Lin et al., 1981; Michaelides et al., 1981; Lei et al., 1982). In addition, immune responses to certain polymers are controlled by genes in both the I-A and I-E subregions, reflecting the components of the I-E molecule (Uhr et al., 1979; Paul, 1984; Schwartz, 1986). Furthermore, it has been reported that an Ir gene defect, which is due to a deletion in the Ea chain gene, has been corrected by transferring a normal Ea gene into the embryo of a deficient strain of mouse (Le Meur et al., 1985). The transfer resulted in tissue-specific expression of an I-E molecule, incorporation into the germline, and correction of the particular immune response gene defect.

1.9.3 Role of the thymus

Experiments with semi-allogeneic, irradiated, chimeric mice suggested that lymphocytes recognize foreign antigen in association with the MHC type of the host environment, and are not limited to the
MHC type of the donor stem cells. This has been shown in vitro for both cytotoxic T cells (Zinkernagel, 1976; Bevan, 1977; von Boehmer et al., 1978) and helper T cells (Sprent & von Boehmer, 1979). Evidence that MHC gene products were responsible has come from studies using MHC mutant strains (Zinkernagel et al., 1978c). Thus, the self-restriction of mature T cells does not appear to be due to genotypic constraints on the T cell receptor repertoire, but appears to be acquired from host elements during ontogeny.

Thymus grafting studies identified the thymus as the host element responsible for imprinting MHC restriction on T cells (Fink & Bevan, 1978; Zinkernagel et al., 1978b), and also suggested that Ir gene expression depends on whether the thymic environment in which T cell precursors develop is of the responder or non-responder type (von Boehmer et al., 1978; Kapler & Marrack, 1978; Miller et al., 1979; Zinkernagel & Doherty, 1979). The theory of adaptive differentiation, which developed from ideas proposed by Katz (1977), suggested that the foreign antigen-receptor pool on precursor T cells must be unlimited since it must be able to adapt to any MHC molecule it encounters during development. However, the receptor pool on mature T cells is limited in terms of which MHC molecules it will recognize.

Two models have been proposed to explain MHC restriction. The first proposes that the T cell receptor for foreign antigen has one binding site which recognizes a complex of foreign antigen and self MHC (Zinkernagel & Doherty, 1974b). The second model proposes that the T cell receptor has two separate binding sites, one for foreign antigen and one for self MHC (von Boehmer et al., 1978; Cohn & Epstein, 1978; Zinkernagel et al., 1978a). The first model is supported by recent findings, including the results of X-ray
crystallography, which have indicated that self MHC molecules may bind foreign antigen and that the most polymorphic parts of the MHC molecule are in the candidate binding site (Bjorkman et al., 1987a; 1987b).

Genes coding for the α and β chains of the putative T cell receptor for foreign antigen have been described (Kronenberg et al., 1986). These T cell molecules are clonally expressed, and their diversity is generated mainly by rearrangement of the α and β chain genes. It may be that during ontogeny, only some T cells from the precursor pool have the appropriate receptors that can bind to MHC antigens expressed on thymic epithelium; all other T cells may die in the thymus (von Boehmer, 1986). The selected T cells would then be restricted by the MHC antigens which selected them. In this way, MHC antigens might also influence the diversity of T cell receptors for foreign antigen.

The hypothesis of thymic learning has been criticized on several grounds (Klein & Nagy, 1982b). There are reservations due to the extensive manipulation that irradiation chimeras undergo and questions concerning which host cells survive in such animals. In addition, there appears to be some degeneracy in MHC restriction; some murine T cells appear capable of recognizing foreign antigen in the context of allogeneic MHC types (Stockinger et al., 1980; Ishii et al., 1981). There are a number of possible explanations for this finding. There is some evidence that an MHC-restricted or alloreactive T cell receptor may "cross-react" with a different MHC molecule, because the receptor recognizes a complex of MHC molecule plus foreign antigen (Zinkernagel & Doherty, 1979; Sredni & Schwartz, 1980; Hunig & Bevan, 1982). Alternatively, the restricting epitope and the associated
serologically defined antigen may be distinct sites on the same molecule. A single MHC molecule appears to contain multiple restriction sites (Lin et al., 1981; Hurwitz et al., 1983; Frelinger et al., 1984). Within an individual immune response, different T cell clones might be restricted by different MHC epitopes, some of which might be shared with other allelic MHC molecules. Finally, it is possible that the degeneracy observed in MHC restriction occurs because the hypothesis of MHC restriction is incorrect.

Chimerism of lymphoid cells and tolerance of foreign MHC determinants can occur naturally, as evidenced by the high incidence of naturally-occurring haemopoietic chimeras in cases of dizygotic twins in cattle. This chimerism occurs due to the development of a common placental circulation (Owen, 1945; Owen et al., 1946). These chimeric calves show no evidence of reaction to their co-twin's lymphocytes by in vivo or in vitro assays (Emery & McCullagh, 1980b; 1980c), although they may display attenuated reactivity against grafts of the co-twin's skin (Emery & McCullagh, 1980a).

1.9.4 Proposed different roles for MHC class I and class II molecules

It has been suggested that MHC molecules might act as markers which different subpopulations of T cells recognize in conjunction with foreign antigen on the surface of antigen-presenting cells (Doherty & Zinkernagel, 1975; Cohn & Epstein, 1978). Cytotoxic and suppressor T cells appear to recognize class I molecules. Since most cells of the body express class I antigens, they could act as antigen-representing cells for cytotoxic and suppressor T cells (Zinkernagel, 1979).
T cells recognizing class II molecules appear to act as helper T cells in antibody production, produce delayed-type hypersensitivity effects and activate macrophages (Katz et al., 1975; Miller, 1978; Zinkernagel et al., 1977). The stimulation of sensitized helper T cells in vitro seems to require the processing and presentation of foreign antigen by a class II-positive accessory cell (Unanue, 1984). Some kind of class II-positive macrophage/monocyte is considered to be the main cell type capable of such antigen presentation in the mouse and in man (Thorsby, 1982; Unanue, 1984). Other class II-positive cell types are also capable of presenting antigen to sensitized helper T cells in vitro (Stingl et al., 1978a; Sunshine et al., 1980; Grey & Chestnut, 1985; Lanzavecchia, 1985).

Strictly speaking, the MHC antigen class recognized by a T cell appears to correlate more closely with the Lyt or OKT (CD) phenotype of the T cell, rather than with its biological activity (helper or cytotoxic function) (Swain, 1981; Meuer et al., 1982). It has been suggested that the OKT4 and OKT8 structures on human T cells (and their equivalents in the mouse) might stabilize cell-cell interactions by binding to non-polymorphic parts of their counterpart MHC molecules on antigen-presenting cells (Reinherz et al., 1983).

1.9.5 MHC polymorphism

One of the most striking features of the MHC is its extreme polymorphism compared with that of other polymorphic loci such as enzyme loci (Bodmer & Bodmer, 1978; Klein & Figueroa, 1981). Over 95% of wild mice are heterozygous at both the K and D class I regions and over 85% are heterozygous at both the Aα and Eα class II loci (Klein & Figueroa, 1981). More than 80% of humans are heterozygous at
all class I and class II loci (Bodmer & Bodmer, 1978). Estimates have put the number of alleles at both the H-2K and H-2D class I loci of wild mice at least 100, and the Aβ and Eβ class II loci may be just as polymorphic. In addition, MHC alleles generally differ from each other by a large number of amino acids, whereas enzyme polymorphisms generally differ from each other by a small number of amino acids (Klein & Figueroa, 1981).

One hypothesis suggests that this polymorphism is maintained by selective forces in the environment, which favour heterozygosity in the individual and diversity at the population level to protect a species against disease epidemics (Zinkernagel & Doherty, 1979; Klein & Figueroa, 1981). Certain combinations of foreign antigen and a particular MHC background apparently fail to be recognized by T lymphocytes, as evidenced by MHC-associated Ir gene defects. A high degree of MHC polymorphism may ensure that within a species there are always some individuals able to respond to a foreign antigen. There may be a tendency for new variants to be at a selective advantage, which may drive the polymorphism (Bodmer & Bodmer, 1978). Multiple MHC loci of each class would increase the MHC diversity within each individual (Zinkernagel & Doherty, 1979; Klein, 1981). Such selective pressure may only operate strongly when a species is subjected to a drastic change in its mode of life (as may occur in the evolution of a new species) (Klein, 1987).

However, there is little published evidence that disease epidemics preferentially affect individuals with certain MHC types. A significant decrease in the frequency of antigen HLA-B7 has been reported in a population surviving a typhoid and yellow fever epidemic (de Vries & van Rood, 1978). The Syrian hamster has extremely limited
class I polymorphism (see Section 1.8) and to date, no specific pathogenic virus has been demonstrated for this species (Streilein et al., 1981). Its attenuated social structure and limited climatic habitat may mitigate against the emergence of Syrian hamster-specific viruses, and thereby greatly reduce evolutionary pressure for class I polymorphism (Streilein et al., 1984).

MHC polymorphism may be generated at least partly by a mechanism called gene conversion (Lew et al., 1986). This term was originally used to describe the meiotic non-reciprocal exchange of information between genes which occurs in fungal spores (Lindegren, 1952). Some geneticists have criticized the use of the term in relation to MHC polymorphism, and maintain that most MHC polymorphism characterized by clustered variation is consistent with the accumulation of multiple point mutations under selective pressure (Klein, 1984). However, there is evidence that some form of copy/substitution does occur within the MHC (Pease, 1985). Sequences present in one part of the genome appear to be copied and substituted in a non-reciprocal fashion onto another MHC gene, either on the homologous chromosome or the same chromosome. Apparent donor DNA sequences have been described for some mouse MHC genes, especially in relation to recent MHC variant genes (Mellor et al., 1983; Denaro et al., 1984; Steinmetz et al., 1984; Nathenson et al., 1986).

Other genetic mechanisms, such as multiple point mutations and small deletions are probably also involved (Gustafsson et al., 1984; Pease, 1985; Rask et al., 1985). Hybrid class II molecules provide an additional mechanism for generating MHC polymorphisms (see Section 1.9.1). The production of multiple products from a single gene, either by post-translational modification or by alternative splicing
of mRNA, has been suggested, but the significance of these events is yet to be determined (Loube et al., 1983; Bell et al., 1985; Lew et al., 1986).

1.9.6 Transfection of MHC genes in vitro

Human and mouse class I genes have been successfully transfected into mouse L cells (Barbosa et al., 1982; Goodenow et al., 1982). The transfected mouse genes have been capable of functioning as restriction elements for cytotoxic T cells when the L cells are infected with virus (Mellor et al., 1982). There have been varying results when transfecting human class I genes into mouse L cells. In some cases, transfected L cells have been lysed by alloreactive cytotoxic T cells (Cowan et al., 1985), and transfected and infected L cells have been lysed by virus-specific cytotoxic T cells (Gomard et al., 1986).

Class II genes from both species have been successfully transfected (Malissen et al., 1983; Rabourdin-Combe & Mach, 1983), and have been reported to function as restriction elements in proliferation assays involving antigen-specific T cells (Germain & Norcross, 1983; Trowsdale et al., 1985).

Coupled with exon shuffling and site-directed mutagenesis, transfection experiments should allow closer examination of the relationship between the structure and function of the products of individual MHC loci. Some mouse and human class I determinants recognized by antibodies and T cells appear to be conformational and involve interaction between the α1 and α2 domains of the class I molecule (Allen et al., 1984; Arnold et al., 1984; Jordan et al., 1985). There is also evidence that some class II determinants
recognized by T cells are conformational and are formed by the interaction of both the \( \alpha \) and \( \beta \) chains of the molecule (Germain & Malissen, 1986).

1.9.7 Possible non-immunological functions of the MHC

The absence of class I polymorphism in the Syrian hamster suggests that the polymorphism seen in mouse and man may reflect a selective advantage via an immunological function, which is secondary to another function of MHC molecules (McGuire et al., 1985). Speculative non-immunological functions of MHC molecules include a general role in inter-cellular adhesion and recognition (Bartlett & Edidin, 1978; Zeleny et al., 1978), a role in directing cell associations during embryogenesis and organogenesis (Ohno, 1977), and some association with hormone receptors on the cell surface (Lafuse & Edidin, 1980; Schreiber et al., 1984; Simonsen et al., 1985).

1.10 MHC ASSOCIATIONS WITH DISEASE

A number of human diseases have been associated with class I and class II polymorphisms of the HLA complex (Solheim et al., 1982; Svejgaard et al., 1983). The strongest association described has been between class I antigen B27 and ankylosing spondylitis (Brewerton et al., 1973; Svejgaard et al., 1983).

Several theories have been proposed to explain HLA-disease associations (Svejgaard et al., 1975; Bodmer & Bodmer, 1978). These include cross-reactivity between MHC antigens and pathogen antigens ("molecular mimicry") and the effects of Ir genes (Svejgaard et al., 1983). In addition, MHC molecules might act as cell surface uptake
sites ("receptors") for certain pathogens, just as the human Duffy red blood cell antigen appears to promote susceptibility to the invasion of red cells by the malarial parasites *Plasmodium knowlesi* and *Plasmodium vivax* (Miller et al., 1975; 1976). However, there is little evidence of MHC molecules acting as pathogen receptors, with the exception of the results observed with the in vitro infection of human and mouse cells by Semliki Forest virus and lactate dehydrogenase virus (Helenius et al., 1978; Inada & Mims, 1984). One variation of the receptor model, which has been suggested in the case of B27-associated ankylosing spondylitis, is the modification of an MHC gene product by a bacterial agent (Geczy et al., 1983). Disease associations may also be due to undetected alleles at closely-linked "disease" loci which are in linkage disequilibrium with the associating MHC alleles (McDevitt & Bodmer, 1974). Some HLA-disease associations appear to have a non-immunological basis mediated by linked genes; for example, haemochromatosis (Fauchet et al., 1980) and congenital adrenal hyperplasia (Dupont et al., 1980).

Possible reasons for the incomplete nature of the HLA-disease associations include disease heterogeneity, linkage disequilibrium, non-HLA genetic factors and environmental factors (Bodmer & Bodmer, 1978). In addition, the disease association may not be with the gene or with the complete HLA molecule, but may be with a single discrete determinant which may be common to a number of other alleles (Silver & Goyert, 1985).

In other species, associations have been described between the MHC and resistance or susceptibility to disease-causing pathogens. In most cases, resistance or susceptibility appears to be under polygenic influences, with both MHC genes and non-MHC genes playing a part. In
the mouse, such diseases include Friend virus leukaemia (Lilly, 1968; Chesbro et al., 1974), Gross virus leukaemia (Lilly, 1971), lymphocytic choriomeningitis virus infection (Oldstone et al., 1973) and Moloney leukaemia virus infection (Debre et al., 1979). MHC-linked genes have also been implicated in the outcome of infection of inbred strains of mice with some human pathogens (Rosenstreich et al., 1982). It should be borne in mind, however, that there is a risk of oversimplifying the explanations of phenomena when disease-resistance studies are done using inbred strains of animals.

Among the domestic animal species, a strong association exists between the B21-haplotype of the chicken MHC and resistance to Marek's disease (Pazderka et al., 1975; Longenecker & Mossman, 1981; Briles et al., 1983). MHC-disease associations have also been described in the chicken in the outcome of Rous sarcoma virus infection (Collins et al., 1977; Schierman et al., 1977), and in the outcome of exposure to low doses of the bacterium Pasteurella multocida (Lamont et al., 1987).

1.11 METHODS OF DEFINING MHC POLYMORPHISMS

Serological methods have been the principal means used to define MHC polymorphisms to date and these will be dealt with more fully in the body of this thesis. Alloantisera may lack the discriminatory power needed to distinguish between antigens encoded by genes in linkage disequilibrium, or between multiple antigens on a single MHC molecule. Alternative tools for defining polymorphisms include monoclonal antibodies (discussed in Chapter 5), the mixed lymphocyte reaction (MLR), the primed lymphocyte test (PLT; a secondary MIR)
(Sheehy et al., 1975), the cell-mediated lympholysis reaction (CML) (Kristensen & Grunnet, 1975), alloreactive or antigen-specific T cell clones, biochemical definition and definition of DNA polymorphisms by restriction fragment length polymorphism (RFLP) analysis. Much of the discussion below deals with the use of these alternative methods in HLA class II definition.

In the MLR, PLT and OML assays, MHC antigens are recognized by T cells as allogeneic histocompatibility determinants. Because the results of these assays may be due to a mosaic of MHC determinants, it is difficult to define component determinants. Nevertheless, some serologically defined HLA-DR antigens appear to be subdivided by MIR typing (Reinsmoen & Bach, 1982; Termijtelen et al., 1982; Schreuder et al., 1984).

Cloned alloreactive T cells offer a more reductionist approach, as they are reagents of a single specificity (Inouye et al., 1980). Cloned T cells which are antigen-specific and MHC-restricted have also been used. When screened against allogeneic, antigen-presenting cells, these cloned T cells have been used to recognize and define the MHC determinant to which they are restricted. Specificities defined by cloned T cells include those that are associated with serologically defined HLA specificities, those that correlate with MIR types, and those that do not correlate with known specificities (Fleischer et al., 1984; Michon et al., 1986; Rosen-Bronson et al., 1986).

MHC molecules immunoprecipitated by appropriate antibodies have been defined biochemically by two-dimensional gel electrophoresis and one-dimensional isoelectric focusing. Some biochemically defined polymorphisms in HLA-DR β chains have been associated with those subdivisions of serologically defined DR specificities which correlate
with MIR types (Groner et al., 1983; Nepom et al., 1983). Other biochemical polymorphisms have suggested further subdivision of the recognized DQ antigens (Goyert et al., 1983).

RFLPs detect polymorphism at restriction endonuclease sites in DNA (Botstein et al., 1980). Correlations have been found between RFLPs or clusters of RFLPs and both DR and DQ serological specificities (including the supertypic specificities DRw52 and DRw53) (Cohen et al., 1985; Moller et al., 1985; Kohonen-Corish & Serjeantson, 1986; Carlsson et al., 1987). Most RFLPs defined to date appear to lie outside the gene coding for the related serological specificity, and the correlations observed are probably due to strong linkage disequilibrium (Paul et al., 1984; So et al., 1984). Correlations have also been described with MIR types (Cohen et al., 1986), and with DP primed lymphocyte test types (Bodmer et al., 1987; Hyldig-Nielsen et al., 1987). Some RFLPs appear to subdivide the serologically defined specificities (Bosch et al., 1985; Kohonen-Corish & Serjeantson, 1986; Carlsson et al., 1987), while others do not correlate with known specificities (Cohen et al., 1985).

Good general agreement has been reported between RFLP analysis and serological analysis of class II polymorphisms in mice (Bukara et al., 1985). In the domestic animal species, limited class II RFLP analysis has been done in pigs (Chardon et al., 1985b; Vaiman et al., 1986), sheep (Chardon et al., 1985a; Scott et al., 1987), cattle (Andersson et al., 1986a; 1986b; Vaiman et al., 1986; Andersson & Rask, 1988) and horses (Vaiman et al., 1986).
1.12 MHC OF CATTLE

1.12.1 Class I loci

Antigens of the bovine lymphocyte antigen system (BoIA) have been serologically defined in cattle by several independent groups (Caldwell et al., 1977; Amorena & Stone, 1978; Spooner et al., 1978; Stear et al., 1982). Evidence that these antigens are encoded by the MHC has come from studies showing that sharing of antigens significantly affects skin graft survival time (Amorena & Stone, 1978, 1980; Adams & Brandon, 1986). The antigens of this system are probably analogous to the class I antigens of other species, as they are carried on glycoprotein molecules of molecular weight 44,000 daltons which are associated with a polypeptide of molecular weight 12,000 daltons (Hoang-Xuan et al., 1982b). Family and population studies have suggested that at least two linked loci code for BoIA class I antigens, and this is supported by the observation of independent capping of some BoIA class I antigens (Stear et al., 1982). Limited RFLP analysis of bovine class I genes has been carried out using human DNA probes (Vaiman et al., 1986).

Specificities recognized by some alloreactive bovine cytotoxic lymphocytes have been reported to correlate with serologically defined BoIA class I antigens (Spoon & Teale, 1985; Teale et al., 1985). Bovine cytotoxic T cells, generated in vivo during Theileria parva immunization and subsequently stimulated in vitro, have been assayed for in vitro lysis of target cells infected with Theileria parva. The cytotoxic activity of individual clones of these T cells has been restricted to target cells of a particular BoIA class I type (Goddeeris et al., 1986).
1.12.2 MIR and class II loci

The demonstrated association between BoLA class I antigens and the MIR suggested the existence of a class II region in cattle (Usinger et al., 1981; Newman et al., 1982b). Evidence of a B lymphocyte alloantigen system, absent from the majority of T cells, was provided by Newman et al. (1982a). The evidence that this system represented the products of the class II region in cattle included its high degree of polymorphism, its restricted cellular distribution, and the suggestion of linkage with BoLA class I antigens. The production of alloantisera recognizing bovine class II antigens has also been reported by Davies & Antczak (1987).

Class II molecules, consisting of polypeptide chains of molecular weights of 34,000, 31,000 (probably the equivalent of the invariant chain in other species) and 27,000 daltons, have been demonstrated on the surface of bovine lymphocytes using mouse alloantisera and anti-HLA monoclonal antibodies (Hoang-Xuan et al., 1982a). Limited RFLP analysis using human DNA probes has demonstrated extensive polymorphism of bovine class II genes and has indicated that the number of class II genes varies between haplotypes (Andersson et al., 1986a; 1986b; Vaiman et al., 1986; Andersson & Rask, 1988). Alloreactive bovine T cell clones recognizing class II determinants have been described (Teale & Kemp, 1987).

1.12.3 Associations between the MHC and immune responsiveness and disease resistance

Variations between breeds of cattle in their susceptibility to natural diseases is known to occur with infectious bovine keratoconjunctivitis (Doct, 1977), cattle tick (Boophilus microplus)
infestation (Utech et al., 1978), and trypanosomiasis (Murray et al., 1982). In addition, resistance to ticks has been found to be responsive to selection and increased resistance is heritable (Utech & Wharton, 1982). One of the purposes of defining blood and serum groups of cattle is to subsequently search for associations between polymorphisms of marker loci and productivity traits, including resistance to production-limiting diseases. Because of its apparent central role in the immune response, the MHC is a logical marker to use in looking for associations with disease resistance, while bearing in mind that the primary factor limiting the response to a pathogen or vaccine might not be immunological.

The search for associations between defined BoLA class I antigens and disease states has so far been quite cursory. Preliminary results indicate that cattle with BoLA antigen CA2 (W6) may have an increased susceptibility to tick infestation and ocular squamous cell carcinoma, while cattle with CA27 (W16) may have an increased resistance to tick infestation and gastro-intestinal helminth infestation. In addition, cattle with CA12 (EU28) may be more likely to develop persistent lymphocytosis (an advanced subclinical stage associated with bovine leucosis virus infection) than cattle without CA12 (Stear et al., 1984; 1985a).

In an independent study of bovine leucosis virus infection (Lewin & Bernoco, 1986), a lower incidence of B cell proliferation and lymphocytosis was found to be associated with locally-defined BoLA class I antigen DA7, while a higher incidence of B cell proliferation and lymphocytosis was found to be associated with Class I antigen DA12.3. Typing for antigen EU28 was not done.
Lie & Solbu (1981) (cited by Lie et al., 1986) described an association between high responsiveness to human serum albumin (HSA) and susceptibility to mastitis, and BoIA antigen W16 has been associated with both high responsiveness to HSA (Lie et al., 1986) and susceptibility to mastitis (Solbu et al., 1982, cited by Lie et al., 1986). Conversely, antigen W2 has been associated with low responsiveness to HSA (Lie et al., 1986) and resistance to mastitis (Solbu et al., 1982, cited by Lie et al., 1986).

1.13 SCOPE OF THIS THESIS

The experiments described in this thesis are concerned with the identification of MHC polymorphisms in cattle. The principal purpose of these experiments was to define the class II polymorphisms in cattle. Further definition of the class I polymorphisms is also described. A serological approach as been used in all of these studies.
CHAPTER 2

MATERIALS AND METHODS

CATTLE. While blood and sera samples were usually obtained from the jugular vein, but occasionally small samples were taken from the tail vein. For small samples, sterile 10 ml and 20 ml blood collection tubes were used with or without EDTA anticoagulant (Falk, Dickson, Richardson, N.J., U.S.A.). Where larger quantities of blood were required, evacuated 250 ml bottles were used when an anticoagulant was required, a 2.7% EDTA-TEA solution was used in the ratio of 1 part EDTA-TEA to 4 parts blood.

Blood was obtained by allowing blood to clot at room temperature and centrifuging the clot-free liquid at 1,500 g for 30 min at 4°C. All sera were heat-inactivated at 56°C for 30 min and stored at -20°C.
2.1 **EXPERIMENTAL ANIMALS**

**Cattle.** The cattle used were held on pasture at the University's "Spring Valley" farm outside Canberra, A.C.T., the Lockhart Pastoral Co. property at Bungendore, N.S.W., the McLachlan Group Pty. Ltd. property at Moss Vale, N.S.W., the Hillside Charolais Stud property at Mittagong, N.S.W. and the Mandalong Investments Pty. Ltd. property at Moss Vale, N.S.W.

**Rabbits.** Outbred rabbits used for the production of antisera and as a source of complement were bred and maintained by the Animal Breeding Establishment, J.C.S.M.R.. They were housed indoors individually, and fed a diet of commercially prepared rabbit pellets (Doust & Rabbidge Pty. Ltd., Sydney, Australia) and water ad libitum.

2.2 **COLLECTION OF BLOOD SAMPLES**

**Cattle.** Whole blood and serum samples were usually obtained from the jugular vein, but occasionally small samples were taken from the tail vein. For small samples, sterile 10 ml and 20 ml blood collection tubes were used with or without EDTA anticoagulant (Becton-Dickinson, Rutherford, N.J., U.S.A.). Where larger quantities of blood were required, evacuated 250 ml bottles were used; when an anticoagulant was required, a 2.7% EDTA-PBS solution was used in the ratio of 1 part EDTA-PBS to 4 parts blood.

Serum was obtained by allowing blood to clot at room temperature and centrifuging the clot-free liquid at 1,500xg for 20 min at 4°C. All sera were heat-inactivated at 56°C for 30 min and stored at -25°C.
**Rabbits.** Blood was obtained from the marginal ear vein. Serum which was to be used as a source of complement was removed after leaving the blood at room temperature for 2-3 hr. The serum was centrifuged, and stored in portions at -25°C. Antisera were obtained from whole blood by the method described above for cattle serum and were heat-inactivated at 56°C for 30 min. The antisera were stored at -25°C.

### 2.3 SOLUTIONS

All chemicals used were of analytical reagent grade.

#### 2.3.1 Physiological solutions

**Phosphate buffered saline, pH 7.3 (PBS):** One litre of solution contained 8.0 g NaCl, 1.2 g Na₂HPO₄·2H₂O, and 0.39 g NaH₂PO₄·2H₂O dissolved in distilled water.

**PBS/Foetal calf serum (PBS/FCS):** Foetal calf serum (Flow Laboratories, Catalogue No. 29-101-54, Sydney, Australia) was added to PBS at 5% v/v or 1% v/v. PBS/FCS was freshly prepared before use.

**0.9% NaCl solution (normal saline):** One litre of solution contained 9 g NaCl in distilled water.

**Hank's balanced salt solution:** One litre of solution contained 8.0 g NaCl, 0.84 g KCl, 0.1 g MgSO₄·7H₂O, 0.1 g MgCl₂·6H₂O, 0.14 g CaCl₂, 0.06 g Na₂HPO₄, 0.06 g KH₂PO₄, 1.0 g glucose and 20.0 mg phenol red dissolved in distilled water. The solution was sterilized by autoclaving, and the pH was adjusted to 7.4 (red colour) by dropwise addition of 5% NaHCO₃ solution.
2.3.2 Other solutions

2.7% EDTA-PBS: One litre of solution contained 27 g EDTA-Na₂ dissolved in PBS. The pH was adjusted to 7.4 with 5M NaOH.

0.5% EDTA-saline: One litre of solution contained 5 g EDTA-Na₂ and 9 g NaCl in distilled water. The pH was adjusted to 7.4 with 5M NaOH.

Erythrocyte shock lysis solution ("shocking solution"): One litre of solution contained 8.0 g NH₄Cl, 0.1 g KH₂PO₄, and 1.0 g EDTA-Na₃. The pH was adjusted to 7.2.

Citrate-saline solution: A 0.1M sodium citrate (C₆H₆O₇Na₃.2H₂O) solution in normal saline was added to a 0.1M citric acid (monohydrate) solution in normal saline until the pH reached 3.0.

Formaldehyde-PBS solution for fixing platelets: A 1% solution of formaldehyde was made by adding 2.5 ml of 40% w/v formaldehyde solution to 90 ml PBS. The pH was adjusted to 7.4 with 1M NaOH.

Formaldehyde-PBS solution for fixing the reaction in the microlymphocytotoxicity test: A 16% solution of formaldehyde was made by adding 40 ml of 40% w/v formaldehyde solution to 60 ml PBS. The pH was 6.6.

Eosin dye solution: 5 g Eosin Yellowish (Fluka AG, Buchs, Switzerland) was dissolved in 100 ml PBS, filtered through Whatman No. 1 filter paper, and stored at 4°C.

Acridine orange/ethidium bromide solution: Separate stock solutions of each dye were prepared by dissolving 10 mg acridine orange (Sigma Chemical Co., St Louis, MO., U.S.A.) in 100 ml PBS, and 10 mg ethidium bromide (Sigma Chemical Co.) in 100 ml PBS. The stock solutions were stored frozen at -25°C. A working solution (each dye
at 1 µg/ml) was prepared by adding 100 µl of each solution to 10 ml PBS; the working solution was stored in the dark at 4°C.

2.4 GENERAL ANALYTICAL METHODS

2.4.1 Protein determination

The concentration of protein in antibody solutions was estimated either by measuring the absorbance at 280 nm using a spectrophotometer (Hitachi, Model No. 100-10, Hitachi, Tokyo, Japan) or by the Coomassie Brilliant Blue method (Bradford, 1976).

For the calculation of protein concentration from absorbance measurements at 280 nm, extinction coefficients (1%, 1 cm) used were 14.0 for rabbit IgG and 15.0 for F(ab')2 fragments of rabbit IgG (Little & Donahue, 1968).

For the estimation of protein concentration using the Coomassie Brilliant Blue method, the BioRad Protein Assay kit (BioRad Laboratories, Richmond, CA., U.S.A.) was used. The concentrated dye reagent was diluted 1:4 with deionized distilled water and filtered through Whatman No. 1 filter paper. One hundred µl of each sample was added to 5 ml diluted dye reagent, and the preparation was gently mixed with a vortex mixer. Absorbance at 595 nm was measured against a reagent blank (sample buffer), 5 min to 1 hr after mixing. Protein concentrations were estimated from a standard curve prepared using bovine IgG.
2.4.2 **Agar gel immunoelectrophoresis**

**Solutions & Buffers.**

Tris-barbiturate buffer, pH 8.6 was made up from pre-measured packets (IKB, No. 2207-101, Bromma, Sweden). Each packet contained 4.43 g Tris, 2.24 g diethylbarbituric acid, 0.065 g sodium azide, and 0.053 g calcium lactate. Two packets were dissolved in 1 litre of distilled water.

Amido black staining and rinsing solutions: to 675 ml distilled water were added 675 ml methanol and 150 ml acetic acid. Amido black (9.0 g) was dissolved in this mixture. The mixture without the amido black was used as a rinsing solution for removing excess stain.

**Method.** A 1% solution of agar was prepared by adding 1.0 g Special Agar Noble (Difco Laboratories, Detroit, MI., U.S.A.) to 25 ml Tris-barbiturate buffer and 75 ml distilled water containing 0.001% thiomersal, and heating in a boiling water bath. Microscope slides were coated with the 1% agar solution and transferred to a humid chamber for 30 min until the agar had set. Antigen wells were formed in the agar and antigen was added to the wells.

The electrode chambers of the electrophoresis apparatus (IKB, Model No. 6800 A) were filled with Tris-barbiturate buffer. Electrophoretic separation of antigen samples took place at a constant voltage of 250 volts over a period of 1 hr. Troughs were formed in the agar for the placement of the antisera; the antisera were added and allowed to react for 24 hr in a humid chamber. Slides were washed in normal saline for 24 hr with one change of solution, and then rinsed with distilled water. Slides were covered with strips of blotting paper and allowed to dry for 48 hr at room temperature. The paper was removed and the slides were washed with running tap
water. Slides were stained by immersing them (with gentle agitation) for 1 min at room temperature in amido black staining solution, followed by 4 rinses in the same solution without amido black.

2.5 IMMUNOPRECIPITATION

2.5.1 Radiolabelling of cell-surface proteins

Peripheral blood lymphocytes were prepared as in Section 2.7.1. The cell membranes were labelled with I\textsuperscript{125} using the lactoperoxidase method as described by Goding (1983). Fifty μl lactoperoxidase (Sigma Chemical Co., St Louis, MO., U.S.A.) at 0.2 mg/ml, and 500 μCi I\textsuperscript{125} (Amersham International Ltd., No. IMS.30, Amersham, Buckinghamshire, England) were added to each preparation of 5 x 10\textsuperscript{7} lymphocytes in 200 μl of PBS. Ten μl of hydrogen peroxide in PBS at dilutions of 1/27,000, 1/9,000, 1/3,000, 1/1,000 were added in that order at 1 min intervals. One min after the last addition, the cells were washed twice in 10 ml PBS containing 1mM tyrosine by centrifuging them between washes at 600xg for 5 min. Each cell preparation was resuspended in 0.25 ml of a solution containing 10mM Tris and 0.15M NaCl (pH 7.2), and cells were pooled into lots of 10\textsuperscript{8} cells in 0.5 ml of this buffer.

2.5.2 Preparation of the cell lysate

The method used to solubilize the plasma membrane was based on that described by Johnstone & Thorpe (1982a). The cell lysis buffer contained 1% NP-40 (Nonidet P-40, Sigma Chemical Co.), 10mM Tris, and 0.15M NaCl, pH 7.2. Lymphocytes (10\textsuperscript{8}) were solubilized by the addition of 0.5 ml of cell lysis buffer and 1 μl 0.1M
phenylmethylsulfonyl fluoride (Sigma Chemical Co., Lot No. 113F-0326) in acetone. The reaction was allowed to proceed for 1 hr on ice. The mixture was centrifuged at 600xg for 10 min, and the supernatant was collected and centrifuged at 12,000xg for 15 min at 4°C. This supernatant represented the labelled cell lysate.

2.5.3 Immunoprecipitation

The method used was based on that described by Johnstone & Thorpe (1982d). The labelled cell lysate was first treated to reduce non-specific binding to Protein A. The source of Protein A was a 10% suspension of *Staphylococcus aureus* cells (Pansorbin, Behring Diagnostics, Lot No. 545001, La Jolla, CA., U.S.A.). The suspension was washed twice in buffer (containing 0.5% NP-40, 10mM Tris, 0.5M NaCl, pH 7.3) and made up as a 25% suspension in this buffer. One hundred and fifty µl of this suspension, and 10 µl of a 10% w/v solution of ovalbumin (Sigma Chemical Co., Grade VII) in 0.5% NP-40 buffer, were added to the labelled cell lysate. The mixture was incubated for 30 min at 4°C on a rotator at 11 revolutions/min (Multipurpose Rotator, Model 120, Scientific Industries Inc., Springfield, MA., U.S.A.). After centrifugation at 12,000xg for 5 min, the supernatant was collected.

The ascites form of monoclonal antibody TH81A5 was used in the immunoprecipitation (for a description of this antibody, see Chapter 5); 2.5 µl of the antibody and 10 µl of a 10% ovalbumin solution in 0.5% NP-40 buffer were added to 1 ml portions of the labelled cell lysate. The mixture was incubated overnight at 4°C. An appropriate control immunoprecipitation was done, using a monoclonal antibody which did not react with bovine lymphocytes.
One hundred µl of a washed 25% suspension of *Staphylococcus aureus* was added to the antibody/cell lysate mixture, and incubated for 1 hr at 4°C. The mixture was centrifuged at 12,000xg for 5 min, and the precipitate was washed 4 times in 0.5% NP-40 buffer, once in the same buffer containing 0.5% SDS, and once in 10mM Tris, pH 7.3. Immune complexes were dissociated by boiling the precipitate for 5 min in 100 µl SDS sample buffer (see below), and the preparation was centrifuged at 12,000xg for 5 min. The supernatant was collected, re-centrifuged, and examined by SDS polyacrylamide gel electrophoresis (Laemmli, 1970).

2.5.4 Electrophoresis

Solutions.

Stacking gel buffer: 6.06 g Tris base and 0.4 g SDS (Sodium dodecyl sulphate, BioRad Laboratories, Richmond, CA., U.S.A.) were added to approximately 80 ml distilled water and the pH was adjusted to 6.8 with concentrated HCl. The total volume was made up to 100 ml with distilled water.

SDS sample buffer: This buffer was made up as a x2 concentrated stock. To 25 ml stacking gel buffer were added 20 g glycerol, 10 g SDS, 4.5 ml saturated bromophenol blue solution (BioRad Laboratories), and 5 ml of 20mM disodium EDTA solution. Dithiothreitol was added to a concentration of 0.1M in the final volume. This x2 concentrated stock solution was diluted 1:1 with distilled water to produce the sample buffer.

Resolving gel buffer: 18.17 g Tris base and 0.4 g SDS were added to approximately 80 ml distilled water and the pH was adjusted
to 8.8 with concentrated HCl. The total volume was made up to 100 ml
with distilled water.

Electrode (reservoir) buffer: 3.03 g Tris, 14.41 g glycine and
1 g SDS were added to distilled water and the total volume was made up
to 1 litre with distilled water. The pH was 8.3

Acrylamide stock solution (30% total acrylamide, of which 4% consisted of the cross-linker; i.e. "4% C"): 28.8 g acrylamide (BioRad Laboratories) and 1.2 g N,N'-methylene bisacrylamide (BioRad Laboratories) were dissolved in distilled water to a total volume of 100 ml.

Light gel solution (12.5% total acrylamide): 5.2 ml acrylamide stock solution was mixed with 3.0 ml resolving gel buffer and 3.8 ml deionized distilled water. Gas was removed from the gel, and immediately before the gel solution was added to the gradient maker, 2.5 µl of N,N,N',N'-tetramethylethylene diamine (TEMED) (BioRad Laboratories) was added, followed by 12.5 µl of a 10% w/v ammonium persulphate solution.

Heavy gel solution (17.5% total acrylamide): 7.0 ml acrylamide stock solution was mixed with 3.0 ml resolving gel buffer, 1.8 g sucrose, and 0.88 ml deionized distilled water. Gas was removed from the gel, and immediately before the gel solution was added to the gradient maker, 2.5 µl of TEMED was added, followed by 12.5 µl of a 10% w/v ammonium persulphate solution.

Stacking gel solution (5% total acrylamide): 2.0 ml acrylamide stock solution was mixed with 3.0 ml stacking gel buffer and 7.0 ml deionized distilled water. Gas was removed from the gel, and immediately before the stacking gel solution was poured into the mould.
above the resolving gel, 12 µl of TEMED was added, followed by 36 µl of a 10% w/v ammonium persulphate solution.

Molecular weight marker proteins: 5 µl of a solution of low molecular weight markers (Sigma Chemical Co., Catalogue No. MW-SDS-70L, Lot No. 103F6173) and 5 µl of a solution of high molecular weight markers (Sigma Chemical Co., Catalogue No. MW-SDS-200, Lot No. 103F6187) were mixed and made up to a total volume of 40 µl in SDS sample buffer.

Method. Samples were electrophoresed in linear gradient SDS slab gels (8.5 cm long x 13.5 cm wide x 1 mm thick) containing 12.5-17.5% total acrylamide (4% C); a stacking gel (1 cm long) containing 5% total acrylamide (4% C) was used.

Forty, 20, 10, and 5 µl portions of the sample were made up to a total volume of 40 µl with SDS sample buffer, and were electrophoresed at a constant 25 mA (with cooling) for about 7 hr until a coloured 18,400 dalton molecular weight marker (Bethesda Research Laboratories, Catalogue No. 6040SA, Gaithersburg, MD., U.S.A.) had reached the bottom of the gel.

2.5.5 Gel staining and autoradiography

Gels were stained for protein by incubating them in 3-5 volumes of Coomassie Blue stain solution for 5-6 hr on an orbital shaker (Johnstone & Thorpe, 1982b). The staining solution consisted of 0.025% Coomassie Brilliant Blue R-250 (Sigma Chemical Co.) in 50% v/v methanol and 5% v/v acetic acid. The background stain was then removed with a solution consisting of 7.5% w/v acetic acid and 5% w/v methanol.
After soaking in a solution of 3% glycerol, 7.5% acetic acid and 20% methanol for 30 min, gels were dried using a slab gel drier (BioRad Laboratories, Model No. 1125B). The dried gels were then exposed for autoradiography (at -70°C for 7 days) using Medical Grade 35 cm x 43 cm x-ray films (Fuji Photo Film Co. Ltd., Japan), and Cronex Quanta III intensifying screens (Du Pont, Towanda, N.Y., U.S.A.). Standard marker proteins (Sigma Chemical Co.) were used for the determination of molecular weights. The position of each marker protein on the gel was measured, and the percentage polyacrylamide concentration at each position was calculated. Log\(_{10}\) molecular weight was plotted against log\(_{10}\) polyacrylamide concentration for each of the marker proteins (Hames, 1981), and a straight line graph was obtained. Molecular weights of the sample were then determined from this standard curve.

2.6 PREPARATION OF RABBIT ANTI-BOVINE IMMUNOGLOBULIN REAGENT

2.6.1 General remarks

Samples were dialysed at 4°C against a volume of buffer at least 100 times the sample volume. Dialysis was done 3 times against this volume of buffer, for at least 3 hr at a time.

The flow of buffer was controlled during column chromatography by a peristaltic pump (12000 Varioperpex, LKB, Bromma, Sweden) and the fractions were collected automatically with a fraction collector (Ultro Rac 7000, LKB).

Affinity purified antibody fractions and F(ab')\(_2\) fragment fractions were concentrated in dialysis tubing by osmotic pressure concentration using Aquacide II (Calbiochem-Behring, La Jolla, CA.,
Other antibody fractions were concentrated under pressure with nitrogen gas in a Diaflo apparatus (Amicon Corp., Lexington, MA., U.S.A.) using XM50 Diaflo membranes (Amicon). These membranes retain material with a molecular weight above 50,000 daltons.

Preparations were agitated gently with a rotator which rotated vertically at 11 revolutions/min (Multi-purpose Rotator, Model 120, Scientific Industries Inc., Springfield, MA., U.S.A.).

### 2.6.2 Xenoimmunization

The bovine immunoglobulin preparation used to immunize rabbits was a gift from Dr M.J. Stear. A brief description of its preparation is as follows. Serum was collected from a steer 14 days after an intramuscular injection of $10^9$ killed *Bordatella pertussis* bacteria (Commonwealth Serum Laboratories, 40 I.O.U., Batch No. 027-1, Melbourne, Australia). The IgG fraction was obtained by gel filtration on a G-200 Sephadex column. The agglutinating antibody titre of this fraction to *Bordatella pertussis* was determined using a standard bacterial agglutination assay (Grant, 1978). The bovine IgG was mixed with *Bordatella pertussis* suspension in conditions of antibody excess for 2 hr at room temperature. The sensitized *Bordatella pertussis* cells were washed 6 times and resuspended in normal saline.

One ml of this suspension was emulsified in 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, MI., U.S.A.), and injected intramuscularly into the thigh of a rabbit. Three different rabbits were used. A second immunization was given about 8 weeks after the first. Serum was taken at fortnightly intervals. Antibody was partially characterised by immunoelectrophoresis against bovine
serum. The antiserum showed strong reactivity against bovine IgG1, IgG2 and IgM (Figure 2.1).

2.6.3 Ammonium sulphate fractionation of rabbit IgG

The method used was based on that described by Heide & Schwick (1978). Ammonium sulphate was added slowly to heat-inactivated whole rabbit serum in the ratio of 1.9 g NH₄SO₄ to 10 ml of serum; this produced a 33% solution of ammonium sulphate. The mixture was stirred gently for 1 hr and left overnight at 4°C. The precipitate was collected after centrifugation at 1,000xg for 15 min and resuspended in a volume of PBS equal to the original volume of rabbit serum. This crude IgG fraction was subjected to a second round of ammonium sulphate precipitation, using 1.9 g NH₄SO₄ to 10 ml of IgG solution. The precipitate was resuspended in PBS, and the protein concentration was estimated. This IgG fraction was dialysed against PBS/0.1% sodium azide and stored frozen at -25°C until further use.

2.6.4 Affinity chromatography

Solutions & Buffers.

Coupling buffer: One litre of solution contained 8.4 g NaHCO₃ (0.1M) and 29.22 g NaCl (0.5M) in distilled water. The pH was adjusted to 8.3 with 5M NaOH.

Low pH washing buffer: 130 ml of 0.6M sodium acetate (CH₃COONa) was mixed with about 375 ml of 0.6M acetic acid (CH₃COOH) and 58.44 g NaCl was dissolved in this solution. The pH of the solution was adjusted to 4.0 with 0.6M acetic acid, and the total volume was made up to 1 litre with distilled water.
Figure 2.1 Immunoelectrophoretic pattern showing the specificity of an antiserum produced by injection of bovine IgG into a rabbit. The antigen in both upper and lower wells was bovine serum.

a = IgM.
b = IgG₁.
c = IgG₂.
Sample buffer: One litre of solution contained 5 ml polyoxyethylenesorbitan monooleate (Tween 80, Sigma Chemical Co., St Louis, MO., U.S.A.) added to 21.22 g NaCl dissolved in PBS. The concentration of NaCl was thus 0.5M.

Eluting buffer No. 1: 5.63 g glycine was added to 375 ml distilled water. The pH was adjusted to 2.5 with 0.2M HCl, and the solution was made up to 750 ml with distilled water.

Eluting buffer No. 2: Diethylene dioxide (Dioxane, BDH Chemicals Ltd., Poole, England) was added to eluting buffer No. 1 at a concentration of 10% w/v.

Regenerating buffer No. 1: 21.92 g NaCl was dissolved in a mixture of 582 ml of 0.1M Tris and 168 ml of 0.1M HCl; the pH was adjusted to 8.5 with 6M HCl.

Regenerating buffer No. 2: 98 ml of 0.6M sodium acetate (CH$_3$COONa) and 21.92 g NaCl were added to 326 ml distilled water. The pH of the solution was adjusted to 4.5 with 0.6M acetic acid (CH$_3$COOH) and the total volume of the solution was made up to 750 ml with distilled water.

Preparation of the immunoadsorbent. The methods used were based on those recommended by Pharmacia: 315 mg bovine IgG (Silenus Laboratories, Batch No. 466, Melbourne, Australia) was coupled to 12 g CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Product No. 17-0430-01, Batch No. KC 35237, Uppsala, Sweden). Less than 1% of the bovine IgG remained unbound to the gel, giving an estimated concentration of 7.5 mg bovine IgG per ml of gel.

Remaining active groups on the Sepharose 4B were blocked by adding coupling buffer containing 0.2M glycine, pH 8.0 to the gel.
The gel was washed alternately with high pH buffer solution (coupling buffer) and low pH buffer solution (described above) in several stages. The gel was then washed with PBS containing 0.02% w/v thiomersal, and was packed into a column under gravity. Packing was completed by washing the column with 130 ml PBS/0.02% thiomersal, at a flow rate of 30 ml/hr. The volume of the gel was 32 ml. The immunoadsorbent was stored and used at 4°C.

**Purification of rabbit anti-bovine IgG.** The methods used were based on those recommended by Pharmacia. Before each operation, the column was washed with 130 ml eluting buffer No. 1 followed by 130 ml sample buffer (Johnstone & Thorpe, 1982c). The IgG solution (18 mg/ml), prepared from hyperimmune rabbit serum by ammonium sulphate precipitation, was dialysed twice against PBS and twice against the sample buffer. Sample volumes of 35-50 ml (representing the equivalent of 70-100 ml hyperimmune rabbit serum) were centrifuged at 10,000xg for 10 min and applied to the column. The flow of sample buffer was stopped for 30 min after the sample was applied to the column. The unbound protein was then washed from the column with 350 ml sample buffer, at a flow rate of 25 ml/hr. The absorbance at 280 nm of the eluate was monitored against a sample buffer reference solution to ensure that it returned to zero.

Bound antibodies were eluted by washing the column with 150 ml eluting buffer No. 1 at a flow rate of 20 ml/hr. The eluate was collected in 5 ml fractions in tubes containing 1 ml 1M Tris solution, pH 9.0 (Johnstone & Thorpe, 1982c). The absorbance at 280 nm of the eluate was monitored against the appropriate reference solution. Positive fractions were pooled, and the pH was adjusted to 8.5 with 1M
Tris solution, pH 9.0. The eluted antibody was concentrated and dialysed against PBS/0.1% sodium azide.

Elution of the bound antibodies was completed by washing the column with 90 ml eluting buffer No. 2 (containing dioxane) and collecting the eluate as above. All fractions were pooled, the pH was adjusted to 8.5, and the eluate was concentrated and dialysed against PBS/0.1% sodium azide. The antibody solutions from both stages of the elution were pooled, centrifuged (2,000xg for 20 min), and the supernatant was collected. The protein concentration of the affinity purified antibody was determined and the antibody was stored frozen at -25°C until it was used.

The gel was regenerated by washing the column with 400 ml regenerating buffer No. 1, 400 ml regenerating buffer No. 2 and 400 ml PBS/0.02% thiomersal, and stored in PBS/0.02% thiomersal at 4°C.

2.6.5 F(\(\text{ab}'\))\(_2\) fragment preparation

The method used was based on that described by Hudson & Hay (1980). A solution of pepsin was freshly prepared by dissolving 110 mg pepsin (3x Crystalline Pepsin, Nutritional Biochemicals Corporation, Lot No. 2215, Cleveland, OH., U.S.A.) in 5 ml of 0.1M sodium acetate solution.

The affinity purified rabbit IgG solution was adjusted to 14 mg/ml, and 12 ml of this solution was dialysed against a solution of 0.1M sodium acetate (CH\(_3\)COONa). The pH of the rabbit IgG solution was adjusted to 4.5 with glacial acetic acid (CH\(_3\)COOH), and 160 µl of the pepsin solution was added to the rabbit IgG solution (representing 2 mg pepsin per 100 mg of rabbit IgG). Digestion of the rabbit IgG was allowed to proceed for 20 hr at 37°C. The digest was then centrifuged
at 2,000xg for 20 min. Digestion was stopped by adjusting the pH of the collected supernatant to 7.4, using 1M NaOH and then 0.03M NaOH. The digest was dialysed against PBS/0.02% sodium azide, and fractionated on a G-100 Sephadex column equilibrated with PBS/0.02% sodium azide. The procedures for swelling the gel and packing the column (2.5 cm diameter x 100 cm long) were those recommended by Pharmacia. A sample volume of 16 ml (which resulted from the above digestion) was applied to the column. A flow rate of 15 ml/hr was maintained, and the eluate was collected in 1.8 ml fractions.

The elution profile, as shown in Figure 2.2, consisted of 3 peaks: F(ab')2 fragments, pFc', and small polypeptides. The F(ab')2 peak occurred after the void volume of the column. There were no undigested IgG or Fab' peaks. Fractions from the ascending side of the F(ab')2 peak were selected (a total volume of 56 ml), and concentrated. The protein concentration was determined using the Coomassie Blue method. The solution was dialysed against PBS/0.1% sodium azide, sterilized through a disposable 0.22 μ filter (Millipore Corporation, Bedford, MA., U.S.A.), and stored frozen at -25°C until used.

2.7 PREPARATION OF CELLS

2.7.1 Preparation of lymphocytes from peripheral blood

Lymphocytes were prepared by density gradient centrifugation, based on the method described by Boyum (1968). Whole blood was layered onto Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) in 10 ml glass centrifuge tubes; 5 ml blood was layered onto 4 ml Ficoll-Paque. This preparation was centrifuged at 1,500xg for 20 min.
Figure 2.2 Elution profile following G-100 Sephadex gel filtration chromatography of a pepsin digest of rabbit IgG.

- **a** = F(ab')$_2$ fragments.
- **b** = pFc'.
- **c** = small polypeptides.
The layer of lymphocytes which formed at the interface was aspirated with a Pasteur pipette and washed once in PBS, centrifuging at 1,500xg for 6 min.

Platelets were removed by differential centrifugation of the lymphocyte suspension: 3 washing steps were done, using sufficient centrifugation (150xg for 6 min) to sediment the majority of the lymphocytes but not the platelets. In order to remove residual red blood cells, a shocking solution was used in the first of these washing steps. The lymphocytes were then washed twice in PBS, and resuspended in PBS at the required concentration.

Where higher numbers of lymphocytes were required (e.g. for panning), 40 ml whole blood, containing 1 part in 5 of 2.7% EDTA-PBS, was centrifuged at 1,500xg for 30 min. The buffy coat was removed with a Pasteur pipette, resuspended in 10 ml PBS and layered onto Ficoll-Paque (5 ml buffy coat suspension onto 4 ml Ficoll-Paque). After centrifugation at 1,500xg for 30 min, the lymphocytes at the interface were aspirated and washed once in PBS (centrifuging at 1,500xg for 6 min). Platelets were removed by centrifuging at 160xg for 8 min during 3 washing steps; the first wash was in shocking solution and the remaining 2 washes were in PBS.

The purity of lymphocyte preparations was monitored by preparing cytocentrifuge stained smears using a modification of the Wright Stain technique (Diff-Quik Stain Set No. 64851, Harleco AHS/Australia Pty. Ltd., Sydney, Australia). Granulocyte contamination was never greater than 2%.

Viability of lymphocyte preparations was determined by staining with acridine orange/ethidium bromide solution, based on the method described by Mishell & Shiigi (1980b). One part of the lymphocyte
suspension at approximately $1 \times 10^6$/ml was mixed with one part of a working solution of acridine orange/ethidium bromide (both reagents at 1 µg/ml). Cells were placed in a haemocytometer and examined by fluorescent microscopy under x400 magnification. An Orthoplan fluorescent microscope was used (described in Section 2.8). The number of green (viable) and orange (non-viable) cells was determined, using both fluorescent and visible illumination.

2.7.2 Preparation of thymocytes

The method used was based on that described by Mishell & Shiigi (1980a). Thymic tissue was obtained from a freshly slaughtered calf. The tissue was cut into small pieces which were gently teased through a stainless steel sieve. The dissociated cells were collected into PBS at room temperature, and any clumps were allowed to settle for 5 min at room temperature. The supernatant was removed and the cells were pelleted by centrifugation at 200xg for 5 min and resuspended in 50 ml PBS. After further centrifugation at 200xg for 5 min, the cells were washed once in 50 ml shocking solution and twice in 50 ml PBS. The cells were resuspended in 50 ml PBS, filtered through 80 µ nylon mesh to remove aggregates, and counted. These preparations contained at least 90% viable cells.

2.7.3 Preparation of buffy-coat leucocytes

Whole blood, containing 1 part in 5 of 2.7% EDTA-PBS, was centrifuged at 1,500xg for 30 min. The buffy-coat was removed with a Pasteur pipette, and resuspended in a volume of PBS equal to the volume of blood used. After further centrifugation at 1,500xg for 30 min, the buffy-coat was again removed leaving most of the red blood
cells in the pellet. This preparation was washed once in PBS, once in shocking solution and twice in PBS (centrifuging between washes at 1,500xg for 20 min).

2.7.4 Preparation of platelets

Platelets were prepared by differential centrifugation of fresh blood, based on the methods described by Shulman et al. (1964) and Colombani (1976). Whole blood, containing 1 part in 5 of 2.7% EDTA-PBS, was centrifuged in 50 ml tubes, which were filled to the 30 ml mark. Tubes were centrifuged at either 300xg for 30 min or 1,300xg for 5 min.

The platelet-rich supernatant was aspirated, care being taken not to disturb the buffy-coat below. The supernatant was pooled into 50 ml centrifuge tubes and centrifuged at 200xg for 30 min. The platelet-rich supernatant was aspirated to within about 1 cm of the pellet of cells at the bottom of each tube, care being taken not to disturb this pellet. The supernatant was pooled into 50 ml tubes, and platelets were pelleted by centrifuging at 1,500xg for 30 min. The platelets were resuspended in 0.5% EDTA-saline, transferred to 15 ml tubes, and washed 3 times in 0.5% EDTA-saline, once in shocking solution, and twice in PBS (centrifuging between washes at 2,500xg for 15 min).

Platelets were counted by diluting the platelet suspension 1/100 in PBS, and adding this suspension to a haemocytometer. The platelets were allowed to settle on the slide for at least 15 min in a humid environment (Schalm et al., 1975) and were counted under x400 magnification using phase contrast objectives. At least 200 platelets were counted. The relationship between platelet number and the volume
of packed platelets was established, and the volume of packed platelets was then used as a guide to estimate platelet number.

Leucocyte contamination was determined by mixing 50 µl of the platelet preparation with 50 µl of acridine orange/ethidium bromide solution (both reagents at 1 µg/ml). Two min later, the suspension was added to a haemocytometer, and leucocytes were counted under x100 magnification using the Orthoplan fluorescent microscope described in Section 2.8.

After the final wash in PBS, platelets were fixed by resuspending them in 1% formaldehyde in PBS (Newman et al., 1982a) (1.5 ml of 1% formaldehyde was used per ml of packed platelets). The platelets were stored in the fixative overnight, washed 3 times in PBS/0.1% sodium azide the following day, and resuspended in PBS/0.1% sodium azide at a concentration of not less than 5 x 10^9/ml. Tubes containing platelet preparations were sealed and stored in the dark at 4°C.

2.8 IDENTIFICATION OF B LYMPHOCYTES BY INDIRECT IMMUNOFLOUORESCENCE

Lymphocytes were resuspended in PBS at a concentration of 10 x 10^6/ml. A sample of at least 0.5 ml was incubated for 30 min in a 37°C water bath. The cells were then washed twice in 37°C PBS, centrifuging between washes at 500xg for 6 min at room temperature. The washed cells were counted and adjusted to 10 x 10^6/ml in PBS/0.2% sodium azide.

The first step reagent antiserum consisted of F(ab')_2 fragments of rabbit IgG with anti-bovine IgG activity, prepared as described in
Section 2.6. The second step reagent antiserum consisted of fluorescein-conjugated F(ab')\textsubscript{2} fragments of goat IgG with anti-rabbit IgG F(ab')\textsubscript{2} fragment-specific activity (Cappel Laboratories, Catalogue No. 1312-0111, Lot No. 19277, West Chester, PA., U.S.A.). Reagent antisera were diluted to appropriate concentrations in PBS/0.2% sodium azide immediately before use, and centrifuged in a Beckman microfuge (i.e. at about 9,000xg) for 5 min (Model B, Beckman Instruments Inc., Palo Alto, CA., U.S.A.).

A 150 µl sample of the lymphocyte suspension was mixed with 75 µl of the first step reagent antiserum in a 1.5 ml capacity microfuge tube, and incubated for 30 min at room temperature. The cells were washed twice in PBS/0.2% sodium azide, centrifuging between washes at 500xg for 4 min. The supernatant was aspirated after each washing step. Seventy-five µl of the second step reagent antiserum was then added to the cell pellet, and after incubation for 30 min at room temperature, the lymphocytes were washed twice in PBS/0.2% sodium azide.

The labelled cell pellet was resuspended in 15 µl of a fixative solution consisting of 0.8% formaldehyde in PBS (de Luca, 1982), and stored in the dark until examined (no more than 2 hr after fixing). Fifteen µl of the fixed lymphocyte suspension was transferred to a microscope slide and mounted with a coverslip. Slides were examined immediately after mounting, using an Orthoplan microscope (Ernst Leitz GMBH, Wetzlar, West Germany) fitted with a Ploemopak 2.1 fluorescence vertical illuminator, and the appropriate filter block (I2). A BG-38 red suppression filter and HBO 200/W4 mercury lamp were installed in the light system. Lymphocytes were examined under x630
magnification, using x100 phase contrast oil immersion objectives, and x6.3 eyepieces as recommended (Nairn, 1976; de Luca, 1982).

Lymphocytes in a particular field were first examined under phase contrast and then under fluorescent light. The percentage of stained lymphocytes was established after counting at least 400 cells from a wide area of the slide. Only cells that appeared morphologically intact under phase contrast were scored. Dead cells which took up the fluorescent antisera non-specifically and had a diffuse homogeneous blue appearance were excluded from the count (Moller, 1961; Nairn, 1976).

2.9 B LYMPHOCYTE ENRICHMENT

2.9.1 Lymphocyte separation by sheep red blood cell (SRBC) rosetting procedures

The method used in the rosetting of T lymphocytes with SRBC was based on that described by Outteridge & Dufty (1981).

A suspension of SRBC was prepared from sheep blood collected and stored in an equal volume of Alsever's medium for up to 2 weeks. SRBC were prepared by centrifuging blood at 2,000xg for 10 min. The plasma and buffy coat were aspirated and discarded. SRBC pellets were washed 5 times at 4°C in normal saline.

A Ficoll solution was prepared by dissolving 17 g Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 100 ml normal saline by gentle heating, and 1.7 ml preservative-free heparin was added (Heparin Injection B.P., 5000 Units/ml, Commonwealth Serum Laboratories, Melbourne, Australia). A 2% v/v suspension of packed
SRBC was prepared in the Ficoll solution; this suspension was stored at 4°C for up to 5 days.

Lymphocytes were resuspended at $10 \times 10^6/\text{ml}$ in Hank's balanced salt solution. When rosetting was done to measure the frequency of rosette-forming lymphocytes, 100 µl of the lymphocytes suspension was mixed with 200 µl of the 2% SRBC suspension in 1.5 ml capacity microfuge tubes. When rosetting was done to enrich for B lymphocytes, one volume of lymphocyte suspension was mixed with 2 volumes of the 2% SRBC suspension in 15 ml glass centrifuge tubes. Cells were kept on crushed ice throughout all manipulations. As soon as mixing was completed, the preparations were centrifuged at 200xg for 15 min at 4°C, and incubated at 4°C overnight.

After incubation, rosettes were resuspended and counted. All rosetted preparations were kept on crushed ice or at 4°C. The bulk of the supernatant was aspirated from preparations in the microfuge tubes, and the remainder of the preparation was very gently resuspended in an equal volume of acridine orange/ethidium bromide solution, using a Pasteur pipette, the tip of which was broken off near the barrel. To count the rosettes, one drop of the stained preparation was placed on a coverslip, which was then mounted onto a microscope slide. Slides were examined immediately, under x400 magnification; the same fluorescent microscope was used as described in Section 2.8. A minimum of 200 live lymphocytes was counted. Lymphocytes with 5 or more SRBC attached were scored as rosettes.

B lymphocyte enrichment was attempted by fractionating the non-rosetted lymphocytes from the preparations in the 15 ml tubes. The preparations were gently resuspended and underlayered with x 0.75 their volume of Ficoll-Paque. Underlayering was done with a
disposable syringe and 18G needle directed onto the bottom of the centrifuge tube. After centrifugation at 1,500xg for 25 min, the lymphocyte layer at the interface was harvested using a Pasteur pipette. If the interface was not very clear and the Ficoll-Paque was cloudy, the top one third of the Ficoll-Paque was also collected. The harvested lymphocytes were washed once in PBS, once in shocking solution and twice in PBS before being resuspended in PBS and counted.

2.9.2 Lymphocyte separation by panning

Bacteriological grade plastic petri dishes (90 mm diameter, Nunc, Roskilde, Denmark) were incubated with 10 ml antibody solution either overnight at 4°C or for 2 hr at room temperature. The antibody solution was affinity purified anti-bovine IgG (H & L chain specific) (KPL Laboratories, Lot No. BB05, Gaithersburg, MD., U.S.A.), diluted to 5 µg/ml in PBS.

Dishes were washed up to 30 min before the lymphocytes were added: after swirling, the antibody solution was collected, and dishes were washed 5 times by pouring on 5-10 ml PBS per wash, swirling, and decanting. Dishes were kept aside at room temperature until the cells were added. The unbound antibody solution could be reused twice without any reduction in the efficiency of B lymphocyte enrichment.

The lymphocyte suspension was adjusted to 20 x 10^6/ml in PBS/5% FCS, and 80 x 10^6 cells (4ml) were added to each dish. If less lymphocytes were available, they were added in a volume of 4 ml. Dishes were lightly shaken to distribute the cell suspension evenly over the surface of the dish. Dishes were incubated for 1 hr at room temperature, and given a 30 second swirl after 30 min.
After incubation, non-adherent cells were decanted and collected, and the dishes were washed to remove residual non-adherent cells. Washing was done by gently pouring medium onto the dish, swirling, and then decanting. Dishes were washed 4 times with PBS and once with PBS/1% FCS, using 5-10 ml of medium per wash. After washing, dishes were flooded with 20-25 ml PBS/1% FCS.

Adherent cells were removed by systematically flushing the surface of each dish, in strips 1-2 cm wide, using a Pasteur pipette. The medium was then decanted from each dish into a 50 ml centrifuge tube. For B-enriched lymphocyte preparations, only fresh polypropylene plastic ware or siliconized glassware was used. Each dish was flooded with another 20-25 ml PBS/1% FCS, and the flushing process was repeated. Adherent and non-adherent cells were pelleted by centrifugation at 800xg for 5 min and were resuspended in PBS and counted.

2.10 PRODUCTION OF ALLOANTISERA

2.10.1 Buffy-coat leucocyte immunization

Buffy-coat leucocytes were prepared from 500 ml of whole blood from donor animals, as described in Section 2.7.3. This volume of blood usually produced 1.0-2.5 ml of packed leucocytes, which were resuspended to a volume of 10 ml in PBS. Recipient cattle were immunized with this freshly isolated leucocyte suspension. The first immunization was given intravenously, and subsequent immunizations were given intramuscularly.

Serum samples were taken before immunization and at weekly intervals thereafter. Sera were screened in a standard class I
microlymphocytotoxicity test (as described in Section 2.12.1) against donor peripheral blood lymphocytes. Immunizations were continued until the cytotoxic titre was at least 32 (which usually required 3-6 immunizations at weekly intervals).

2.10.2 Skin implant immunization

The method used was based on that described by Pringnitz et al. (1982).

Animals were restrained in a head bail, and sedated with 2% Xylazine (Rompun, Bayer Australia Ltd., Sydney, Australia), used at 0.05 mg/kg. The operative site was anaesthetized locally: in the case of skin donors, 3-4 ml of 2% lignocaine hydrochloride (Anacaine, VR Laboratories, Sydney, Australia) was injected to block innervation at the base of the ear. In the case of skin recipients, 2 ml of 2% lignocaine hydrochloride was used for a field block above the incision site on the lateral aspect of the neck or in the paralumbar fossa. Operative sites were clipped, scrubbed with soap, shaved and prepared for surgery by swabbing first with 70% alcohol and then with 0.5% chlorhexidine gluconate (Hibitane, ICI Australia Ltd., Sydney, Australia) in 70% alcohol.

Surgical procedures were done as aseptically as possible. Donor skin was taken from the dorsal side of the ear close to the base. A piece of skin about 2 cm square was removed and placed on a sterile gauze swab moistened with PBS. The swab was placed in a covered sterile disposable plastic petri dish. Pressure was briefly applied to the excision site with a swab to reduce any bleeding, and the site was treated with chloramphenicol spray (Chloropel Footrot Spray, Troy Laboratories, Sydney, Australia).
Recipient cattle were steers aged between 18 months and 3 years and unmated heifers aged 8-10 months. A horizontal skin incision 3-4 cm long was made in the middle of the lateral aspect of the neck or in the middle of the paralumbar fossa, and a subcutaneous pocket (about 3 cm square) was formed by blunt dissection ventral to this incision. A piece of skin was inserted in this pocket, and the incision was closed with nylon sutures. The incision site was treated with chloramphenicol spray. Donor and recipient wounds were monitored and were briefly treated with chloramphenicol spray if there was excess exudate. Otherwise, all wounds healed uneventfully.

Serum samples were collected from recipient animals before immunization and at weekly intervals thereafter. Sera were screened in a standard class I microlymphocytotoxicity test (as described in Section 2.12.1) against donor peripheral blood lymphocytes. Recipients which had not responded with a cytotoxic titre of at least 64 received a second implant 4-6 weeks after the initial immunization.

2.11 ABSORPTION OF ALLOANTISERA

2.11.1 Platelet absorption

Platelets were used for absorption at least 48 hr but not more than 4 weeks after preparation (Colombani, 1976; Colombani et al., 1976). Platelets were washed twice in PBS, centrifuging between washes at 2,500xg for 15 min.

The supernatant was aspirated as completely as possible after the last wash, and the platelets were thoroughly resuspended to 4 times their volume of PBS. Appropriate volumes of the platelet
suspension were added to 1.5 ml capacity microfuge tubes. Each platelet preparation was divided into 2 equal lots; one lot was used for the absorption of a serum at room temperature, and the other lot was used for the subsequent absorption of that serum at 4°C.

The platelet preparation for the room temperature absorption was centrifuged in a Beckman microfuge (i.e. at about 9,000×g) for 5 min. The PBS supernatant was aspirated completely with a Pasteur pipette. The platelet pellet was recentrifuged, and any additional supernatant was aspirated. The appropriate volume of serum, which had previously been heat-inactivated at 56°C for 30 min, was added to its respective platelet pellet. The ratio of packed platelets to serum was never more than 2:1. Serum and platelets were mixed thoroughly using a fine glass rod and a vortex mixer, and were incubated for 1 hr at room temperature on a slow rotator.

The mixture was centrifuged in a microfuge for 5 min, and the absorbed serum was aspirated with a Pasteur pipette. The platelet pellet was recentrifuged, and any additional serum was aspirated. The collected serum was added to the second half of its respective platelet preparation, which had been centrifuged twice in a microfuge to remove PBS. Serum and platelets were mixed thoroughly and incubated for 1 hr at 4°C on a slow rotator.

The absorbed serum was aspirated after centrifugation in a microfuge. Collected sera were placed in fresh microfuge tubes and cleared of residual platelets by centrifuging in a microfuge for 5 min. Sera were aspirated with a Pasteur pipette, care being taken to avoid any platelet pellet present. The harvested sera were immediately dispensed into the wells of 60-well microtest plates (Nunc, Catalogue No. 163118, Roskilde, Denmark) for future
cytotoxicity testing; otherwise, sera were stored frozen at -25°C until further use.

2.11.2 Buffy-coat leucocyte absorption

Buffy-coat leucocytes were prepared as described in Section 2.7.3 and were transferred to 1.5 ml capacity microfuge tubes. After centrifugation in a microfuge for 5 min, the supernatant was aspirated, and an appropriate volume of serum was added to the leucocyte pellet. Absorption was carried out with the buffy-coat leucocytes from 4-20 ml of blood per 100 µl of serum. If leucocytes from more than 8 ml of blood were used per 100 µl of serum, the leucocytes were divided into 2 lots, and the absorption was done in 2 stages. Sera had previously been absorbed with platelets.

Serum and leucocytes were mixed thoroughly using a micropipette and a vortex mixer and incubated for 1 hr at room temperature on a slow rotator. The mixture was centrifuged in a microfuge for 5 min, and the absorbed serum was aspirated with a Pasteur pipette. Collected sera were placed in fresh microfuge tubes and were cleared of residual leucocytes by centrifuging in a microfuge for 5 min. The sera were carefully aspirated with a Pasteur pipette and immediately dispensed into the wells of microtest plates for future cytotoxicity testing; otherwise, sera were stored frozen at -25°C until further use.
2.12 DETECTION OF BOVINE MHC ANTIGENS

2.12.1 Standard class I microlymphocytotoxicity test

Tests were done in 60-well microtest plates (Nunc, Catalogue No. 163118, Roskilde, Denmark). All test sera had previously been heat-inactivated at 56°C for 30 min. Test sera and pooled non-reactive bovine serum (see Section 2.12.3 below) were centrifuged in a microfuge (i.e. at about 9,000 x g) for 5 min. Appropriate dilutions of test sera were made in non-reactive bovine serum.

The method used was based on the standardized HLA typing method (Mittal, 1978). One µl of test serum was added to each well of the microtest plates with a Hamilton dispenser (Hamilton Co., Reno, NE., U.S.A.). Wells were covered with liquid paraffin within 3 min of the addition of the sera. Plates were stored frozen at -25°C for up to 3 months. Before testing, plates were thawed at room temperature for at least 30 min.

Peripheral blood lymphocytes were adjusted to a concentration of 2 x 10⁶/ml in PBS, and 1 µl of the lymphocyte suspension was added to each well of the microtest plates, using a Hamilton dispenser. The room temperature was checked (tests were done at 20-25°C), and the plates were incubated for 30 min at this temperature.

Five µl of rabbit serum was added to each well as a source of complement (see Section 2.12.3 below), and the plates were incubated for a further 50 min at room temperature.

One µl of eosin dye was added to each well, followed 10 min later by 5 µl of fixative (16% formaldehyde in PBS).
2.12.2 Extended microlymphocytotoxicity test

To provide greater sensitivity in the detection of class II antigens, longer incubation periods were used than for class I typing (Bodmer et al., 1976). The method used was based on the Seventh International HLA Workshop standard typing technique for Ia serology (Bodmer et al., 1978). After the addition of lymphocytes, microtest plates were incubated for 60 min at room temperature, and after the addition of complement, plates were incubated for a further 120 min.

2.12.3 General remarks

Pooled rabbit serum was used undiluted as a source of complement. Sera from a number of rabbits were screened for non-reactivity in a microlymphocytotoxicity test against lymphocytes from 10 unrelated cattle. All non-reactive sera were pooled (at least 6 rabbit sera to a pool), divided into portions and stored frozen at -25°C. Frozen portions were thawed no more than 30 min before addition to microtest plates. Thawed, unused complement was discarded.

Pooled non-reactive bovine serum was used as a diluent for test sera and to determine background levels of lymphocyte death. Sera from a number of calves were heat-inactivated at 56°C for 30 min and screened for non-reactivity in a microlymphocytotoxicity test against lymphocytes from unrelated cattle. All non-reactive sera were pooled, and stored frozen at -25°C.

Lymphocytes were allowed to settle for at least 24 hr and preferably for 48 hr, before the plates were read. Plates were read under x100 magnification using an inverted microscope and phase contrast objectives (Diavert, Ernst Leitz GMBH, Wetzlar, West
Live lymphocytes appeared white-coloured and normal in size whereas dead lymphocytes appeared dark and enlarged, due to the uptake of eosin dye (Figure 2.3).

Tests were scored according to the percentage of dead lymphocytes, based on the scoring system described by Mittal (1979):

<table>
<thead>
<tr>
<th>% of Dead Cells</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 19</td>
<td>-</td>
</tr>
<tr>
<td>20 - 29</td>
<td>2</td>
</tr>
<tr>
<td>30 - 49</td>
<td>4</td>
</tr>
<tr>
<td>50 - 79</td>
<td>6</td>
</tr>
<tr>
<td>80 - 94</td>
<td>8</td>
</tr>
<tr>
<td>95 - 100</td>
<td>9</td>
</tr>
</tbody>
</table>

Negative control wells containing non-reactive bovine serum were included to determine background levels of lymphocyte death.

Cytotoxic endpoint titres were measured by testing doubling dilutions of antisera in non-reactive bovine serum. The endpoint titre of an antiserum was defined as the reciprocal of the highest dilution displaying at least 80% of the maximum cytotoxic reaction of that antiserum.

2.13 STATISTICAL METHODS

Standard statistical methods used were those described by Snedecor & Cochran (1980).

Relationships between lymphocyte typing sera were analysed from 2 x 2 contingency tables using Chi-square ($X^2$) analysis (van Rood &
**Figure 2.3**

Appearance of lymphocytes in the microlymphocytotoxicity test.

a. Test reaction of <20% dead cells (score -).

b. Test reaction of ≥95% dead cells (score 9).

Magnification x125.
van Leeuwen, 1963), with Yate's correction because of the small number of animals tested (Pickbourne et al., 1978b). From the $X^2$ parameter, the correlation coefficient ($r$) was derived (Bodmer & Payne, 1965; Dausset et al., 1965). The correlation coefficient represents $X^2$ corrected for the number of individuals tested, and is a measure of the strength of the association between antisera.

The formulae used for calculating these parameters were:

$$X^2 = \frac{[(ad-bc)-n/2]^2}{(a+c)(a+b)(b+d)(c+d)}$$

$$r = \pm \sqrt{\frac{X^2}{n}}$$

where $a$ is the number of common positive reactions, $d$ is the number of common negative reactions, and $b$ and $c$ are the number of reactions positive with only one antiserum or the other. The positive or negative sign to $r$ indicates whether the reactions of 2 antisera tend to overlap or to present a contrasting distribution.

Computer analysis was used to obtain $r$ values and to cluster antisera on the basis of these $r$ values; the program is described by Pickbourne et al. (1978b).

Because of the small number of animals tested, the 2 x 2 contingency tables of antisera reactions were also analysed using a two-tailed Fisher's exact test (Klein, 1975; Snedecor & Cochran, 1980; Dr W. Bodmer, personal communication). This test yields the probability of observing a 2 x 2 table that gives at least as much evidence of association as the table actually observed. With row and
column margins considered fixed, a value of \( p \) was calculated for each possible table that gave at least as much evidence of association that was in the same direction as that of the observed table, using the formula:

\[
p = \frac{(a+b)! \; (c+d)! \; (a+c)! \; (b+d)!}{n! \; a! \; b! \; c! \; d!}
\]

The \( p \) value of each table was added and this summed \( p \) value was multiplied \( \times 2 \) to give the two-tailed probability (Armitage, 1971).

Antigen frequencies were calculated as the number of positive cattle divided by the number tested. Gene frequencies (\( p \)) were estimated from antigen frequencies (\( f \)) by the formula:

\[
p = 1 - \sqrt{(1 - f)}, \quad (\text{Mattiuz et al., 1970}).
\]

Standard errors of gene frequencies (\( SE \)) were calculated from the formula:

\[
SE = \sqrt{pq / 2n},
\]

where \( p \) is the gene frequency, \( q \) is the frequency of all other alleles at that locus (i.e. \( 1 - p \)), and \( n \) is the number of cattle tested.

Population associations between antigens were analysed using the \( \chi^2 \) test for independence calculated from the corresponding 2 x 2 contingency tables (Andresen et al., 1963; Bodmer et al., 1969; Klein,
1975). Because of the small number of animals tested, these tables were also analysed using a two-tailed Fisher's exact test.

The likelihood of linkage between 2 loci was investigated using the sequential probability ratio test (Morton, 1955). The parameter calculated was the LOD score (z). The LOD score is the logarithm of the probability ratio formed by dividing the probability under a chosen recombination frequency by the probability under a recombination frequency of 0.5 (i.e. independence of loci). The method of calculating LOD scores was the same as that used by Hines et al. (1981), when examining the linkage relationships among loci of polymorphisms of blood and milk constituents of cattle. The LOD score was calculated as:

\[ z = \log_{10} 2^{s-1} \left[ RF^a d (1-RF)^b c + RF^b c (1-RF)^a d \right] \]

where \( s \) is the total number of informative matings (i.e. the family size), \( RF \) is the chosen recombination frequency, and \( a, b, c \) and \( d \) are the numbers of the different kinds of paired allelic combinations transmitted by the sire (i.e. are the numbers in the 2 x 2 table of the traits under consideration).
CHAPTER 3

PREPARATION OF CELL PANELS

FOR THE IDENTIFICATION OF ANTI-CLASS II ANTIBODIES

Only expressing class II antigens are a minority in properly
mixed lymphocytes (Fujita et al., 1974; Kojima et al., 1976). It would
be noted that IFN preparations will readily be
contaminated with mixed lymphocytes and the extent of this
contamination will depend on the method of preparation of the IFN.
The identification of anti-class II antibodies using the
microlymphocytotoxicity technique and IFN requires that these cells
pools be enriched for class II-bearing cells (van Rood et al., 1979).
Alternatively, the class II-bearing cells may be distinguished from
other cells, as in the indirect fluorescence assay (van Rood et
al., 1979).

Since B lymphocytes, unlike class II-bearing cells,
produce antibodies, pools of
wells seeded with each type of lymphocyte
in the microwell assay (van Rood et al., 1979; Tsuchida et al., 1979; Bigg et
al., 1979; Bigg et al., 1979) are tested.

Lines of the B cell lines, including splenic and bone-
transformed cell lines, have also been used (Salmon, et al., 1976).-
Splenic cells have been used as target cells in the detection of many
class II antigens (Kawabata et al., 1978).

Antigen identification of B lymphocytes is a prerequisite for
their enrichment from a completerate cell population. B lymphocytes
are defined as those lymphocytes having mostly B cells from
membrane immunoglobulin (mIg), which is synthesized by the cell
carrying it and which is distinguishable from external antigens
attached to the cell membrane. B lymphocytes in enriched cells can
and must form reactive with sheep red blood cells (SRBC).
Cells expressing class II antigens are a minority in peripheral blood lymphocytes (PBL) (Winchester & Kunkel, 1979; Kaufman et al., 1984). It should be noted that PBL preparations will usually be contaminated with some monocytes, and the extent of this contamination will depend on the method of preparation of the PBL. The identification of anti-class II antibodies using the microlymphocytotoxicity technique and PBL requires that test cell panels be enriched for class II-bearing cells (van Rood et al., 1975). Alternatively, the class II-bearing cells may be distinguished from the other cells, as in the two colour fluorescence assay (van Rood et al., 1976).

Since B lymphocytes express surface class II antigens, panels of cells enriched for B lymphocytes have been widely used as target cells in the serological definition of HIA class II antigens (van Rood et al., 1975; Terasaki et al., 1978; Lowry et al., 1979). Lymphoid cell lines of the B cell variety, including Epstein Barr virus-transformed cell lines, have also been used (Bodmer, J., 1978a). Spleen cells have been used as target cells in the definition of mouse class II antigens (Colombani et al., 1976).

Accurate identification of B lymphocytes is a prerequisite for their enrichment from a conglomerate cell population. B lymphocytes are defined as those lymphocytes having readily demonstrable surface membrane immunoglobulin (smIg), which is synthesized by the cell carrying it and which is distinguishable from external cytophilic Ig attached to the cell membrane. T lymphocytes in contrast lack smIg, and most form rosettes with sheep red blood cells (SRBC) (WHO/IARC,
Mouse and human B lymphocytes usually express IgM and IgD on their surface; most of the IgG and IgA carried by lymphocytes is thought to be externally attached to cell surface receptors via the Fc piece of Ig ("cytophilic Ig") (Winchester et al., 1975b; Parkhouse et al., 1976; Pettersson et al., 1978).

In the identification of B lymphocytes, erroneously high values can be due to: (a) non-specific antibody activity in the anti-Ig antiserum; (b) binding of the Fc piece of the anti-Ig reagent to Fc receptors on non-B cells; or (c) the presence of cytophilic immunoglobulin on non-B cells.

The specificity of an anti-Ig reagent can be checked by titrating the reagent, choosing an appropriate working dilution, and testing the reagent against a negative control cell panel such as thymocytes (Raff et al., 1970).

In the mouse and in man, Fc receptors are present on B lymphocytes, some T lymphocytes, and cells of the monocyte/macrophage lineage (LoBuglio et al., 1967; Dickler, 1976; Unkeless et al., 1981). The binding of antibody reagents to Fc receptors can be prevented by the use of F(ab')2 fragment reagents rather than whole IgG reagents (Winchester et al., 1975b). The most widely used method to promote the removal of cytophilic immunoglobulin is the incubation of cells in serum-free medium for up to 30 min at 37°C (Johnson et al., 1978). There have been some claims that a longer period of incubation is necessary for the complete removal of cytophilic Ig from all lymphocytes and monocytes (Alexander et al., 1978; Shaala et al., 1979; Wilson et al., 1984). A suggested alternative method of removing cytophilic Ig is the washing of whole blood with PBS prior to separating the lymphocytes on Ficoll-Hypaque (Alexander et al., 1978).
T lymphocytes from man (Brain et al., 1970; Lay et al., 1971; Jondal et al., 1972) and a number of animal species (Johansen et al., 1974; Escajadillo & Binns, 1975) bind SRBC to form clusters called E rosettes. The percentage of E rosette-forming cells in PBL from healthy human donors varies from 30-80%, depending on the conditions of the test (Hayward, 1981). Because E rosettes are fragile, technical modifications which enhance the stability of rosettes result in greater numbers of E rosettes being observed (Jondal, 1976). Methods of enhancing E rosette formation by human lymphocytes include the pre-treatment of SRBC with neuraminidase or AET (2-aminomethylisothiouronium bromide) (Bentwich et al., 1973; Weiner et al., 1973; Pellegrino et al., 1975), and the supplementation of the incubation medium with Dextran or high concentrations of foetal calf serum (Brown et al., 1975; Woody & Sell, 1975). Where studied, E rosette formation has proved to be a property of T lymphocytes, although certain enhancing conditions may enable some B lymphocytes to form rosettes (Bentwich et al., 1973).

Bovine E rosettes have been reported, using a variety of the enhancing conditions used for human E rosetting (Grewal et al., 1976; Higgins & Stack, 1977; Wardley, 1977; Binns, 1978; Grewal & Babiuk, 1978; Reeves & Renshaw, 1978; Paul et al., 1979). The highest mean percentage of E rosette-forming PBL reported in adult cattle is 83% (Outeridge & Dufty, 1981), when rosette formation was enhanced by incorporating Ficoll in the incubation medium and rosettes were defined by 5 or more SRBC attached to a lymphocyte. Bovine E rosette formation has been shown to be a property mainly of T lymphocytes (Binns, 1978; Grewal & Babiuk, 1978; Paul et al., 1979), although a
few B lymphocytes may form rosettes when certain enhancing conditions are used (Usinger & Splitter, 1981).

Separation of rosetted and non-rosetted cells by density gradient centrifugation has been used as a means of enriching human T and B lymphocytes (Mendes et al., 1973; Wahl et al., 1976). T lymphocyte rosetting and nylon wool separation are 2 widely used methods of T and B lymphocyte separation for HLA class II typing (Terasaki et al., 1978; Lowry et al., 1979; Danilovs et al., 1980).

T and B lymphocytes may also be separated by a technique called panning, using plastic petri dishes coated with an anti-Ig antibody reagent (Mage et al., 1977; Wysocki & Sato, 1978). Dishes are coated by adding the antibody solution to the dish and allowing the antibody molecules to be adsorbed onto the plastic. B lymphocytes bind to the surface of the dish by virtue of their smlg, while T lymphocytes remain unbound and can be decanted. The adherent cells are then collected from the surface of the dish (usually mechanically). To weaken the binding of the adherent cells to the dishes and facilitate their detachment, the specific antibody reagent is diluted with non-specific antibody solution (Mage et al., 1977; Wysocki & Sato, 1978) or buffer solution (Maryanski et al., 1985; Zola et al., 1985).

When mouse spleen cells (40-70% smlg positive) have been panned on anti-Ig coated dishes, adherent cell preparations containing >90% smlg positive cells have been reported (Mage et al., 1977; Wysocki & Sato, 1978). Minor cell subpopulations (2.5% positive cells) can also be efficiently enriched (Wysocki & Sato, 1978). Human B lymphocyte-enriched preparations containing an average of 70-80% smlg positive cells have been produced by panning of PBL on dishes coated with anti-
Ig antisera or anti-B cell monoclonal antibodies (Fong et al., 1981; Tsoi et al., 1982; Smeland et al., 1985).

Panning has been used to enrich for B lymphocytes in a number of domestic animal species, including the sheep (Miyasaka et al., 1983; Chasset et al., 1985), the pig and cattle. Pig B lymphocytes adhere non-specifically to ovalbumin-coated dishes or uncoated dishes (Vaiman et al., 1983; Chasset et al., 1985). Panning of cattle PBL has been done to prepare non-adherent cells enriched for T lymphocytes (Splitter & Everlith, 1982; Williams et al., 1986). Ovalbumin-coated dishes have also been used for panning of cattle PBL, and the adherent cells have been reported to contain usually >90% B lymphocytes (Spooner & Ferrone, 1984).

In the experiments reported in this chapter, the effect of incubation at 37°C on the percentage of bovine PBL identified as smIg positive was examined, and was compared with the effect of pre-washing whole blood with PBS prior to separating the lymphocytes. T lymphocyte rosetting and panning were examined as methods of B lymphocyte enrichment in cattle. The aim was to consistently obtain cell panels containing >60% B lymphocytes, based on the recommendations of Bodmer, J. (1978b) who discussed requirements for HLA class II typing.

3.2 EXPERIMENTAL METHODS

An affinity purified rabbit F(ab')2 anti-bovine IgG reagent (Section 2.6) was used in an indirect immunofluorescence assay for the identification of bovine B lymphocytes (Section 2.8). Both first and second step reagents in the assay were titrated in a checkerboard
fashion against PBL from 4 cattle. The specificity of the assay was checked against a negative control cell panel consisting of bovine thymocytes.

The effect of incubation at 37°C on the percentage of PBL identified as smIg positive was examined. PBL were incubated at 37°C for 0, 30, 60 or 120 min, and washed twice with PBS at 37°C before staining. Preparations which were not incubated at 37°C were washed twice at 4°C before staining.

To examine the effect of pre-washing whole blood on the staining result, PBL were prepared by first washing whole blood 3 times with 10x volume of PBS and then subjecting the cells to Ficoll-Hypaque density gradient centrifugation. These PBL were not incubated at 37°C.

Rosetting was done as described in Section 2.9.1, based on the method described by Outteridge & Dufty (1981). Initially, techniques for the handling of rosetted preparations were established to ensure minimum disruption of rosettes.

In panning method A, tissue culture grade dishes (88 mm diameter, Kayline Plastics, Product No. PDS8813, Adelaide, Australia) were incubated with the antibody solution. This solution contained one part of F(ab')2 rabbit anti-bovine IgG reagent (Section 2.6) at 100 µg/ml and 9 parts of normal rabbit IgG at 100 µg/ml. The normal rabbit IgG was prepared by subjecting normal rabbit serum to 2 rounds of ammonium sulphate precipitation (Section 2.6.3). The remainder of the procedure was as described in Section 2.9.2

In panning method B (Section 2.9.2), the use of bacteriological grade petri dishes and whole IgG antibodies diluted in PBS was evaluated. To examine the effect of temperature on the purity of
adherent cell preparations, PBL were incubated on dishes at 4°C or room temperature.

3.3 RESULTS

3.3.1 Identification of B lymphocytes

The endpoint titre of the affinity purified anti-bovine IgG first step reagent varied from 1.25–2.5 µg/ml when tested against the PBL of different animals. Therefore, a working dilution of 10 µg/ml was chosen. The fluorescein-conjugated second step reagent (anti-rabbit F(ab')₂), at a concentration of 2x its working dilution, was by itself non-reactive when tested against bovine PBL.

The first step reagent was screened at a number of dilutions against calf thymocytes, with the second step reagent being employed at its usual working dilution of 1/10. At the highest concentration of first step reagent tested (40 µg/ml), 0.25% of cells were positive.

PBL from 4 animals were incubated at 37°C for 0, 30, 60 and 120 min. The mean percentages of smIg positive cells subsequently detected in these preparations were 37.9%, 23.0%, 24.6% and 27.4% respectively. The preparations incubated at 37°C for 120 min showed evidence of lower cell viability (generally <80%) and lower cell recovery compared with preparations incubated for 0, 30 or 60 min. As there may have been a selective loss of lymphocyte subpopulations, preparations incubated for 120 min could not be compared validly with the other preparations. PBL from 8 animals were incubated at 37°C for 0, 30 and 60 min. There were significantly less smIg positive cells (p < 0.05) in the preparations incubated for 30 min compared with preparations which were not subjected to incubation, but there was no
significant difference between preparations incubated for 30 min and 60 min (Table 3.1).

There was no significant difference between the percentage of smIg positive cells in pre-washed cell preparations (average of 19.7% smIg positive cells) and preparations incubated at 37°C for 30 min (average of 21.6% smIg positive cells) (n=4). The average yield after pre-washing was about 30% of the yield after conventional separation. Because there may have been a selective loss of lymphocyte subpopulations associated with this low yield, the results obtained after pre-washing could not be compared validly with those obtained after 37°C incubation.

The percentage of B lymphocytes in PBL was determined for 16 healthy cattle, which were from 3 breeds. All animals were older than 15 months. These values were obtained using the standardized conditions (see above). Cell viability in control preparations was generally >90% when PBL were prepared from fresh blood. The mean percentage of B lymphocytes found in these animals was 17.4%, with a range of 4.3%-30.8%.

### 3.3.2 Rosetting

The results obtained by subjecting PBL from 5 cattle (all older than 6 months) to E rosetting are shown in Table 3.2. The percentage of E rosettes varied from 3-60%. Substantial numbers of lymphocytes had bound only 2, 3 or 4 SRBC. When the preparations were centrifuged on Ficoll-Hypaque after rosetting, 6-25% of the lymphocytes subjected to E rosetting were recovered at the interface. In only one of 5 experiments did these non-rosetted interface cells contain >50% B lymphocytes (Table 3.2).
Table 3.1  Effect of incubation at 37°C on estimates of the percentage smIg positive cells in PBL.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Time of Incubation at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>K26</td>
<td>31.5 a</td>
</tr>
<tr>
<td>K35</td>
<td>30.0</td>
</tr>
<tr>
<td>GC20</td>
<td>42.0</td>
</tr>
<tr>
<td>452</td>
<td>48.0</td>
</tr>
<tr>
<td>848</td>
<td>12.8</td>
</tr>
<tr>
<td>812</td>
<td>62.5</td>
</tr>
<tr>
<td>163</td>
<td>31.5</td>
</tr>
<tr>
<td>949</td>
<td>22.5</td>
</tr>
<tr>
<td>Mean</td>
<td>35.1</td>
</tr>
<tr>
<td>SE</td>
<td>5.5</td>
</tr>
</tbody>
</table>

p < 0.05

a = percentage smIg positive cells.
### Table 3.2: E rosetting of bovine PBL.

<table>
<thead>
<tr>
<th>Animal</th>
<th>%smIg +ve</th>
<th>%E</th>
<th>%smIg +ve</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td>Rosettes</td>
<td>Cells</td>
<td></td>
</tr>
<tr>
<td>848</td>
<td>18.8</td>
<td>6</td>
<td>58.5</td>
<td>16</td>
</tr>
<tr>
<td>949</td>
<td>11.0</td>
<td>3</td>
<td>45.5</td>
<td>11</td>
</tr>
<tr>
<td>K26</td>
<td>32.0</td>
<td>24</td>
<td>45.8</td>
<td>25</td>
</tr>
<tr>
<td>K35</td>
<td>24.5</td>
<td>25</td>
<td>26.0</td>
<td>11</td>
</tr>
<tr>
<td>K58</td>
<td>22.0</td>
<td>60</td>
<td>27.8</td>
<td>6</td>
</tr>
</tbody>
</table>

- **a**: E rosette was defined as a lymphocyte with 5 or more SRBC attached.
- **b**: Lymphocytes in non-rosetted preparation as percentage of total number of lymphocytes subjected to E rosetting.
- **c**: Percentage of E rosettes observed if E rosette was defined as a lymphocyte with 2 or more SRBC attached.
3.3.3 Panning

Table 3.3 shows the results of panning on tissue culture grade petri dishes coated with \( F(ab')_2 \) fragments of rabbit anti-bovine IgG (method A). B lymphocyte purity after one round of panning (56.6 ± 16.3\%) was not sufficient for class II typing, especially taking into account those preparations at the lower end of the range. The B lymphocyte purity of the adherent cells was not improved by applying smaller numbers of PBL to each dish (as few as 25 x 10^6), increasing the concentration of the specific antibody reagent (to a final concentration as high as 100 \( \mu \)g/ml), or diluting the anti-Ig reagent in PBS rather than normal rabbit IgG. A substantial number of adherent cells was recovered from control dishes which were coated with normal rabbit IgG.

Subjecting adherent cells to a second round of panning produced preparations of adequate B lymphocyte purity (72.2 ± 6.8\%). However, the efficiency of the second round of panning was lower; the average B lymphocyte enrichment factor was only x1.3 compared with a factor of x3.5 during the first round of panning. On average, about 80\% of the available B lymphocytes were collected after one round of panning; the equivalent figure for the second round was about 60\% (giving a cumulative B lymphocyte yield of about 50\%). Although panning twice was quite time-consuming, this procedure was used to prepare B-enriched lymphocyte panels for much of the early work in the screening of platelet-absorbed alloantisera which is described in Chapter 4.

The use of bacteriological grade petri dishes coated with IgG of a different affinity purified rabbit anti-bovine IgG reagent (method B) resulted in greater enrichment of B lymphocyte preparations after one round of panning (Table 3.4). An IgG concentration of 5 \( \mu \)g/ml
Table 3.3  Panning: Results obtained using F(ab')\textsubscript{2}-coated tissue culture grade dishes (method A).

<table>
<thead>
<tr>
<th>% smIg +ve Cells</th>
<th>Cell Yield \textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>16.4 ± 7.1 \textsuperscript{b}</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>Adherent Cells,</td>
<td>56.6 ± 16.3</td>
</tr>
<tr>
<td>Round 1</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>Adherent Cells,</td>
<td>72.2 ± 6.8</td>
</tr>
<tr>
<td>Round 2</td>
<td>(n = 5)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} = yield of adherent cells as percentage of total number of PBL added to dishes.

\textsuperscript{b} = all values are shown as mean ± SD; (n) = number of experiments for which the particular parameters were measured.
Table 3.4  Panning: Comparison of the use of F(ab')$_2$-coated tissue culture grade petri dishes (method A) with IgG-coated bacteriological grade petri dishes (method B).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>%smIg +ve Cells</th>
<th>Yield $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBL</td>
<td>23.0</td>
<td>100</td>
</tr>
<tr>
<td>Method A</td>
<td>58.7</td>
<td>13</td>
</tr>
<tr>
<td>Method B</td>
<td>70.2</td>
<td>5</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBL</td>
<td>7.9</td>
<td>100</td>
</tr>
<tr>
<td>Method A</td>
<td>25.2</td>
<td>13</td>
</tr>
<tr>
<td>Method B</td>
<td>63.0</td>
<td>10</td>
</tr>
</tbody>
</table>

$^a$ = yield of adherent cells as percentage of total number of PBL added to dishes.
was sufficient to produce maximum B lymphocyte enrichment. Using method B, there was no significant difference between the B lymphocyte purity of the adherent cells obtained by incubating lymphocytes on the dishes at 4°C (average of 81.8% B cells) or at room temperature (average of 82.4% B cells) (n=5).

The purity of B-enriched cell preparations obtained from PBL of 15 cattle using method B are shown in Table 3.5. The 15 cattle were all older than 18 months and were from 3 breeds. The average purity of such preparations was 78%; even preparations at the lower end of the range (63% B lymphocytes) were sufficiently enriched for class II typing.

3.4 DISCUSSION

Capping and subsequent loss of smIg can occur during immunofluorescence assays due to cross-linking of smIg by the anti-Ig reagents. To minimize capping, some investigators handle cells at 4°C and in the presence of sodium azide (Johnson et al., 1978). In the present experiments, sodium azide was used and the cells were handled at room temperature rather than at 4°C, as the experiments of Loor et al. (1972) suggested that the use of sodium azide alone is sufficient to inhibit capping.

The present experiments suggested that 30 min incubation at 37°C was sufficient to produce maximum removal of cytophilic Ig from bovine PBL. The alternative method of washing the blood before lymphocyte separation suggested by Alexander et al. (1978) was no more effective than 37°C incubation. To produce maximum removal of cytophilic Ig from human lymphocytes, a number of studies have suggested that cells
Table 3.5  Panning: Results obtained using IgG-coated bacteriological grade petri dishes (method B) at room temperature.

<table>
<thead>
<tr>
<th>Animal</th>
<th>PBL</th>
<th>Adherent Cells</th>
<th>Yield $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% smIg +ve Cells</td>
<td>% smIg +ve Cells</td>
<td></td>
</tr>
<tr>
<td>341</td>
<td>7.9</td>
<td>63.0</td>
<td>10</td>
</tr>
<tr>
<td>T131</td>
<td>6.4</td>
<td>69.0</td>
<td>8</td>
</tr>
<tr>
<td>163</td>
<td>23.0</td>
<td>70.2</td>
<td>6</td>
</tr>
<tr>
<td>SV15</td>
<td>24.9</td>
<td>71.3</td>
<td>13</td>
</tr>
<tr>
<td>98</td>
<td>6.9</td>
<td>73.3</td>
<td>6</td>
</tr>
<tr>
<td>19</td>
<td>30.0</td>
<td>76.5</td>
<td>13</td>
</tr>
<tr>
<td>K57</td>
<td>12.5</td>
<td>77.0</td>
<td>14</td>
</tr>
<tr>
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<td>79.0</td>
<td>13</td>
</tr>
<tr>
<td>K105</td>
<td>25.0</td>
<td>79.7</td>
<td>14</td>
</tr>
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<td>K101</td>
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<td>6</td>
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<td>13</td>
</tr>
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<td>595</td>
<td>17.9</td>
<td>86.1</td>
<td>14</td>
</tr>
<tr>
<td>L22</td>
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<td>15</td>
</tr>
<tr>
<td>Mean</td>
<td>17.3</td>
<td>77.7</td>
<td>12</td>
</tr>
<tr>
<td>SD</td>
<td>8.1</td>
<td>7.4</td>
<td>4</td>
</tr>
</tbody>
</table>

$a$ = yield of adherent cells as percentage of total number of PBL added to dishes.
should be incubated for 15-20 min at 37°C and washed at 37°C after incubation (Kumagai et al., 1975; Lobo et al., 1975; Pettersson et al., 1978). Human lymphocytes which have shed immunoglobulin can readсорb IgG when exposed to serum (Kumagai et al., 1975; Pettersson et al., 1978). Therefore, the decrease in the percentage of smIg positive cells seen after 37°C incubation is probably not due simply to a selective loss of B lymphocytes.

In studies involving cattle PBL, Johansson & Morein (1983) found that 2 hr incubation at 37°C produced a significant decrease in the percentage of PBL identified as smIg positive, but it isn't clear whether they also examined other times of incubation. Gershwin et al. (1983) found that incubation at 37°C had no effect on the percentage of cattle PBL identified as smIg positive, but they appear to have studied cells from only one animal.

Although the reported range of normal values of B lymphocytes in human PBL varies from 4-34% (Johnson et al., 1978; Pettersson et al., 1978; Warr, 1980), values closer to the lower end of this range have been reported when 37°C incubation and F(ab')2 fragments have been included in the method (Winchester et al., 1975b; Pettersson et al., 1978). Reported mean values of B lymphocytes in PBL of healthy cattle have ranged from 11.3-30.0%, with a range of 4.5-41.0% in individual animals (Kumar et al., 1978; Reeves & Renshaw, 1978; Paul et al., 1979; Outeridge & Dufty, 1981; Usinger & Splitter, 1981; Gershwin et al., 1983; Johansson & Morein, 1983; Lewin et al., 1985). When both 37°C incubation and F(ab')2 fragments have been used, reported mean values of B lymphocytes in cattle PBL have included 20.7% and 27.0% (Usinger & Splitter, 1981; Johansson & Morein, 1983).
Fewer E rosette-forming cells were observed than were reported by Outteridge & Dufty (1981) for cattle older than 6 months. Possible reasons for this difference include differences in the populations being sampled, variation between individual sheep in the suitability of their RBC for E rosetting (Smith et al., 1975; Reeves & Renshaw, 1978), the fragile nature of E rosettes and inadequate handling procedures (Jondal, 1976). There is a general lack of agreement regarding the optimum conditions for E rosetting in cattle. The range of normal values reported may reflect the fragile nature of bovine E rosettes and variations in the definition of a rosette. In many reports of E rosetting of cattle PBL, a rosette has been defined by as few as 2-3 SRBC attached to a lymphocyte.

Although panning with F(ab')2-coated tissue culture grade petri dishes produced encouraging results, non-specific cell binding to these dishes was high. The reduced efficiency of the second round of panning could have been due to some degree of capping and subsequent loss of lymphocyte smIg by the action of the immobilized anti-Ig reagent during the first round, as is suggested to occur (Mage et al., 1981; Tsoi et al., 1982). Capping of smIg was evident when adherent cells were examined by immunofluorescence.

Wysocki & Sato (1978) reported that non-specific cell binding to dishes is reduced if bacteriological grade petri dishes are used rather than tissue culture grade dishes, which may account for the improved B lymphocyte enrichment obtained with method B. The antibody reagent used to coat the dishes in method B was also different. It is possible that the F(ab')2 fragments used in method A may not bind as well to plastic as do IgG molecules.
Cells of the monocyte/macrophage lineage adhere non-specifically to glass and plastic surfaces in a temperature-dependent fashion (Mosier, 1967; Shortman et al., 1971), and may contaminate adherent cells during panning (Tsoi et al., 1982; Hoover et al., 1985). As well as using bacteriological grade petri dishes, Wysocki & Sato (1978) also incubated the cells at 4°C. Some investigators have suggested that non-specific monocyte adherence is minimal if panning is done at 4°C (Tsoi et al., 1982; Smeland et al., 1985). However, Hoover et al. (1985) found significant monocyte contamination in B-enriched adherent cells prepared by panning at 4°C, if steps were not taken to remove monocytes prior to panning. In my experiments, panning at 4°C did not improve B-enriched cell purity. Although the energy-dependent, non-specific binding by monocytes may be reduced by panning at 4°C, monocytes (and some T lymphocytes) may nevertheless bind to the dishes by way of cytophilic Ig attached to their Fc receptors.

Adherent cells prepared by panning method B were 63-90% smIg positive by immunofluorescence, and yet were consistently >80%, and usually >90% class II positive by cytotoxicity (see Chapter 5). Many of the non-B cells in these preparations were probably monocytes. Virtually all human peripheral blood monocytes express DR class II antigens, although only a subpopulation expresses DQ class II antigens (Gonwa et al., 1983; Nunez et al., 1984). In the mouse, a subpopulation of macrophages and peripheral blood monocytes expresses class II antigens; the size of this subpopulation increases after infection or immunization (Beller et al., 1980; Nussenzweig et al., 1981). Mouse macrophages expressing class II antigens do not uniformly express both I-A and I-E antigens (Cowing et al., 1978).
Because monocytes vary in their expression of class II loci, they are not the ideal cell for defining class II antigens. In addition, there is evidence that human monocytes do not react as strongly to anti-DR antisera in the cytotoxic test as do B cells (Cicciarelli et al., 1978). Monocytes could be removed prior to panning by incubating PBL on uncoated plastic petri dishes, or by incubating PBL or whole blood with carbonyl iron particles and removing those cells which ingest iron (Boyum, 1982). However, some lymphocytes will also be lost using these methods and this loss may involve a selective loss of B lymphocytes (Fernandez & Macsween, 1977).

Adherent cell panels produced by panning method B were used for the subsequent detection of class II antigens in cattle.
CHAPTER 4

PREPARATION OF ANTISERA AS ANTI-CLASS II REAGENTS

Alternatively, class I antigens may be stripped from the surface of target cells using anti-β2-microglobulin antisera (Price et al., 1979), or they may be incubated with anti-β2-microglobulin of donor origin (Bertocci et al., 1979). Another option is to enrich target cells with α-enriched (α) and β-depleted (β) lymphocytes from a large number of third party individuals. Some of these individuals may be positive for the class II antigen but negative for the class I antigen recognised by the antisera. Basophil anti-class II reagents may be overlaid using this method if required.
4.1 INTRODUCTION

Alloantisera may contain antibodies to both class I and class II antigens if raised between individuals which differ at both the class I and class II regions of the MHC (Sachs & Cone, 1975; Shreffler & David, 1975). There are a number of ways of detecting the anti-class II antibodies. The anti-class I antibodies can be absorbed selectively from the antisera by cells which bear class I but not class II antigens, leaving the anti-class II antibodies uncontaminated. In studies in the mouse and the rat, platelets and RBC have been used for this purpose (Colombani et al., 1976; Staines et al., 1976; Radka et al., 1977; Shinohara et al., 1977). Platelets have also been used for the absorption of anti-class I antibodies from alloantisera of a number of other species including man (Van Rood et al., 1975), the Rhesus monkey (Roger et al., 1976) and cattle (Newman et al., 1982a). Platelets are obtained usually by differential centrifugation of whole blood (Colombani, 1976). In HLA class II definition, antisera are often absorbed with pooled platelets obtained from blood banks (Bodmer et al., 1976).

Alternatively, class I antigens may be stripped from the surface of target cells using anti-β2-microglobulin antisera (Jones et al., 1975), or they may be blocked with anti-β2-microglobulin of avian origin (Bernoco et al., 1976). Another option is to screen antisera against B-enriched (B+) and B-depleted (B-) lymphocytes from a large number of third party individuals. Some of these individuals may be positive for the class II antigens but negative for the class I antigens recognized by an antiserum. Useful anti-class II reagents may be overlooked using this method if appropriate class II-
positive/class I-negative target cells are not available (Vassalli et al., 1979: Engelfriet et al., 1982).

In mice, rats, humans and Rhesus monkeys, immunogens used in the production of alloantisera have included leucocytes, lymphocytes, skin or whole blood, either used alone or in a variety of primary and boosting combinations (Klein, 1975; Radka et al., 1977; Belvedere et al., 1978; Roger et al., 1980). Conventional sources of alloantisera used for BoLA class I typing have been sera from parous cows, sera from cattle immunized with allogeneic lymphocytes and sera from skin graft recipients (Caldwell et al., 1977; Spooner et al., 1978; Amorena & Stone, 1980; Stear et al., 1982). The use of subcutaneous skin implants to produce high titre class I typing reagents has been reported by Pringnitz et al. (1982).

After alloimmunization, peak cytotoxic titres of anti-class II antibodies generally coincide with peak cytotoxic titres of anti-class I antibodies (Belvedere et al., 1978; Newman & Stear, 1983). The best source of HIA class II typing reagents are generally those alloantisera with anti-class I activity (Vassalli et al., 1979; Borelli et al., 1982; Engelfriet et al., 1982).

In the experiments reported in this chapter, alloantisera were raised by buffy-coat leucocyte immunizations and skin implant immunizations. The antisera were absorbed with platelets and screened to evaluate their potential as class II typing reagents.
4.2 EXPERIMENTAL METHODS

4.2.1 Immunizations

A number of alloantisera raised by immunization with buffy-coat leucocytes were donated by Dr M.J. Stear; their titres against donor peripheral blood lymphocytes (PBL) in the standard microlymphocytotoxicity test ranged from 1 to 256. Subsequently, I prepared 11 alloantisera by buffy-coat leucocyte immunizations, as described in Section 2.10.1. All of these immunizations were matched for one haplotype (i.e. parent into offspring, or half-sibling into half-sibling), in an attempt to obtain antisera of restricted specificity.

Twenty-eight alloantisera were raised by skin implant immunizations, as described in Section 2.10.2; 18 immunizations were matched for one haplotype, and 10 were mismatched. Twenty-one of the recipient animals were given booster immunizations (generally if the titre against donor PBL in the standard microlymphocytotoxicity test was ≤64).

Serum samples were collected from 240 parous cows, and were screened undiluted in a standard microlymphocytotoxicity test against PBL from 16 cattle. Antisera were selected for further screening if they did not give cytotoxic reactions of ≥80% with PBL from any of these cattle. All of the class I antigens that could be detected by the Canberra antisera were represented in these 16 cattle. Selected antisera were then screened in an extended microlymphocytotoxicity test against B(+) and B(−) cells from 20 cattle.
4.2.2 Anti-class II type activity

An antiserum was described as displaying anti-class II type activity at a particular dilution if it reacted preferentially with B(+) cell panels compared with B(-) cell panels. This was defined as follows: if the antiserum gave a reaction of ≥80% cell death against B(+) cells from some animals, and <50% cell death against B(-) cells from all animals, it had anti-class II type activity. An antiserum was described as showing a weak anti-class II type reaction at a particular dilution if it gave a reaction of 50-80% cell death against a B(+) cell preparation, and <30% cell death against the corresponding B(-) cell preparation. It should be noted that the anti-class II nature of this activity was not confirmed until later, and is discussed in Chapter 9.

4.2.3 Platelet preparation and absorption

Platelets were prepared by differential centrifugation of whole blood, as described in Section 2.7.4. In an effort to optimize platelet yield, a variety of centrifugation procedures were examined. Optimum platelet yield was obtained by centrifuging blood at 300xg for 30 min or 1,300xg for 5 min. Unless stated otherwise, the platelets used for absorption of antisera were from the tissue donor in the alloimmunization. Platelet absorptions were done as described in Section 2.11.1.

The effect of fixing (using 1% formaldehyde) on the absorptive capacity of platelets was examined. The absorptive capacity of reused platelets was also examined, in an effort to reduce the workload in preparing fresh platelets. Platelets which had been used for absorption were immediately washed twice in PBS. Absorbed antibody
was then eluted by resuspending the platelets in freshly made 0.1 M citrate buffer, pH 3.0 (Newman & Stear, 1983). One ml of buffer was used per $1 \times 10^{10}$ platelets, and the platelets were mixed with the buffer on a slow rotator for 15 min at room temperature. The platelets were pelleted by centrifugation, washed 3 times in PBS/0.1% sodium azide, and stored in the dark at 4°C. Separate samples of antisera were absorbed with fresh and reused platelets, and the cytotoxic titres of the absorbed antisera were determined.

Initially, antisera were subjected to "complete" platelet absorption: an antiserum was repeatedly absorbed with donor platelets until a reaction of <80% cell death was observed when the antiserum was tested undiluted in the extended microlymphocytotoxicity test against donor PBL. Anti-class II type activity was then evaluated.

Antisera subjected to "partial" platelet absorption were absorbed until the cytotoxic titre in an extended microlymphocytotoxicity test against donor PBL or B(-) cells was 8 or less. Dilutions higher than this were then examined for anti-class II type activity.

4.3 RESULTS

4.3.1 Buffy-coat leucocyte immunization

Eleven animals were immunized with buffy-coat leucocytes from a haplotype-matched donor. All immunized animals responded, although weekly immunizations for 3-6 weeks (average of 4 immunizations) were required to produce a cytotoxic titre of $\geq 32$ against donor PBL. The peak cytotoxic titres ranged from 32 to $\geq 512$, with a median value of 256.
4.3.2 Skin implant immunization

The results of 28 skin implant immunizations are shown in Table 4.1 and summarized in Table 4.2. All recipients responded to the initial immunization, though some peak titres were quite low, especially where immunizations were matched for one haplotype (a median titre of 8). With mismatched immunizations, peak titres were significantly higher (median titre 64) \(p < 0.05\); Wilcoxon's rank sum test). The peak titres were first observed either 14 or 21 days after immunization (serum samples were taken at weekly intervals).

Twenty-one of the immunized animals were given a second skin implant. Titres had started to decrease in 17 of these recipients by the time the second immunization was given; 2 typical examples are shown in Figures 4.1 and 4.2. However, some animals had titres which were still at a peak at the time they were reimmunized (Figure 4.3). The secondary challenge usually produced a substantial increase in the peak titre (Tables 4.1, 4.2). In the 15 haplotype-matched immunizations which were boosted, the median peak titre rose from 8 to 128, and in the 6 mismatched immunizations which were boosted, the median peak titre rose from 32 to 128. Not all peak titres were increased by secondary immunization (e.g. recipient L31). As was the case after the initial immunizations, peak titres occurred about 2 or 3 weeks after reimmunization.

Titres did not remain at a peak for long after immunization. The median peak titre observed after final immunizations (whether a boosting immunization, or in cases where only one immunization was done) was 128 (range 32-2256). By 41-47 days after final immunizations, the median titre was 32 (range 4-128), and by 82-93 days, the median titre was 8 (range 1-16). Some examples of the
Table 4.1  Skin implant immunizations.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Initial Immunization</th>
<th>Secondary Immunization b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak Titre a</td>
<td>First Day of Peak Titre</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Haplotype-matched:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K31</td>
<td>1 c</td>
<td>21</td>
</tr>
<tr>
<td>K85</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>K18</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>L1</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>H19</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>K21</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>K84</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>K83</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>M2</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>L33</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>L22</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>L30</td>
<td>16</td>
<td>14</td>
</tr>
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<td>L25</td>
<td>32</td>
<td>21</td>
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<tr>
<td>L29</td>
<td>64</td>
<td>14</td>
</tr>
<tr>
<td>L31</td>
<td>128</td>
<td>21</td>
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<tr>
<td>L2</td>
<td>64</td>
<td>14</td>
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<td>K109</td>
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<td>K107</td>
<td>128</td>
<td>14</td>
</tr>
<tr>
<td>Mismatched:</td>
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<td></td>
</tr>
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<td>L3</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>K28</td>
<td>32</td>
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</tr>
<tr>
<td>L28</td>
<td>32</td>
<td>21</td>
</tr>
<tr>
<td>L36</td>
<td>32</td>
<td>14</td>
</tr>
<tr>
<td>L34</td>
<td>64</td>
<td>21</td>
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<tr>
<td>M3</td>
<td>64</td>
<td>14</td>
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<td>H11</td>
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<td>L12</td>
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<td>L32</td>
<td>128</td>
<td>14</td>
</tr>
<tr>
<td>L26</td>
<td>128</td>
<td>14</td>
</tr>
</tbody>
</table>

a = peak titre against donor PBL in the standard microlymphocytotoxicity test.

b = reimmunization was done either 29 or 40 days after the initial immunization.

c = titre "1" refers to the antiserum tested undiluted.
Table 4.2 Summary of skin implant immunizations.

<table>
<thead>
<tr>
<th>Number of Recipients</th>
<th>Peak Titre ( ^a )</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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</tr>
<tr>
<td><strong>Initial Immunization:</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Haplotype-matched</td>
<td>18</td>
<td>1 - 128</td>
<td>8</td>
</tr>
<tr>
<td>Mismatched</td>
<td>10</td>
<td>16 - 128</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After Secondary Immunization:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haplotype-matched</td>
<td>15</td>
<td>32 - ≥256</td>
<td>128</td>
</tr>
<tr>
<td>Mismatched</td>
<td>6</td>
<td>64 - ≥256</td>
<td>128</td>
</tr>
</tbody>
</table>

\( ^a \) = peak titre against donor PBL in the standard microlymphocytotoxicity test.
Figure 4.1  Cytotoxic antibody response against donor PBL after immunization with implanted allogeneic skin (standard microlymphocytotoxicity test). Animal K84. A booster immunization was given on day 40.
Figure 4.2  Cytotoxic antibody response against donor PBL after immunization with implanted allogeneic skin (standard microlymphocytotoxicity test). Animal K83. A booster immunization was given on day 40.
Figure 4.3  Cytotoxic antibody response against donor PBL after immunization with implanted allogeneic skin (standard microlymphocytotoxicity test). Animal K85. A booster immunization was given on day 40.
Figure 4.4  Cytotoxic antibody response against donor PBL after immunization with implanted allogeneic skin (standard microlymphocytotoxicity test). Animal K107. No booster immunization was given. Animal L29. A booster immunization was given on day 29.
decrease in titres observed after immunizations are shown in Figures 4.1, 4.2, 4.3 and 4.4.

4.3.3 Platelet preparation

The average yield of platelets from fresh blood was $0.91 \times 10^{10}/100$ ml of blood, with a range of $0.34-1.88 \times 10^{10}/100$ ml ($n = 78$). Leucocyte contamination varied from one leucocyte per $10^7$ platelets to one per $10^5$ platelets. It was important to use freshly-collected blood for platelet preparation; leucocyte contamination of platelets prepared from blood which had been collected 24 hr previously was unacceptably high (in excess of one per $10^5$ platelets).

4.3.4 Complete platelet absorptions

Large numbers of platelets were required when antisera were subjected to complete platelet absorption (Table 4.3) and the absorption often had to be repeated several times (up to 5 times). Formaldehyde fixation did not alter the absorptive capacity of platelets. Platelets which had not been fixed were found to clump more than fixed platelets when mixed with the antisera during the absorption. In addition, antisera absorbed with unfixed platelets were noticeably cloudier than antisera absorbed with fixed platelets, even after centrifugation. Reused platelets did not have the absorptive capacity of fresh platelets, although their use achieved some reduction in the cytotoxic titre against PBL. Therefore, all subsequent absorptions were done with fresh platelets.

A relatively high number of platelets was required to complete the absorption of an antiserum once the titre had been reduced to
<table>
<thead>
<tr>
<th>Titre of Antiserum a</th>
<th>Number of Antisera Absorbed</th>
<th>Platelet Number Used b</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>2</td>
<td>9 - 12</td>
</tr>
<tr>
<td>32</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>64</td>
<td>3</td>
<td>9 - 36</td>
</tr>
<tr>
<td>128</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>256</td>
<td>4</td>
<td>11 - 30</td>
</tr>
<tr>
<td>≥ 512</td>
<td>2</td>
<td>12 - 23</td>
</tr>
</tbody>
</table>

a = against donor PBL in the standard microlymphocytotoxicity test.

b = platelet numbers x 10^10 per ml of antiserum absorbed (fresh platelets only).
about 8 or less. Three of the more extreme examples of this finding are shown in Figure 4.5.

4.3.5 Partial platelet absorptions

Separate samples of 23 antisera were subjected to partial and complete platelet absorption. After partial absorption, 13 antisera displayed anti-class II type activity. For these 13 antisera, the average number of doubling dilutions which displayed anti-class II type activity was 2.8. After complete absorption, 10 antisera displayed anti-class II type activity, and the average number of doubling dilutions displaying this activity was 1.5.

All subsequent platelet absorptions were partial rather than complete. Table 4.4 shows the reactions of 22 partially absorbed antisera in an extended microlymphocytotoxicity test against donor B(+) and B(-) cells. These are the best results obtained for each antiserum, in terms of anti-class II type activity. For most of the antisera, a number of separate absorptions were done, using a range of platelet numbers. In some cases it was possible to obtain a more useful reagent by slightly varying the platelet number used for the absorption. A number of antisera did not appear to have any useful anti-class II type activity after an initial absorption, and in most such cases, anti-class II type activity could not be revealed by varying the number of platelets used for absorption. Absorption of lower titre antisera (titre ≤32 against donor PBL) compared favourably with absorption of higher titre antisera (titre ≥64) from the same immunization in terms of anti-class II type activity produced, and required fewer platelets.
Figure 4.5 Complete platelet absorption: the decrease in cytotoxic antibody titres against donor PBL observed after absorption of alloantisera with donor platelets. Three examples of absorption carried out on antisera which had high cytotoxic titres against donor PBL (standard microlymphocytotoxicity test).

Figure 4.5(a) Antiserum G12.
Figure 4.5(b)  antiserum G25.
Platelet absorption. 

Figure 4.5(c) antiserum K30.
Table 4.4  Partial platelet absorptions: reactions of absorbed antisera in an extended microlymphocytotoxicity test against donor B(+) and B(-) cells. Reactions are shown only at dilutions which displayed anti-class II type activity; reactions of <50% cell death against B(+) cells are not shown.

<table>
<thead>
<tr>
<th>Absorbed Antiserum</th>
<th>Cell Panel</th>
<th>Doubling Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>K9</td>
<td>B+</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>L30</td>
<td>B+</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>L36</td>
<td>B+</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>K84</td>
<td>B+</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>L22</td>
<td>B+</td>
<td>9</td>
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<tr>
<td></td>
<td>B-</td>
<td>4</td>
</tr>
<tr>
<td>H6</td>
<td>B+</td>
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<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>G30</td>
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<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>H28</td>
<td>B+</td>
<td>/</td>
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<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>K19</td>
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<td></td>
<td>B-</td>
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<tr>
<td>L2</td>
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<td></td>
<td>B-</td>
<td>4</td>
</tr>
<tr>
<td>372</td>
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<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>H11</td>
<td>B+</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
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</tr>
</tbody>
</table>
Table 4.4 (continued).

<table>
<thead>
<tr>
<th>Absorbed Antiserum</th>
<th>Cell Panel</th>
<th>Doubling Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
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<tr>
<td>H32</td>
<td>B+</td>
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</tr>
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<td>K83</td>
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<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>K107</td>
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</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>K109</td>
<td>B+</td>
<td>/</td>
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<td></td>
<td>B-</td>
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<tr>
<td>L28</td>
<td>B+</td>
<td>/</td>
</tr>
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<td></td>
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<td>L31</td>
<td>B+</td>
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<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>H19</td>
<td>B+</td>
<td>/</td>
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<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>L3</td>
<td>B+</td>
<td>/</td>
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<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>L12</td>
<td>B+</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
<tr>
<td>L34</td>
<td>B+</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>B-</td>
<td>/</td>
</tr>
</tbody>
</table>

/ = the antiserum was not sufficiently absorbed at this dilution; i.e. gave a reaction of >50% cell death against B(-) cells.
Nine alloantisera with a titre of 128 in the standard microlymphocytotoxicity test against donor PBL were absorbed. The platelet number required to reduce the titre to 4 ranged from 6-16 x 10^{10}/ml of antiserum. Four antisera with a titre of 64 were absorbed; the platelet number required to reduce the titre to 4 ranged from 4-9 x 10^{10}/ml of antiserum. Twenty-one antisera with a titre of 32 were absorbed. The platelet number required to reduce the titre to 4 ranged from 2-6 x 10^{10}/ml of antiserum, and the platelet number required to reduce the titre to 2 ranged from 2-11 x 10^{10}/ml of antiserum. As was the case when complete absorptions were done, the absorption of residual cytotoxic activity against donor PBL became increasingly difficult once the titre of an antiserum had been reduced to about 4 or 8.

Six other alloantisera of low titre (1-32 in the standard microlymphocytotoxicity test against PBL), raised by leucocyte immunizations, were absorbed with appropriate class I-matched platelets (tissue donors from the alloimmunizations were not available). In some cases, platelets from more than one animal were required for the absorption. Only one of the 6 antisera displayed anti-class II type activity; this activity was weak and the antiserum was rejected as a class II typing reagent.

4.3.6 Comparison of buffy-coat leucocyte and skin implant immunization: Anti-class II type activity

The 2 methods of immunization gave similar results, both in the frequency of antisera displaying anti-class II type activity after platelet absorption, and in the average number of dilutions at which anti-class II type activity was displayed (Table 4.5). Overall, 56%
of the immunizations produced antisera containing anti-class II type activity. Within this group of potential anti-class II reagents, the number of doubling dilutions displaying anti-class II type activity ranged from 1-5, with an average value of 2.7.

Table 4.5  Partial platelet absorptions: Comparison of anti-class II type activity in antisera produced by buffy-coat leucocyte immunization and skin implant immunization.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Number of Antisera</th>
<th>Percentage of Antisera Displaying Anti-class II Type Activity</th>
<th>Average Number of Doubling Dilutions Displaying Anti-class II Type Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffy-coat Leucocytes</td>
<td>15</td>
<td>53%</td>
<td>3.0</td>
</tr>
<tr>
<td>Skin</td>
<td>26</td>
<td>58%</td>
<td>2.5</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>56%</td>
<td>2.7</td>
</tr>
</tbody>
</table>
4.3.7 Parous sera

Of the 240 parous sera screened against PBL, 130 were selected and screened against B(+) and B(-) cells. Five of these antisera were further selected on the basis of giving preferential reactions against B(+) cells, but were subsequently rejected as anti-class II reagents either because of poor repeatability or significant residual reactivity with B(-) cells. After absorption with appropriate platelets, no anti-class II type activity was displayed.

4.4 DISCUSSION

The yield of platelets in these experiments compared favourably with yields of human platelets reported when low levels of leucocyte contamination were specified (Bialek et al., 1966; Svejgaard & Kissmeyer-Nielsen, 1968). Leucocyte contamination could contribute to loss of anti-class II activity from platelet-absorbed antisera. The maximum level of leucocyte contamination which I allowed (one per $10^5$ platelets) was based on the strictest guideline that I was able to find in the literature (Prou et al., 1980).

Newman et al. (1982a) found that fixing bovine platelets in 1% formaldehyde made them more durable during absorption and prevented the occasional serum from clotting after absorption. In my experiments, fixing did not adversely affect the absorptive capacity of platelets. The cloudy appearance of antisera after absorption with unfixed platelets may have been due to platelet debris or immune complexes.

When complete platelet absorptions were done, the platelet numbers used were often higher than reported elsewhere for cattle and
other species (in cases where specific rather than pooled platelets were used) (Colombani et al., 1976; Shinohara et al., 1977; Belvedere et al., 1978; Guimezanes et al., 1979; Newman et al., 1982a). Even when partial absorptions were done in the present experiments, the platelet numbers required to reduce cytotoxic titres to 2-4 were in some cases higher than reported elsewhere. It is possible that the expression of class I antigens on platelets varies between species, with expression on bovine platelets being relatively weak. Within a species, variation may exist. In the HLA system for example, certain class I antigens appear to vary in their expression on the surface of platelets, while some class I antigens are poorly expressed on platelets (Aster et al., 1977; Mueller-Eckhardt et al., 1980).

The greatest difficulty with platelet absorption was in removing residual cytotoxic activity against PBL, once absorption had reduced the titre to about 8 or less. This is not what one would expect from a simple consideration of the relationship between the endpoint titre of an antiserum and the amount of antibody activity in that antiserum. It may be that the antisera contained some non-MHC lymphocytotoxic antibodies, which recognized products present on the surface of lymphocytes but absent from the surface of platelets. A variety of non-HLA lymphocytotoxic antibodies with specificities for red blood cell ABH and Lewis antigens have been described (Curtoni et al., 1980; Oriol et al., 1980; Hudelson et al., 1981; Nielsen et al., 1983). Alternatively, residual lymphocytotoxic antibodies may have been of low affinity and therefore not bound by platelets (Shulman et al., 1964), but these antibodies may have reacted with lymphocytes in a subsequent cytotoxic test.
The presence of anti-RBC lymphocytotoxic antibodies could be monitored by RBC absorptions. A platelet absorption step at 37°C may be needed to improve the efficiency of absorption of anti-class I antibodies. However, the experiments of Heinrich et al. (1974) indicated that within the range of 10-37°C, the temperature at which platelet absorption is done is not critical.

Partially absorbed antisera displayed cytotoxic activity against B(-) cells at lower dilutions. Therefore, the concept of endpoint titre of anti-class II type activity had no meaning. I have referred to the number of doubling dilutions, beyond the endpoint titre against B(-) cells, at which an antiserum displayed anti-class II type activity. In principle, the use of high dilutions of partially absorbed antisera as anti-class II reagents is no different to the use of antisera which have been "completely" absorbed. In both cases, reagents are used at a specific dilution, at which anti-class II activity is present but cytotoxic activity against B(-) lymphocytes is undetectable. "Completely" absorbed antisera almost certainly still contain anti-class I antibodies, but at an undetectable concentration when the antiserum is tested undiluted. Potential anti-class II reagents reported in this thesis were always screened at a number of dilutions, against the B(+) and B(-) cells of all animals tested. This was done to ensure that dilutions described as displaying anti-class II type activity showed no evidence of reactivity with B(-) cells from any animals.

Non-specific loss of anti-class II activity may occur with exhaustive platelet absorption. This may explain why more anti-class II type activity was evident after partial platelet absorptions than after complete platelet absorptions. For partially absorbed antisera,
the number of doubling dilutions displaying anti-class II activity compared favourably with other results reported in cattle (Newman, 1981) and in man (Belvedere et al., 1978). Stronger anti-class II reagents have been reported after platelet absorption of mouse and rat alloantisera (Colombani et al., 1976; Shinohara et al., 1977; Guimezanes et al., 1979; Ohhashi et al., 1981).

Antisera of very high cytotoxic titre against PBL may not be the most useful source of anti-class II reagents. Such antisera may contain proportionately more contaminating antibody activity which is difficult to remove by platelet absorption. This may explain why absorption of antisera with a cytotoxic titre of $\leq 32$ compared favourably with absorption of higher titre antisera in the present experiments.

In the production of alloantisera, the skin implant method has some advantages over skin grafting. Less surgical skill is required, the skin does not need to be processed prior to implantation and it can be stored for up to 2 weeks and transported without special handling and without loss of immunogenicity (Pringnitz et al., 1982). In the present experiments, all skin implant recipients responded with lymphocytotoxic antibody to the primary immunization, compared with a 79% response reported by Pringnitz et al. (1982), and 62% and 100% responses to primary skin grafting in cattle reported by Spooner et al. (1979b) and Newman & Stear (1983) respectively. The peak titres obtained after the initial skin implant were generally lower than those reported by Pringnitz et al. (1982) and Newman & Stear (1983), possibly due to differences in the size of the piece of skin transferred. The timing of the peak antibody titres and the pattern of antibody decay after skin implant immunization were similar to
those reported elsewhere for cattle after both skin implant immunization and skin grafting (Newman, 1981; Pringnitz et al., 1982; Newman & Stear, 1983).

The antibody response after buffy-coat leucocyte immunizations and skin implant immunizations varied widely between individuals. No attempt was made to compare the 2 immunization methods statistically, as the sample sizes were small and individual responses may depend on the combinations of donor and recipient MHC types (which were not similar in the 2 groups of immunizations). There appears to be no simple explanation as to why haplotype-matched antisera were of generally lower titre than mismatched antisera. The difference may have been due to the particular MHC antigens possessed by donors and recipients in individual immunizations. Immune response gene effects have been observed in the antibody response of mice to MHC antigens (Stimpfling & Durham, 1972).

Fewer successive skin implant immunizations were required to produce antisera with cytotoxic titres comparable with those observed after buffy-coat leucocyte immunizations. Both methods produced very similar results in terms of anti-class II type activity. However, the skin implant method has the advantage of being less time-consuming per immunization.

In the production of anti-class II antibodies, skin has been widely used as an immunogen. Studies in mice, Rhesus monkeys and cattle have suggested that skin is a more effective class II immunogen than leucocytes or lymphocytes (Gotze, 1976; Roger et al., 1980; Newman & Stear, 1983). Gunther et al. (1978) found no difference between these immunogens in the production of rat anti-class II alloantibodies. There may be a greater concentration of class II
antigen in skin, possibly due to epidermal Langerhans cells which are class II-positive, at least in man, in the mouse and in the guinea pig (Klæreskog et al., 1977; Rowden et al., 1977; 1978; Stingl et al., 1978b).

No anti-class II reagents were produced from the parous sera. There are a number of drawbacks to using parous sera. Firstly, they are generally weak, which may explain the problems with repeatability when using parous sera in the present experiments. Secondly, for the absorption of parous sera, it is often difficult to obtain platelets from the specific father(s) of the relevant offspring. On a similar note, no anti-class II reagents were produced from the 6 other sera absorbed with non-donor platelets in the present experiments. All platelet absorptions, other than those done with these 6 antisera and the parous sera, were done with platelets from the tissue donor of the relevant alloimmunization. The aim was to ensure that those dilutions of an absorbed antiserum which displayed anti-class II type activity would not display significant anti-class I activity when tested against a wider panel of animals. A third disadvantage of parous sera is the complexity of these sera when they are obtained from multiparous females, especially if there have been a number of different fathers (as is often the case with cattle).

When raising anti-class II antibodies, it may be possible to reduce or eliminate the need to absorb alloantisera with platelets by immunizing between individuals which share class I antigens, but which differ in their class II regions. The class II differences could be detected by mixed lymphocyte reactivity (Albrechtsen et al., 1977), or by differences in restriction fragment length polymorphisms revealed using class II DNA probes. Davies & Antczak (1987) have reported the
production of anti-class II alloantisera in cattle by immunizing between animals which were class I compatible but which differed in their mixed lymphocyte reactions.

The percentage of lymphocytotoxic alloantisera which displayed anti-class II type activity in the present experiments was 56%. This compares favourably with one other result reported in cattle, where 41% of antisera displayed anti-class II activity (Newman et al., 1982a). Percentages reported in mice and humans have ranged from 30-80% (Terasaki et al., 1975; Colombani et al., 1976; Decary et al., 1979; Vassalli et al., 1979; Borelli et al., 1982), with the occasional report of a higher percentage (Belvedere et al., 1978).
Monoclonal antibodies have some advantages over alloantisera in the definition of MCA class II antigens. They are uncontaminated by anti-class I antibodies and are more specific, whereas alloantisera often contain a mixture of antibody populations. Monoclonal alloantibodies have identified new MCA class II specificities previously defined by alloantisera. They have also been useful in identifying new specificities, some of which are subdivisions of specificities defined by alloantisera (Pohler, J. et al., 1984).

Many cases of species reactivity of anti-class II monoclonal antibodies appear to involve structurally or broadly polymorphic determinants (Van Noorden & Barlow, 1987; Delius et al., 1987). Some monoclonal antibodies which react with inbred lymphocytes (Van Noorden & Barlow, 1987; Delius et al., 1987) also react with bovine lymphocytes (Barlow & Pohler, 1984) and with cells in culture (Barlow & Pohler, 1984; Delius et al., 1987). Other monoclonal antibodies which recognize a narrowly polymorphic MCA class II antigen have been reported to react in a polymorphic fashion in culture (Barlow & Pohler, 1984). The production of a number of anti-class II monoclonal antibodies and polyclonal alloantisera in antigen class II antigens has been reported (Heuvel et al., 1987; Lewis et al., 1987; Lewis et al., 1987).

In the experiments reported in this chapter, a series of monoclonal antibodies were used. The aim of the experiments was to identify positive control alloantisera for the detection of
5.1 INTRODUCTION

Monoclonal antibodies have some advantages over alloantisera in the definition of MHC class II antigens. They are uncontaminated by anti-class I antibodies and are monospecific, whereas alloantisera often contain a mixture of antibody populations. Monoclonal xenoantibodies have identified some HLA class II specificities previously defined by alloantisera. They have also been useful in identifying new specificities, some of which are subdivisions of specificities defined by alloantisera (Bodmer, J. et al., 1984).

Many cases of species cross-reactivity of anti-MHC monoclonal antibodies appear to involve monomorphic or broadly polymorphic determinants (Brodsky & Parham, 1982; Teillaud et al., 1982). Some monoclonal antibodies which recognize monomorphic HLA class II determinants have reacted in a monomorphic fashion with cattle lymphocytes (Brodsky et al., 1981; Teillaud et al., 1982; Chardon et al., 1983), while others have been reported to react in a broadly polymorphic fashion with cattle lymphocytes (Spooner & Ferrone, 1984; Lewin et al., 1985). One monoclonal antibody which recognizes a broadly polymorphic HLA class II antigen has been reported to cross-react in a polymorphic fashion in cattle (Brodsky et al., 1981). The production of a number of other monoclonal antibodies recognizing bovine class II molecules has been reported (Letesson et al., 1983; Lalor et al., 1986; Davis et al., 1987; Emery et al., 1987).

In the experiments reported in this chapter, a number of monoclonal antibodies were studied. The aims of the experiments were twofold: to identify positive control antibodies for the detection of
class II-bearing cells of cattle; and to search for antibodies detecting bovine class II polymorphisms.

5.2 EXPERIMENTAL METHODS

Monoclonal antibodies HIA-D.m1 (Batch No. 421201) and HIA-D.m2 (Batch No. 522002) were a gift from Australian Monoclonal Development Pty. Ltd. (Sydney, Australia). Antibody HIA-D.m1, (also known as B5.1) appears to recognize a monomorphic determinant on HIA class II molecules (Sparrow & McKenzie, 1983). Antibody HIA-D.m2 (also known as MC-26.1) has been described as recognizing a broadly polymorphic determinant on the molecules carrying the HLA-MT3 and MB3 determinants (now known as DRw53 and Dw3 respectively) (Thompson et al., 1983).

Monoclonal antibodies B2VAL7C7 and 44C10 were donated by Dr J.-J. Letesson (Unité de Microbiologie, Facultés Universitaires N.-D. de la Paix, Namur, Belgium). The procedures for producing and cloning the hybridomas have been described (Letesson et al., 1983). Antibody 44C10 reacts with the majority of cells in bovine B-enriched lymphocyte preparations, and reacts strongly in an ELISA assay with purified class II molecules (Dr J.-J. Letesson, personal communication).

Monoclonal antibodies H34A, H42A, TH4B, TH14B, TH21A and TH81A5 were donated by Dr W.C. Davis (Department of Veterinary Microbiology & Pathology, Washington State University, Pullman, WA., U.S.A.). The procedures for producing and cloning the hybridomas have been described (Davis et al., 1983; 1987). It has been suggested that these antibodies recognize bovine class II determinants (Lewin et al., 1985; Carter et al., 1986; Davis et al., 1987).
Table 5.1 shows the class, subclass and form of each antibody. Initially, antibody HLA-D.m1 was evaluated as a positive control anti-class II reagent. It was screened in an immunofluorescence assay against peripheral blood lymphocytes (PBL) from cattle; the second-step reagent was fluorescein-conjugated sheep F(ab')2 anti-mouse Ig (New England Nuclear, Lot No. FPD-199, Boston, MA., U.S.A.).

All antibodies were screened in an extended microlymphocytotoxicity test against B-enriched (B+) and B-depleted (B-) cells. The number of animals of each breed against which the antibodies were tested is shown in Table 5.1. All of the BoLA class I antigens that could be detected by the Canberra antisera were represented in the cattle screened. Background cell death in the cytotoxic test was generally <10%, although occasionally it was as high as 30-40%. Antibodies HLA-D.m1, H34A, H42A, TH4B, TH14B, TH21A and TH81A5 were titrated at doubling dilutions from undiluted to 1/128,000. Antibody HLA-D.m2 was titrated at doubling dilutions from 1/80 - 1/80,000. Antibodies B2VAL7C7 and 44C10 were titrated at trebling dilutions from 1/5 - 1/1,215. The endpoint titre of an antibody was defined as the reciprocal of the highest dilution displaying at least 80% of the maximum cytotoxic reaction of that antibody. The problem of carry-over was addressed by strategically inserting negative control wells (containing non-reactive bovine serum) between antibody-containing wells on the test trays.

Immunoprecipitation studies were done with antibody TH81A5, as described in Section 2.5.
Table 5.1  Monoclonal antibodies tested against B(+) and B(-) cells in the extended microlymphocytotoxicity test.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Class &amp; subclass</th>
<th>Form</th>
<th>Charolais</th>
<th>Friesian</th>
<th>Jersey</th>
<th>Brahman</th>
<th>Angus</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-D.m1</td>
<td>IgG2b</td>
<td>P</td>
<td>88</td>
<td>11</td>
<td>10</td>
<td>4</td>
<td>1</td>
<td>114</td>
</tr>
<tr>
<td>B2VAI7C7</td>
<td>IgG1</td>
<td>A</td>
<td>63</td>
<td>11</td>
<td>10</td>
<td>4</td>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td>44C10</td>
<td>IgG2a</td>
<td>A</td>
<td>63</td>
<td>11</td>
<td>10</td>
<td>4</td>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td>H34A</td>
<td>IgG2b</td>
<td>S</td>
<td>32</td>
<td>11</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>57</td>
</tr>
<tr>
<td>H42A</td>
<td>IgG2a</td>
<td>A</td>
<td>32</td>
<td>11</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>57</td>
</tr>
<tr>
<td>TH4B</td>
<td>IgM</td>
<td>S</td>
<td>32</td>
<td>11</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>57</td>
</tr>
<tr>
<td>TH14B</td>
<td>IgG2a</td>
<td>S</td>
<td>32</td>
<td>11</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>57</td>
</tr>
<tr>
<td>TH21A</td>
<td>IgG2b</td>
<td>A</td>
<td>32</td>
<td>11</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>57</td>
</tr>
<tr>
<td>TH81A5</td>
<td>IgG2a</td>
<td>A</td>
<td>116</td>
<td>11</td>
<td>10</td>
<td>4</td>
<td>1</td>
<td>142</td>
</tr>
</tbody>
</table>

* = the Charolais content of these animals varied from 1/2 to 31/32, with the majority being 3/4 or more Charolais.

P = purified from ascites.
A = ascites form.
S = supernatant form.
Antibody HIA-D.ml reacted with lymphocytes from all cattle against which it was tested in both immunofluorescence and cytotoxic assays (114 animals in total). In the cytotoxic test against B(+) cell preparations, HIA-D.ml always reacted with $\geq 80\%$, and often with $\geq 90\%$, of the cells. In B(-) cell preparations, it generally reacted with $<10\%$ of the cells, though the occasional B(-) cell preparation contained as many as 20–30% positive cells (above background).

Antibody TH81A5 reacted with lymphocytes from all cattle against which it was tested in the cytotoxic assay (142 animals). This antibody immunoprecipitated 2 polypeptide chains from the surface of bovine lymphocytes (Figure 5.1). Three separate determinations of the molecular weights were made; on each occasion, the molecular weights of the 2 polypeptide chains immunoprecipitated were approximately 34,000 and 31,000 daltons.

Antibodies HIA-D.ml and TH81A5 were subsequently used as positive control antibodies to detect class II-bearing cells of cattle.

The 2 Belgian antibodies (B2VAL7C7 and 44C10) and 5 of the 6 Washington State antibodies (H34A, H42A, TH14B, TH21A and TH81A5) reacted strongly in the cytotoxic test ( $\geq 80\%$ cell death) with the B(+) cell preparations of all cattle tested (Table 5.2). B(-) cell preparations generally contained $<30\%$ positive cells, though occasionally they contained 30–50% positive cells. In 3 instances, antibody H42A reacted with $\geq 50\%$ of B(-) cells. Some lack of concordance was noted between the reactions of some test antibodies and the reactions of the reference antibody HIA-D.ml with B(-) cell
Autoradiograph of SDS polyacrylamide gel electrophoresis of $^{125}\text{I}$-labelled lymphocyte surface proteins which have been solubilized in detergent and immunoprecipitated with monoclonal antibody TH81A5. The arrows point to the polypeptide chains immunoprecipitated. The position of standard molecular weight markers is indicated.
66 Kd
45 Kd
36 Kd
29 Kd
24 Kd
### Table 5.2 Monoclonal antibodies which reacted with all animals tested.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Number of Animals Tested</th>
<th>Unrelated Animals Tested</th>
<th>Discrepant Reactions (B-depleted Cells)*</th>
<th>Endpoint Titres Range</th>
<th>Median</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-D.ml</td>
<td>114</td>
<td>56</td>
<td>-</td>
<td>1,024 - 4,096</td>
<td>2,048</td>
<td>8</td>
</tr>
<tr>
<td>B2VAL7C7</td>
<td>89</td>
<td>53</td>
<td>-</td>
<td>135 - ≥1,215</td>
<td>≥1,215</td>
<td>89</td>
</tr>
<tr>
<td>44C10</td>
<td>89</td>
<td>53</td>
<td>-</td>
<td>15 - ≥1,215</td>
<td>≥1,215</td>
<td>89</td>
</tr>
<tr>
<td>H34A</td>
<td>57</td>
<td>44</td>
<td>4.4%</td>
<td>64 - 2,048</td>
<td>1,024</td>
<td>20</td>
</tr>
<tr>
<td>H42A</td>
<td>57</td>
<td>44</td>
<td>16.7%</td>
<td>8,000 - ≥128,000</td>
<td>64,000</td>
<td>20</td>
</tr>
<tr>
<td>TH14B</td>
<td>57</td>
<td>44</td>
<td>2.6%</td>
<td>64 - 1,024</td>
<td>256</td>
<td>20</td>
</tr>
<tr>
<td>TH21A</td>
<td>57</td>
<td>44</td>
<td>6.1%</td>
<td>8,000 - 64,000</td>
<td>32,000</td>
<td>20</td>
</tr>
<tr>
<td>TH81A5</td>
<td>142</td>
<td>84</td>
<td>-</td>
<td>4,096 - ≥128,000</td>
<td>64,000</td>
<td>20</td>
</tr>
</tbody>
</table>

* = percentage of reactions which differed from the reactions of antibody HLA-D.ml by ≥20% cell death.

n = number of tests (animals) on which titration data is based.
preparations. These differences were always due to stronger reactions on the part of the test antibodies. The most frequent discrepancies occurred with antibody H42A. Discrepancies of ≥20% cell death compared with the reaction of HIA-D.m1 are summarized in Table 5.2. There was no consistent grouping of particular antibodies, in terms of their discrepant reactions.

Antibody TH4B reacted strongly (≥80% cell death) with B(+) cells from 53 out of 56 cattle tested. In the case of the other 3 cattle tested, only 20-50% of B(+) cells were positive.

With most of the antibodies, the cytotoxic endpoint titres varied widely between individual animals. For each antibody, the range of endpoint titres observed and the median value are shown in Table 5.2. There was no obvious grouping of antibodies in terms of having higher or lower endpoints against particular animals. The endpoint titre of antibody TH4B varied from 1-8 (data based on the results from 20 animals). The endpoint titre of some of these antibodies (especially H42A, TH21A and TH81A5) were not sharply defined against the cells of some animals, and antibody activity dropped off slowly on titration. An example is shown in Figure 5.2.

The cytotoxic endpoint titre of antibody HIA-D.m2 ranged from 160-10,000. When screened at a dilution of 1/80 against cells from 111 cattle, it reacted strongly (≥80% cell death) against B(+) cells from 58.6% of animals, while 27.0% of B(+) reactions were of <30% cell death. However, there was a substantial number (14.4%) of partial reactions of 30-80% cell death. In most B(-) cell preparations, <10% of cells were positive, although occasional reactions as high as 30% were noted. This antibody was not screened at concentrations greater than 1/80.
Figure 5.2

Titration of monoclonal antibody TH81A5 against B-enriched lymphocytes from animal G37 (extended microlymphocytotoxicity test).
<table>
<thead>
<tr>
<th>Cytotoxic Score</th>
<th>Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1 2 5 0 0 0 0 0 0</td>
</tr>
<tr>
<td>8</td>
<td>1 2 4 8 6 2 4 8 1</td>
</tr>
<tr>
<td>6</td>
<td>1 3 6 2 4 8 1 2</td>
</tr>
<tr>
<td>4</td>
<td>3 5 1 2 4 9 0 0 0</td>
</tr>
<tr>
<td>2</td>
<td>8 6 2 4 8 6 0 0 0</td>
</tr>
<tr>
<td>1</td>
<td>0 0 0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>
5.4 DISCUSSION

The heavier polypeptide chain immunoprecipitated by antibody TH81A5 corresponds to the molecular weight of the α chain of mouse and human class II molecules (using reducing conditions) (Kaufman et al., 1984). The position of the lighter chain on the autoradiograph suggested that TH81A5 may react with a class II molecule with a β chain of approximate molecular weight 31,000 daltons, rather than the classically described 25,000-30,000 daltons. The molecular weights observed can be regarded only as approximations, as the polypeptide chains appeared as diffuse bands on the autoradiograph. The diffuse nature of these 2 bands may be due to the presence of a number of partially resolved polypeptide chains within each band. It is unlikely that the 31,000 dalton chain represented co-precipitation of the bovine equivalent of the cytoplasmic class II invariant chain of other species (Jones et al., 1979; Charron & McDevitt, 1980), as lymphocytes were labelled externally by iodination and there were few dead cells present. In other studies of bovine class II molecules using reducing conditions, the β chains immunoprecipitated have been of molecular weights 27,000-28,000 daltons (Hoang-Xuan et al., 1982a; Chardon et al., 1983; Letesson et al., 1983).

The suggestion that antibody TH81A5 recognizes a bovine class II molecule was supported by the preferential B cell reactivity of this antibody, and by the concordance of these reactions with the reactions of the reference anti-class II antibody, HLA-D.m1. Immunoprecipitation studies reported elsewhere have indicated that antibodies HLA-D.m1 (Sparrow & McKenzie, 1983), HLA-D.m2 (Thompson et al., 1983), H42A and TH14B (Carter et al., 1986; Davis et al., 1987),
TH21A (Dr W.C. Davis, personal communication), and B2VAL7C7 (Letesson et al., 1983) all recognize class II molecules. The preferential B cell reactivity of these antibodies observed in the present experiments supported this suggestion of anti-class II activity. The preferential B cell reactivity of antibodies H34A and 44C10 suggested that they also react with class II molecules, but biochemical studies will be needed to confirm this.

The wide variation observed in the endpoint titre of some of the test antibodies may have been due to quantitative variation in antigen density on the cell surface. Alternative explanations include steric hindrance effects mediated by adjacent cell-surface determinants, and low affinity cross-reactions with structurally related determinants. The variation in endpoint titres demonstrates the need to screen an unknown antibody at a number of dilutions before concluding that it recognizes a polymorphic determinant.

Antibody B2VAL7C7 appeared to react with a broadly polymorphic bovine class II determinant when tested in a radioimmunoassay (Letesson et al., 1983). Antibody 44C10 reacted in a similar fashion (Dr J.-J. Letesson, personal communication). However, in the experiments reported in this thesis, both antibodies reacted in a monomorphic fashion with B(+) cells in the cytotoxic assay. A similar discrepancy exists between the results reported in this thesis for antibody TH21A and the results of Lewin et al. (1985), who reported heterogeneity of expression of the determinant recognized by TH21A in a cytotoxic test.

There are a number of possible explanations for these discrepancies. Firstly, the antibodies may recognize broadly polymorphic determinants which were possessed by all cattle in the
population which I tested. It will be necessary to test more animals of more breeds to clarify the activity of antibodies B2VAL7C7, 44C10, and TH21A, and to confirm the apparent monomorphic activity of antibodies HIA-D.m1, H34A, H42A, TH14B and TH81A5. Secondly, different assays may differ in the binding affinity required to detect the binding of a monoclonal antibody to a class II determinant. Thirdly, different forms of the antibodies might have been used in the various population studies. Since endpoint titres may vary widely between individual animals, a particular form or dilution of an antibody may be sufficiently weak for some animals to appear negative (as may have been the case with antibody TH4B in the present experiments).

Although antibody HIA-D.m2 has been described as recognizing a polymorphic HIA class II determinant, its reactions against human B cells are not always clearly positive or negative (Thompson et al., 1983). Its partial reactions against cattle B(+) cells at a dilution of 1/80 suggested that it could react in a monomorphic fashion in cattle, if tested at a high enough concentration. For this reason no class II antigen was defined with this antibody.

The tendency of the test antibodies to recognize monomorphic or broadly polymorphic class II determinants is not surprising, in view of the findings with anti-HLA class II monoclonal antibodies (Guy & van Heyningen, 1983; McKenzie & Zola, 1983; Beckman, 1984). This may be a consequence of immunizing across a species barrier in the production of these monoclonal antibodies. The production of a monoclonal alloantibody recognizing an HLA class II determinant has been reported (Effros et al., 1986). The antibody was produced by taking B lymphocytes from an alloimmunized human, transforming the
cells in vitro with Epstein-Barr virus, and fusing the transformed cells with a human myeloma cell line.

Sequential absorption and immunoprecipitation studies with antibodies H42A, TH14B and TH81A5 have suggested that separate class II molecules exist in cattle (Carter et al., 1986) as in other species (Kaufman et al., 1984). DNA hybridization studies have also suggested the existence of at least 2 class II subregions in cattle (Andersson et al., 1986a; 1986b). This might explain the substantial number of stronger B(-) cell reactions of antibody H42A when compared with the reference antibody HLA-D.m1 or antibody TH81A5. If there are multiple class II molecules in cattle, then the gene products recognized by H42A on the one hand and HLA-D.m1 and TH81A5 on the other hand do not appear to be distributed identically on B(-) cells (consisting of peripheral blood lymphocytes and most probably some monocytes). In the mouse and in man, the different class II subregion products are not distributed identically on cells of the monocyte/macrophage lineage (Cowling et al., 1978; Gorwa et al., 1983; Nunez et al., 1984).
CHAPTER 6

DEFINITION OF BOVINE CLASS II ANTIGENS
6.1 INTRODUCTION

The class II alloantigen system of the MHC which is readily detected on B lymphocytes, has been described in a number of species, including the mouse (David, 1976), man (Bodmer, J., 1978a), the guinea pig (Geczy et al., 1975), the rat (Radka et al., 1977; Gunther et al., 1978), the pig (Vaiman et al., 1975, cited by Ivanyi, 1977), the Rhesus monkey (Roger et al., 1976), the chicken (Ewert et al., 1980), and the rabbit (Knight et al., 1980). In the various species, the genes coding for these antigens have been shown to be linked to the MHC and associated with mixed-lymphocyte reactivity. There is also evidence of a class II alloantigen system in the dog (Krumbacher et al., 1986). In cattle, a class II alloantigen system has been described and appears to be linked with the BoLA class I system (Newman et al., 1982a).

In this thesis, the definition of the HIA system has been taken as a model for the serological definition of an alloantigen system in an outbred species. There are essentially 2 stages in the definition of an antigen system (Bodmer & Payne, 1965). The first stage is the resolution of individual antigens, while the second stage is the elucidation of the mode of inheritance of antigens and the elucidation of the relationships between antigens and with other antigen systems.

Because alloantisera are generally polyspecific rather than monospecific, 2 basic approaches have been adopted when using alloantisera to define individual antigens. One approach is to try and change a complex alloantiserum into a monospecific typing reagent (or at least a reagent of narrower specificity). This can be accomplished in a number of ways, including: (a) absorptions, using
positively reacting cells; (b) elution of antibodies from absorbing cells; (c) titrating antisera and using them at appropriate dilutions, thereby diluting out unwanted antibody activity; and (d) immunizing between individuals which are matched for one MHC haplotype or for some MHC antigens.

Absorptions have been used extensively in the definition of RBC antigens, and to a lesser extent in the definition of MHC antigens of outbred species. The aim is to produce a reagent specific for an individual antigen by removing other antibodies in the antisemum. In a cross-absorption, portions of an antisemum are each absorbed with one of the positively-reacting cell panels, and then re-tested. Problems can occur if all of the cells used for a cross-absorption carry all of the antigens detected by a particular antisemum, or if all test cells lack one of the antigens detected by the antisemum (Bodmer & Payne, 1965).

The likelihood of these problems occurring will be reduced if a large number of cell panels is used in the cross-absorption. Walford & Troup (1967) introduced the concept of "operational monospecificity" to describe an antisemum in which no more than one antibody specificity was detected during 29 or more absorptions with positively-reacting cells. There would then be a 95% confidence that the antisemum is either monospecific or detects antigenic factors showing more than 90% association. Such a standard is very difficult to achieve in practice, and seems to be rarely applied in the definition of HLA antigens. In any case, a more important criterion in evaluating monospecificity may be the number of cell donors against which absorbed antisera are tested, rather than the number of cells used for absorption (Bodmer & Payne, 1965). The risk that all cells
used for absorption carry all of the antigens detected by a particular antiserum will be increased if all cells come from the one population. For this reason, the use of heterogeneous cell panels drawn from different populations has been recommended when screening antisera (Dausset et al., 1965).

An alternative approach in the definition of antigens is to use groups of polyspecific antisera, which share particular antibody specificities. Antisera are first screened against a large panel of cells. The reactions of each antiserum are statistically compared with the reactions of each other antiserum, using 2 x 2 contingency tables (van Rood & van Leeuwen, 1963; Bodmer & Payne, 1965; Dausset et al., 1965). A significant association between 2 antisera may be due to shared antibodies. Alternatively, the antisera may contain antibodies directed against antigens which are associated in the population (Dausset et al., 1965; Bodmer et al., 1969). The latter possibility may be tentatively excluded after testing antisera against heterogeneous cell panels drawn from different populations, and by absorption analysis.

Because most alloantisera are not truly monospecific (even after absorptions and titration), the concordance between antisera is rarely perfect. Therefore, antigens are generally defined by clusters of antisera rather than by a single antiserum.

In practice, both absorption analysis and association analysis have been used in the definition of MHC antigens. Association analysis should ultimately be confirmed by absorption analysis (Bodmer et al., 1969).

In the experiments reported in this chapter, candidate class II typing antisera were prepared from platelet-absorbed alloantisera by
absorption analysis and titration analysis. The candidate typing antisera were screened against B-enriched (B+) and B-depleted (B-) lymphocytes from a large number of cattle, which included both unrelated animals and family members. Class II antigens were tentatively defined, and their inheritance was confirmed by family analysis. The accumulated evidence that MHC class II antigens were being detected is presented in Chapter 9.

6.2 EXPERIMENTAL METHODS

Platelet-absorbed alloantisera were screened against B(+) and B(-) lymphocytes from a group of 20 cattle. Selected antisera were cross-absorbed with buffy-coat leucocytes from positively-reacting animals. The presence of B lymphocytes in the buffy coat leucocyte preparations made it possible to absorb the anti-class II type activity. Each portion of antiserum was absorbed with one of the positively reacting cell preparations and was subsequently re-tested against all of the positively-reacting cell preparations.

Candidate typing antisera included antisera absorbed with platelets only, and antisera sequentially absorbed with platelets and leucocytes. These antisera were screened against B(+) and B(-) lymphocytes from 86 cattle. Selected typing antisera were then screened against a further 31 cattle. Anti-class II type activity is defined in Section 4.2.2. Alloantisera were tested at 3-5 doubling dilutions. The working dilution subsequently chosen in the definition of antigens was either 1 or 2 doubling dilutions before the endpoint.

The 117 cattle tested were from 4 separate herds; 115 were of the Charolais breed or were Charolais-cross (the Charolais content of
these animals varied from 1/2 to 31/32, with the majority being 3/4 or more Charolais). Fifty-eight of the Charolais cattle tested did not share any parents, and were treated as an independent panel of animals in the statistical analysis of associations between antisera. These 58 cattle included 48 animals which did not share any grandparents, and 10 animals which shared a single grandparent with one of the above 48 animals.

Family members were tested to study the inheritance of antigens. The 117 cattle tested included one large paternal half-sibling family, 2 smaller paternal half-sibling families and a number of maternal half-sibling families. Parentages of many (though not all) of the cattle within the family groups had previously been confirmed by typing for RBC antigens (courtesy of Dr K. Bell, Department of Physiology and Pharmacology, University of Queensland, St Lucia, Queensland).

Associations between antisera were examined by computer analysis of the reactions of antisera against the 58 independent panel members, using a program which calculated correlation coefficients (r) and then clustered antisera on the basis of these r values (see Section 2.13). Since \( r = \sqrt{\frac{X^2}{n}} \), when \( n = 58 \), \( r \) must be \( \geq 0.43 \) to attain a significance level of \( p \leq 0.001 \) (Bodmer et al., 1969). Because of the small number of animals tested, the 2 x 2 contingency tables of antisera reactions were also analysed using a two-tailed Fisher's exact test, as described in Section 2.13 (Klein, 1975; Snedecor & Cochran, 1980; Dr W. Bodmer, personal communication).
6.3 RESULTS

6.3.1 Buffy-coat leucocyte absorptions

Buffy-coat leucocyte absorption analysis was done on those platelet-absorbed antisera which displayed 3 or more doubling dilutions of anti-class II type activity and which reacted with more than one animal out of a group of 20.

As examples, the summarized results of 2 buffy-coat leucocyte absorption analyses (antisera K84 and G30) are shown in Tables 6.1 and 6.2 respectively. Antiserum K84 (platelet-absorbed) reacted weakly with animals 11 and 789, and strongly with 4 other animals. Absorption with leucocytes from either 11 or 789 removed reactivity to 11 and 789 only. Cross-absorptions between the other positive animals did not produce reagents of narrower specificity. This suggested that, at the working dilution, antiserum K84 (absorbed 11) and K84 (absorbed 789) were reacting with a single specificity (see specificity B below).

Anti-class II type reactions of antiserum G30 (platelet-absorbed) were split into 2 groups by absorptions with leucocytes from animals 11 and A20 respectively, which suggested that antiserum G30 recognized at least 2 specificities. This was supported by 2 other lines of evidence. Firstly, absorption of antiserum G30 (platelet-absorbed) with leucocytes from animal 71 gave similar (though not identical) results to absorption with 11, and absorption with 163 gave similar results to absorption with A20. Secondly, the activity of antiserum G30 (absorbed A20) was similar to that of antiserum L30 (platelet-absorbed), which suggested that both antisera recognized the same specificity (see antigen E below).
Table 6.1  Leucocyte absorption analysis of antiserum K84 (platelet-absorbed).

<table>
<thead>
<tr>
<th>Test Animals a</th>
<th>Antisera</th>
<th>Unabsorbed</th>
<th>Abs 11</th>
<th>Abs 789</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>850</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>H30</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>G37</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>789</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Reactions are shown at the working dilution of each antiserum.

a = those animals from which leucocytes were used for absorption.

Results are expressed as:

+ = anti-class II type reaction at this working dilution (score '9', '8' or '6' against B-enriched lymphocytes).

- = class II-negative (score '4' or less against B-enriched lymphocytes).
Table 6.2  Leucocyte absorption analysis of antiserum G30 (platelet-absorbed).

<table>
<thead>
<tr>
<th>Test Animals a</th>
<th>Antisera</th>
<th>Unabsorbed</th>
<th>Abs 11</th>
<th>Abs A20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>869</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>341</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>163</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A20</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>789</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>116</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>71</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Reactions are shown at the working dilution of each antiserum (2 doubling dilutions before the respective endpoint).

a = those animals from which leucocytes were used for absorption.

Results are expressed as:

+ = anti-class II type reaction at this working dilution (score '9', '8' or '6' against B-enriched lymphocytes).

- = class II-negative (score '4' or less against B-enriched lymphocytes).
It appears that animals 869 and 341 carried both the specificity recognized by antiserum G30 (absorbed 11) and the specificity recognized by antiserum G30 (absorbed A20). The alternative explanation is that 3 antigens were being recognized. One antigen may have been carried by animals 869 and 341, and been recognized by antiserum G30 and by its leucocyte-absorbed derivatives (i.e. activity against this antigen was not removed by absorption with leucocytes from either 11 or from A20). The second antigen was then carried by animal 11 (and removed by absorption with 11 leucocytes), and the third antigen was carried by animal A20. However, the family data (see Chapter 7) argued against the existence of 3 antigens and in favour of the existence of 2 antigens. The 2 offspring of 869 inherited opposite class I haplotypes. With regard to class II reactions, one of the offspring inherited reactivity to antiserum G30 (absorbed 11) only, and the other offspring inherited reactivity to antiserum G30 (absorbed A20) only. The sole offspring of 341 which was studied inherited reactivity to antiserum G30 (absorbed 11) only.

It was not possible to examine further the number of specificities being recognized by antiserum G30 (platelet-absorbed) by more absorptions, because only a limited quantity of the platelet-absorbed antiserum was available.

6.3.2 Selection of antisera

From the 22 platelet-absorbed antisera listed in Table 4.4, 41 candidate typing antisera were prepared. In some cases, more than one leucocyte-absorbed typing reagent was prepared from a single platelet-absorbed alloantiserum. In addition, multiple versions of some platelet-absorbed alloantisera were tested as typing reagents,
especially in the case of weak anti-class II antisera, and only the best version was selected for class II antigen definition.

After testing against 86 cattle, 13 of the candidate typing antisera were tested against a further 31 cattle. Details of the preparation of these 13 reagents are shown in Table 6.3. Table 6.4 shows the working dilution of each antiserum in relation to the strength of its anti-class II type activity. Reasons for the rejection of antisera as class II typing reagents included consistently weak anti-class II type activity, lack of information demonstrating inheritance of an antigen, and the presence of residual anti-class I activity at dilutions which had previously displayed only anti-class II type activity.

6.3.3 Reliability of antigen assignment

Evidence for reliability of antigen assignment came from 2 sources:

(a) Reproducibility of the cytotoxic test. Seven animals were subjected to repeat testing. The reactions considered were those of the antisera used in the definition of antigens, at each of the dilutions at which they displayed anti-class II type activity. Reproducibility was considered only from the point of view of reactions being either class II-positive or negative. There were 15 discrepancies in 276 repeated tests, giving a reproducibility estimate of 94.6%.

(b) Repeatability of antigen assignment. The antigen assignment was the same in both series of tests done on these 7 animals.
Table 6.3  Details of the preparation of bovine class II typing reagents.

<table>
<thead>
<tr>
<th>Typing Reagent</th>
<th>Parent</th>
<th>Absorptions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiserum</td>
<td>Platelets</td>
</tr>
<tr>
<td>JM-1</td>
<td>H6</td>
<td>71</td>
</tr>
<tr>
<td>JM-2</td>
<td>L36</td>
<td>71</td>
</tr>
<tr>
<td>JM-4</td>
<td>L22</td>
<td>L25</td>
</tr>
<tr>
<td>JM-6</td>
<td>K84</td>
<td>850</td>
</tr>
<tr>
<td>JM-9</td>
<td>H19</td>
<td>850</td>
</tr>
<tr>
<td>JM-11</td>
<td>K109</td>
<td>850</td>
</tr>
<tr>
<td>JM-16</td>
<td>K9</td>
<td>812</td>
</tr>
<tr>
<td>JM-17</td>
<td>K9</td>
<td>812</td>
</tr>
<tr>
<td>JM-18</td>
<td>K9</td>
<td>812</td>
</tr>
<tr>
<td>JM-23</td>
<td>G30</td>
<td>341</td>
</tr>
<tr>
<td>JM-24</td>
<td>G30</td>
<td>341</td>
</tr>
<tr>
<td>JM-25</td>
<td>G30</td>
<td>341</td>
</tr>
<tr>
<td>JM-26</td>
<td>L30</td>
<td>341</td>
</tr>
</tbody>
</table>
Table 6.4  Working dilutions of antisera in relation to the strength of their anti-class II type activity.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>First Doubling Dilution of Anti-class II Type Activity</th>
<th>Working Dilution</th>
<th>Endpoint Dilution of Anti-class II Type Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM-1</td>
<td>1/16</td>
<td>1/32</td>
<td>1/64</td>
</tr>
<tr>
<td>JM-2</td>
<td>1/8</td>
<td>1/16</td>
<td>1/64</td>
</tr>
<tr>
<td>JM-4</td>
<td>1/2</td>
<td>1/8</td>
<td>1/32</td>
</tr>
<tr>
<td>JM-6</td>
<td>1/16</td>
<td>1/16</td>
<td>1/32</td>
</tr>
<tr>
<td>JM-9</td>
<td>1/8</td>
<td>1/8</td>
<td>1/16</td>
</tr>
<tr>
<td>JM-11</td>
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<td>1/16</td>
</tr>
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<td>JM-16</td>
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<td>1/32</td>
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<td>1/16</td>
<td>1/32</td>
</tr>
<tr>
<td>JM-18</td>
<td>1/8</td>
<td>1/16</td>
<td>1/32</td>
</tr>
<tr>
<td>JM-23</td>
<td>1/32</td>
<td>1/32</td>
<td>1/128</td>
</tr>
<tr>
<td>JM-24</td>
<td>1/16</td>
<td>1/16</td>
<td>1/64</td>
</tr>
<tr>
<td>JM-25</td>
<td>1/16</td>
<td>1/16</td>
<td>1/64</td>
</tr>
<tr>
<td>JM-26</td>
<td>1/8</td>
<td>1/16</td>
<td>1/64</td>
</tr>
</tbody>
</table>
6.3.4 **Antigenic specificities**

Antigens were defined by assuming that a specificity, identified by a single alloantiseraum or a group of associated alloantisera, constituted a single antigen. Family data were used to study the inheritance of each antigen. Table 6.5 shows the associations (correlation coefficients and Fisher's exact test p values) between pairs of antisera, which were calculated from the results of testing the 58 independent panel animals. Table 6.6 shows the class II antigens tentatively defined and the reaction frequencies of the defining antisera (at their working dilutions) against the 58 independent panel animals.

The associations between antisera shown in Table 6.5 involve antigens A, B and E only, because in the cases of antigens C and D, pairs of typing antisera used to define a single antigen were leucocyte-absorbed derivatives of a single alloantiseraum (K9 and G30 respectively). Regarding the associations between antisera JM-1, JM-2 and JM-4, the smallest expected value in any cell in each of the 2 x 2 contingency tables was < 2. Although the corresponding $X^2$ values were significant at $p < 0.001$, the $p$ values obtained by a two-tailed Fisher's exact were not significant at $p < 0.001$, but were significant at $p < 0.0025$. In the other 2 x 2 tables of association between antisera used in antigen definition, the smallest expected value in any cell was > 2, and the associations were significant at $p < 0.001$ using either $X^2$ analysis or Fisher's exact test. Animals were described as being positive for an antigen only if their B(+) lymphocytes reacted with all of the antisera defining that antigen. Each antigen is described separately below.
Table 6.5 Associations between anti-class II antisera, calculated from the results of testing 58 independent panel animals.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>JM-1</th>
<th>JM-2</th>
<th>JM-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM-2</td>
<td>r = 0.48</td>
<td>r = 0.56</td>
<td>p = 0.0022</td>
</tr>
<tr>
<td>JM-4</td>
<td>r = 0.51</td>
<td></td>
<td>p = 0.0023</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>JM-6</th>
<th>JM-9</th>
<th>JM-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM-9</td>
<td>r = 0.59</td>
<td>r = 0.56</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>JM-11</td>
<td>r = 0.51</td>
<td></td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>JM-25</th>
<th>JM-26</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM-25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM-26</td>
<td>r = 0.90</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

\( r = \) correlation coefficient calculated from the \( X^2 \) value.

\( p = \) probability of obtaining an association equal to or greater than that observed, calculated using a two-tailed Fisher's exact test.
Table 6.6  Tentatively defined bovine class II antigens.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Defining Antisera</th>
<th>Reaction Frequency a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>JM-1</td>
<td>12.1%</td>
</tr>
<tr>
<td></td>
<td>JM-2</td>
<td>10.3%</td>
</tr>
<tr>
<td></td>
<td>JM-4</td>
<td>5.2%</td>
</tr>
<tr>
<td>B</td>
<td>JM-6</td>
<td>13.8%</td>
</tr>
<tr>
<td></td>
<td>JM-9</td>
<td>27.6%</td>
</tr>
<tr>
<td></td>
<td>JM-11</td>
<td>25.9%</td>
</tr>
<tr>
<td>C</td>
<td>JM-16</td>
<td>1.7%</td>
</tr>
<tr>
<td></td>
<td>JM-17</td>
<td>1.7%</td>
</tr>
<tr>
<td></td>
<td>JM-18</td>
<td>1.7%</td>
</tr>
<tr>
<td>D</td>
<td>JM-23</td>
<td>63.8%</td>
</tr>
<tr>
<td></td>
<td>JM-24</td>
<td>44.8%</td>
</tr>
<tr>
<td>E</td>
<td>JM-25</td>
<td>22.4%</td>
</tr>
<tr>
<td></td>
<td>JM-26</td>
<td>20.7%</td>
</tr>
</tbody>
</table>

a = percentage of class II-type reactions against the independent panel of 58 animals, at the working dilution of each antiserum.
Antigen A. This antigen was defined by reactivity with 3 antisera, JM-1, JM-2 and JM-4. Antiserum JM-1 was used at a working dilution of one doubling dilution before the endpoint, as it had a tail of weak "extra" reactions (compared to the reactions of JM-2 and JM-4) at lower dilutions. Antisera JM-2 and JM-4 were used 2 doubling dilutions before the endpoint. The antisera defining this antigen were raised against tissue from animal 71 and its offspring L25, and 2 of the antisera were raised against the haplotype which carried class I antigen CA24. The one offspring of animal 71 which inherited the CA24 haplotype was positive for antigen A; the 2 offspring which didn't inherit the CA24 haplotype were negative for antigen A.

Antigen B. This antigen was defined by reactivity with 3 antisera, JM-6, JM-9 and JM-11, at the first doubling dilution at which these antisera displayed anti-class II type activity (one doubling dilution before the endpoint). All of the antisera defining this antigen were raised against the haplotype of the bull 850 which carried class I antigen CA30. As well as being carried by the bull 850, antigen B was also carried by the bull 980, and 36 offspring of these 2 bulls were tested. The inheritance of this antigen is shown in Table 6.7.

Antigen C. This antigen was defined by reactivity with 3 antisera, JM-16, JM-17 and JM-18, at one doubling dilution before their respective endpoints. These 3 antisera were leucocyte-absorbed reagents, produced from a single platelet-absorbed alloantiserum (K9). This antigen was relatively rare, being present in only one of the 58 independent panel animals. Antiserum K9 was raised against the
Table 6.7  Inheritance of antigen B.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Number</th>
<th>Parents</th>
<th>Offspring</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>+ x +</td>
<td>1</td>
<td>3</td>
<td>0 : 0</td>
<td>4 : 0</td>
</tr>
<tr>
<td>+ x -</td>
<td>2</td>
<td>20</td>
<td>8 : 7</td>
<td>11 : 5</td>
</tr>
<tr>
<td>- x +</td>
<td>1</td>
<td>1</td>
<td>0 : 0</td>
<td>0 : 1</td>
</tr>
<tr>
<td>- x -</td>
<td>1</td>
<td>6</td>
<td>0 : 1</td>
<td>0 : 6</td>
</tr>
</tbody>
</table>

M = male.
F = female.

"..."
haplotype of animal 812 which carried the class I antigen CA42. The one offspring of animal 812 which inherited the CA42 haplotype was positive for antigen C; the 2 offspring which didn't inherit the CA42 haplotype were negative for antigen C.

Antigen D. This antigen was defined by reactivity with 2 antisera, JM-23 and JM-24, at 2 doubling dilutions before their respective endpoints. Antiserum JM-23 appeared to be di-specific, and JM-24 was a leucocyte-absorbed version of JM-23 which appeared to detect one of these specificities. The inheritance of this antigen is shown in Table 6.8.

Antigen E. This antigen was defined by reactivity with 2 antisera, JM-25 and JM-26, at 2 doubling dilutions before their respective endpoints. Antiserum JM-25 was a leucocyte-absorbed version of the di-specific antiserum, JM-23, and its activity correlated closely with that of antiserum JM-26 ($r = 0.90$). Antiserum JM-26 was produced by a haplotype-matched immunization (against the haplotype of animal 341 which carried class I antigen CA38). Antiserum JM-23 was raised against the entire MHC of animal 341. The inheritance of this antigen is shown in Table 6.9.

Miscellaneous. Nineteen of the 58 independent panel animals (33%) did not carry any of the defined antigens. Although a null allele may exist, it is most likely that more class II antigens will be defined (as in the HLA system).
Table 6.8  Inheritance of antigen D.

<table>
<thead>
<tr>
<th>Parents</th>
<th>Offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>Number</td>
</tr>
<tr>
<td></td>
<td>M</td>
</tr>
<tr>
<td>+ x +</td>
<td>0</td>
</tr>
<tr>
<td>+ x -</td>
<td>1</td>
</tr>
<tr>
<td>- x +</td>
<td>3</td>
</tr>
<tr>
<td>- x -</td>
<td>2</td>
</tr>
</tbody>
</table>

M = male.
F = female.
Table 6.9 Inheritance of antigen E.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Number</th>
<th>Offspring</th>
<th>Totals (M + F)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Parents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ x +</td>
<td>0 0</td>
<td>- : -</td>
<td>- : -</td>
</tr>
<tr>
<td>+ x -</td>
<td>0 0</td>
<td>- : -</td>
<td>- : -</td>
</tr>
<tr>
<td>- x +</td>
<td>3 7</td>
<td>1 : 3</td>
<td>5 : 1</td>
</tr>
<tr>
<td>- x -</td>
<td>4 19</td>
<td>0 : 13</td>
<td>0 : 20</td>
</tr>
<tr>
<td>Offspring</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenotypes</td>
<td></td>
<td>+ : -</td>
<td>+ : -</td>
</tr>
</tbody>
</table>

M = male.
F = female.
6.4 DISCUSSION

There are a number of limitations to the interpretation of absorption analysis:

(a) absorption analysis can under-estimate the number of specificities being detected by an antiserum, if absorbing cells share specificities which are highly associated in a population (Dausset et al., 1965).

(b) a number of serological anomalies may occur (Bodmer et al., 1969). Firstly, an antibody may be absorbed by an antigenic specificity with which it does not react in a cytotoxic test (CYNAP phenomenon). Secondly, an antibody may cross-react with 2 specificities, in that it will react with both and be absorbed by both. Both immunization experiments (where antibodies could be produced which reacted against antigens not present in the immunizing cells) (Thorsby et al., 1970), and absorption experiments (Mittal & Terasaki, 1972) have suggested that a single antibody population may react with more than one HLA antigen. Complicated patterns of uni- and bi-directional absorption have been observed (Mittal & Terasaki, 1972). An antiserum may contain both private and public antibodies, and absorption with cells carrying the public antigen will not remove reactivity to the private antigen, whereas absorption with cells carrying the private antigen will remove both reactivities. This situation may become more complicated where there is more than one public determinant on a molecule (Schwartz et al., 1980). Thirdly, an antiserum may interact with 2 specificities, in the sense that it reacts only when both of the primary specificities are present.
(c) the actual number of class II-positive lymphocytes used for absorptions was not standardized in the present experiments. Therefore, when absorbing 2 separate portions of an antiserum with leucocytes from 2 positively-reacting animals, the titre of a single antibody population may have been reduced by significantly different amounts. Absorptions were done with buffy-coat leucocytes because this was more convenient than preparing class II-positive lymphocytes. Nevertheless, leucocyte absorption analyses were useful in narrowing the specificities of some typing reagents in the present experiments.

It has been noted in the HIA system that reactivity with B lymphocytes and non-reactivity with T lymphocytes are insufficient criteria alone for defining anti-class II activity. Some studies have suggested that B lymphocytes are more sensitive to many anti-HIA class I antibodies than are T lymphocytes (Richiardi et al., 1977; Belvedere et al., 1978). The experience in the HIA system seems to be that the activity of antibodies which react with class I antigens only on B cells is apparent at just a single dilution of the antiserum, becoming negative at the next doubling dilution (Engelfriet et al., 1982). In my experiments, the preferential B cell reactivity of the defining antisera titrated at least 2, and up to 5, doubling dilutions. This would suggest that at these dilutions, the antibodies were specific for B cells, rather than weak antibodies to class I or other lymphocyte antigens. It is unlikely that the antigens described here as class II antigens are in fact red cell antigens which have been passively absorbed by lymphocytes, as it is unlikely that such antigens would be preferentially absorbed by B lymphocytes and not by T lymphocytes. The presence of anti-red cell lymphocytotoxic
antibodies could be monitored by absorptions with red cells, as discussed in Section 4.4.

The essential question that requires resolution is what positive reactions with antisera constitute an antigen? When a group of clustered antisera is used in the definition of an antigen, the distinction between positive and negative individuals is facilitated if there is a bimodal distribution of the numbers of individuals reacting with given numbers of antisera. Such a pattern is taken to indicate that the cluster detects a factor present in some individuals and not present in others (Bodmer et al., 1969). In the present experiments, reaction frequencies in the clusters defining antigens A, B and E were bimodally distributed. Because only small numbers of antisera were clustered in these experiments, it was decided that antigens would be defined only if the individual reacted with all of the antisera defining the cluster. In this way, the likelihood of false positive antigen assignment should have been reduced.

If an animal reacts with only some of the antisera defining a particular antigen and that animal in fact carries the antigen, the negatively-reacting antisera are giving "false negative" reactions (with respect to that antigen). "False negative" reactions may occur due to technical reasons (e.g. weak antisera). Alternatively, some antisera in a cluster may define antibodies corresponding to antigenic components of the main antigenic specificity (Bodmer & Payne, 1965). In an effort to reduce the likelihood of "false negative" reactions due to weak activity, all defining antisera in these experiments were used at least one doubling dilution before their anti-class II endpoint titre.
When using multispecific antisera, MHC antigens are conventionally defined by clusters of associated antisera (Bodmer et al., 1969). In the present experiments, antigens C and D were each defined by the products of a single alloantiserum (antisera K9 and G30 respectively), as there were no associating alloantisera. Given that this study is an early step in the definition of the bovine class II antigen system, it was felt that the reactions of the leucocyte-absorbed derivatives of antisera K9 and G30 were sufficiently informative to tentatively define class II antigens. The reactions of the defining antisera were heritable and segregated consistently with class I haplotypes in families (see Chapter 7). More than one typing reagent (derived from a single alloantiserum) was used to define each of antigens C and D, to help clarify those situations where a single typing reagent gave a weak reaction. Antigens were defined only if the individual reacted with all of the typing reagents derived from the single alloantiserum.

In the small number of family groups studied, each of the defined antigens was observed to be inherited. If a trait is inherited in a Mendelian dominant fashion, then it would be expected that in families with one positive heterozygous parent and one negative parent, there will be approximately a 1:1 segregation of positive to negative offspring ("dominant" referring to the presence of the trait, and "recessive" referring to absence) (Cavalli-Sforza & Bodmer, 1971a). Positive parents are assumed to be heterozygous if they have at least one negative offspring. Not all positive parents from these families could be proven to be heterozygous, as some cows had only a small number of offspring. However, for antigens B, D and E, the pooled data from families with only one positive parent were
consistent with a 1:1 segregation of positive to negative offspring, which is consistent with Mendelian dominant inheritance of these antigens. Because antigens A and C were each present in only one parent/offspring combination, no conclusions can be drawn regarding their inheritance.

There are several lines of evidence which support the definition of the putative class II antigens:

(a) all defining antisera displayed anti-class II type activity over at least 2 doubling dilutions.

(b) for each of antigens A, B and E, different immunizations directed against the one MHC type or haplotype produced a group of alloantisera whose anti-class II type reactions were significantly associated, which suggested that antisera within each group defined a shared class II specificity. When examining associations between antisera for MHC typing, a significant positive association is assumed to exist if, when \( n \geq 100 \), \( p \leq 0.001 \) (Dausset et al., 1965; Klein, 1975). The associations between antisera defining antigen B and between antisera defining antigen E were significant at \( p < 0.001 \). The associations between antisera defining antigen A were significant at \( p < 0.0025 \), and an antigen was tentatively defined as this level of significance is approaching the level conventionally used. The failure to achieve a greater level of significance may have been due to the small sample size.

(c) antigen assignment was reproducible in the few animals subjected to repeat testing.

(d) regular Mendelian inheritance of antigens B, D and E would suggest (though it does not prove) that these antigens were
correctly defined; the absence of Mendelian inheritance would have suggested that antigens may not have been correctly defined.

However, the antigen definition used here can be criticized on a number of grounds. The data should be seen to be preliminary, in that they are based on the serological testing of a relatively small number of animals of one breed, and associations between antisera were examined statistically using only 58 animals. To examine associations between antisera for MHC typing, the use of a large ($n \geq 100$), random panel of test individuals is recommended; in HLA typing, a test panel is generally made up of unrelated individuals (Dausset et al., 1965; Klein, 1975). In choosing a test panel of cattle in these experiments, the aim was to select animals within the Charolais breed which were as unrelated as practically possible. Because of the relatively small gene pool of the Charolais breed in Australia, it was very difficult to find a large number of animals which did not share grand-parents, and so some animals sharing a single grand-parent were included. In addition, there would have been some degree of consanguinity among the other independent panel animals due to sharing of more distant ancestors. The test population would therefore have been less heterogeneous than an unrelated population of individuals.

When the test population is less heterogeneous, there will be a greater chance that the concordance of antisera reactions is due to the detection of antigens which are associated in the population, rather than the detection of a common antigen. The associated antigens may be on different molecules which are the products of linked genes, or may represent different determinants on the one molecule. The testing of a less heterogeneous population could thus
lead to spurious or artificially high correlation coefficients. The more heterogeneous the test population, the greater the likelihood that population associations between antigens will be broken.

While the association between the antisera defining antigen E was relatively strong, the associations between the antisera defining antigen A and between the antisera defining antigen B were relatively weak, and all 3 antigens were defined by only 2 or 3 antisera. In the case of antigen C and antigen D, all typing reagents were derived from a single alloantiserum. The definition of the above antigens therefore remains provisional until more defining antisera have been prepared. As the number of associated antisera in a cluster increases, the probability of an individual reacting with all antisera in the cluster but not carrying the antigen defined by the cluster will decrease (Bodmer et al., 1969). Further immunizations could be directed against other animals identified as positive for the individual antigens. All defining antisera will need to be tested against a larger, more heterogeneous panel of animals. The specificity of antisera should be checked by cross-absorptions with positively-reacting cells (Bodmer et al., 1969). This approach would help confirm the existence of the defined class II antigens or enable these antigens to be "split". It is possible that pairs of associating alloantisera in this study were not detecting a single antigen; instead both antisera in a pair may have contained 2 distinct antibody specificities which were detecting 2 antigens controlled by genes in strong linkage disequilibrium in the animals tested.

The antisera should eventually be tested against different breeds, and associations between antisera should be examined within each breed. Antisera which are associated in one breed may not be
associated in other breeds if, for example, the antisera are detecting antigens controlled by genes in strong linkage disequilibrium. In the HLA system, some antisera which give identical or very similar results in one population have been found to diverge in reactivity when tested in another population (Dausset et al., 1965; Batchelor et al., 1973).
CHAPTER 7

GENETIC RELATIONSHIPS OF BOVINE CLASS II ANTIGENS

The second stage in the definition of an antigen system is the elucidation of the mode of inheritance of antigens, which was dealt with in Chapter 6, and the elucidation of the relationships between antigens and their relationships with other antigen systems. Relationships between antigens may take the form of allelism at a single locus, allelism at closely linked loci, or the antigens may belong to a number of independent loci. The methods normally used to examine these relationships include analysis of associations of antigens in populations, and analysis of the inheritance of antigens in families.

At a population level, a significant positive association between a given antigen and another antigen in the same testing panel is generally taken to suggest that these antigens are allelic. Other explanations are possible, such as departure from random mating. A significant negative association to generally taken to suggest allelism, although negative associations may also exist between non-allelic antigens due to linkage disequilibrium. The hypothesis of allelism would be supported by population data if no individual was found to carry more than 2 allelic antigens, and by family data if no single haplotype was found to carry more than one allelic antigen. Population analysis may also reveal intergroup relationships between antigens.
7.1 INTRODUCTION

The second stage in the definition of an antigen system is the elucidation of the mode of inheritance of antigens, which was dealt with in Chapter 6, and the elucidation of the relationships between antigens and their relationships with other antigen systems. Relationships between antigens may take the form of allelism at a single locus, allelism at closely linked loci, or the antigens may belong to a number of independent loci. The methods normally used to examine these relationships include analysis of associations of antigens in populations, and analysis of the inheritance of antigens in families.

At a population level, a significant positive association between 2 blood group antigens (in a 2 x 2 contingency table) is generally taken to suggest very close linkage of the genes coding for these antigens (Andresen et al., 1963; Andresen & Baker, 1964; Bodmer et al., 1969). Other explanations are possible, such as departures from random mating. A significant negative association is generally taken to suggest allelism, although negative associations could also exist between non-allelic antigens due to linkage disequilibrium (Andresen et al., 1963). The hypothesis of allelism would be supported by population data if no individual was found to carry more than 2 allelic antigens, and by family data if no single haplotype was found to carry more than one allelic antigen (Mattiuiz et al., 1970). Population analysis may also reveal subgroup relationships between antigens.
In the experiments reported in this chapter, the genetic relationships of the putative class II antigens described in Chapter 6 were examined.

7.2 EXPERIMENTAL METHODS

The associations between pairs of class II antigens were analysed in the 58 independent panel animals using a $X^2$ test for independence and a two-tailed Fisher's exact test, as described in Section 2.13. Class I antigens were not included in the population study, because some of the 58 independent panel animals had not been typed for class I antigens.

The 117 cattle tested included one large paternal half-sibling family and 2 smaller paternal half-sibling families. In addition, some of the adult females tested had at least one, and up to 4, offspring which were tested. Nearly all of the animals within family groups had previously been typed for class I antigens, and in most cases parental class I antigens had been assigned to haplotypes by observing the segregation of the antigens in offspring.

The likelihood of linkage was investigated using the sequential probability ratio test (Morton, 1955). Informative matings were those in which one parent was positive and the other parent was negative for the traits under consideration. The additional requirement that the positive parent be heterozygous was satisfied if the parent was found to have at least one negative offspring. The parameter calculated was the LOD score ($\sigma$), as described in Section 2.13.
7.3 RESULTS

There were no significant positive or negative population associations between class II antigens at $p \leq 0.05$. One animal carried more than 2 class II antigens; this animal carried antigens A, B and D.

The inheritance of the class I and putative class II antigens is shown in Tables 7.1 and 7.2. Table 7.1 shows the inheritance in 2 half-sibling families, based on the sires 850 and 980. Table 7.2 shows the inheritance in the remainder of those maternal half-sibling families in which offspring carrying each of the maternal class I haplotypes were found. Some of the dams listed in Table 7.2 also appear in Table 7.1. The possible class I and class II genotypes were deduced from the way in which antigens appeared to segregate. Not all of the 11 matings listed in Table 7.2 are informative according to the definition given Section 7.2. However, I have included them to show that all genotypes can be arranged so that maternal class I and class II antigens appeared to segregate together.

When family data were examined for evidence of segregation of the defined class II antigens, antigen D was observed to segregate from antigen E in 3 matings. With the exception of antigen A, which was inherited with antigen E, no more than one class II antigen was inherited on any single haplotype.

The 2 paternal half-sibling families which were analysed for linkage are those listed in Table 7.1. Only the linkage between class I antigen CA30 and class II antigen B was investigated. The class II type of the sire 980 was inferred from comparisons of the
Table 7.1

Linked inheritance of class I antigen CA30 and class II antigen B in 2 informative paternal half-sibling families.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Paternal Haplotype</th>
<th>Maternal Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>850</td>
<td>CAJO</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>856</td>
<td>CA36</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each animal, the class I antigens are listed above the class II antigens. The prefix "CA" precedes all of the class I antigens. Thus for example, the sire 850 carries class I antigen CA30 and class II antigen B on one haplotype, and class I antigen CA36 on the other haplotype (with no class II antigen being defined on this haplotype).

For each offspring, the paternal haplotype is listed before the maternal haplotype.

- = no class II antigen was defined on this haplotype.

* = homozygosity cannot be excluded.
<table>
<thead>
<tr>
<th>SIRE IDENTITY</th>
<th>POSSIBLE GENOTYPE</th>
<th>DAM IDENTITY</th>
<th>POSSIBLE GENOTYPE</th>
<th>CALF IDENTITY</th>
<th>POSSIBLE GENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>850</td>
<td>30 / 36</td>
<td>71</td>
<td>24 / 47</td>
<td>L25</td>
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</tr>
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</table>


Table 7.2

Linked inheritance of class I and class II antigens in the remainder of those maternal half-sibling families in which offspring carrying each of the maternal class I haplotypes were found.

For each animal, the class I antigens are listed above the class II antigens. The prefix "CA" precedes all of the class I antigens. Thus for example, the sire LION carries class I antigens CA4 and CA12 on one haplotype, and class I antigen CA36 on the other haplotype (with no class II antigens being defined on either haplotype).

For each offspring, the paternal haplotype is listed before the maternal haplotype.

- = no class II antigen was defined on this haplotype.

* = homozygosity cannot be excluded.
<table>
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<tr>
<th>SIRE IDENTITY</th>
<th>POSSIBLE GENOTYPE</th>
<th>DAM IDENTITY</th>
<th>POSSIBLE GENOTYPE</th>
<th>CALF IDENTITY</th>
<th>POSSIBLE GENOTYPE</th>
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<td>L1</td>
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<td>2 / 36</td>
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<td>22 / 27</td>
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<td>/ D</td>
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class II types of the offspring and dams. When all animals were considered only from the point of view of being CA30-positive/negative and class II antigen B-positive/negative, there were 29 informative matings in these 2 families. Class I antigen CA30 and class II antigen B appeared to segregate together in 18 offspring, and one animal (H6) appeared to inherit a recombinant haplotype (CA30-positive/antigen B-negative). The corresponding 2 x 2 table was:

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IDD scores were calculated for a range of recombination frequencies, and the LOD score was at a maximum when recombination frequency = 0.03. This was therefore the best estimate of recombination frequency, and corresponded to the observed recombination frequency. The maximum LOD score was 6.53557, which meant that the odds in favour of linkage between class I antigen CA30 and class II antigen B were $10^6.53557$ to one (i.e. greater than 3 million to one). Conventionally, LOD scores of greater than + 3.0 are assumed to show proof of linkage (Ceppellini et al., 1967).
Maternal half-sibling families which contained offspring carrying each of the maternal class I haplotypes were examined. In all such families, the maternal class II antigens could be assigned in such a way that they segregated "en bloc" with a class I haplotype (with the exception of the recombinant mentioned above).

7.4 DISCUSSION

There were no significant population associations between the defined class II antigens, although existing associations may not have been detected because of the small sample size.

Antigens A and E were inherited jointly by one animal. The joint inheritance of 2 antigens may be explained by poor antigen definition, serological artefact, a single allele coding for both antigens, or separate but linked loci coding for the 2 antigens (for a more detailed discussion, see Section 8.4). Similar explanations may account for one animal carrying 3 antigens. More animals need to be studied to resolve the genetic relationships of antigen A. Antigens D and E behaved as alleles in 3 matings, although more families need to be studied to confirm this finding.

Linkage between antigen systems can be studied by population analysis and family studies. Linkage disequilibrium refers to the difference between the observed frequency of association of 2 alleles in a population (i.e. the haplotype frequency), and their expected frequency of association calculated from their individual allele frequencies and based on the assumption that they are independent (Bodmer & Bodmer, 1978). Linkage disequilibrium is a common finding for HLA antigens at different loci (Albert & Gotze, 1977; Bodmer &
Bodmer, 1978) and its strength can be estimated by calculating the linkage disequilibrium parameter (delta). A value of delta significantly different from zero is generally an indication of relatively close linkage between loci (Bodmer & Payne, 1965; Bodmer et al., 1969). In the present experiments, population analysis was not used to study linkage between the class I antigen system and the defined class II antigens, because some of the independent panel animals had not been typed for class I antigens.

Linkage between antigen systems is best demonstrated by family studies (Mattiuz et al., 1970). In the present experiments, only paternal segregation was formally analysed for linkage. There were too few full siblings to formally examine maternal segregation, and maternal and paternal data were not pooled because recombination rates may differ in males and females (Cavalli-Sforza & Bodmer, 1971b). Data from the 2 sire families indicated highly significant linkage between class I antigen CA30 and class II antigen B. Antigen B could be distinguished from class I antigen CA30 by a recombination. However, the estimate of recombination frequency (0.03) may not be very accurate, as only the one recombinant was observed. Ideally, a large number of families should be analysed to confirm the recombination frequency.

The putative class II antigen B may in fact be a class I antigen. However, this was considered unlikely; the accumulated evidence that antigens A, B, C, D and E are class II antigens is presented in Chapter 9.

The putative class II antigens were inherited as autosomal codominant traits (see Chapter 6) and were inherited jointly with class I haplotypes in families. The only firm conclusion that could be
reached regarding the genetic relationships of the defined class II antigens was that antigen B is closely linked to class I antigen CA30, and that antigen B can thus be assumed to be part of the bovine MHC. Although all other family data were consistent with the hypothesis that antigens A, B, C, D and E are part of a single genetic system which is closely linked to the class I antigen system, more data will be needed to confirm this hypothesis.
Chapter 8

DEFINITION OF BOVINE CLASS I ANTIGENS

Class I antigens have been serologically defined in cattle by several independent groups (Caldwell et al., 1979; Jackson et al., 1978; Jackson et al., 1982; Ozan et al., 1992). Thirty-three lymphocyte antigens have been defined in cattle by studies done at the J.C.R.L. Antigens CRM and CRM are blood group antigens, which are not part of the bovine MHC (Jackson & Bell, 1984; Jones et al., 1988). The other 31 antigens are assumed to be MHC class I antigens because all peripheral blood lymphocytes from positive animals are killed by the appropriate antiserum.

Fifteen antigens at W1, W2, W3, and W4 and 2 subgroup antigens (W6.1 and W6.2) were defined at the First and Second International Bovine Workshop (Bensussan et al., 1994; Bensussan et al., 1997). Five regional antigens (W5, W7, W8, W9, and W10) and 4 subregion antigens of W1 were defined at the 1987 European Bovine Workshop (Lerondel, 1988). Oliver et al. (1991) reported that family studies showed that at least 12 of the Workshop antigens, including W1, W2, W3, W4, W5, W6, W7, W8, W9, W10, and W11, belonged to alleles at a single locus and this finding has been confirmed by others (Lerondel, 1993).

Of the 33 bovine class I antigens (Table 8.1), 12 correspond to International Workshop antigens and 8 correspond to European Workshop antigens. 14 of these Workshop antigens also correspond to antigens defined by a group at Perthville in Australia. The other 15 antigens have not been defined at any workshop, although a comparison to antigens defined by the group at Perthville (Smith et al., 1985),

size family studies have demonstrated that 30 of the 33 proposed class I antigens are inherited as autosomal co-dominant traits. They
8.1 INTRODUCTION

Class I antigens have been serologically defined in cattle by several independent groups (Caldwell et al., 1977; Amorena & Stone, 1978; Spooner et al., 1978; Stear et al., 1982). Thirty-three lymphocyte antigens have been defined in cattle from studies done at the J.C.S.M.R.. Antigens CA16 and CA19 are blood group antigens, and are not part of the bovine MHC (Stear & Bell, 1984; Stear et al., 1985b). The other 31 antigens are assumed to be MHC class I antigens because all peripheral blood lymphocytes from positive animals are killed by the appropriate antisera.

Fifteen antigens (W1 - W13, W16 and W20) and 2 subgroup antigens (W6.1 and W6.2) were defined at the First and Second International BoLA Workshops (Spooner et al., 1979a; Anonymous, 1982). Five regional antigens (EU1, EU2, EU12, EU27 and EU28) and a subgroup antigen of W9 were defined at the 1982 European BoLA Workshop (Leveziel, 1983). Oliver et al. (1981) reported that family studies showed that at least 15 of the Workshop antigens, including W6 and W11, behaved as alleles at a single locus and this finding has been confirmed by others (Leveziel, 1983).

Of the 31 Canberra class I antigens (Table 8.1), 13 correspond to International Workshop antigens and 2 correspond to European Workshop antigens (4 of these Canberra antigens also correspond to antigens defined by a group at Parkville in Melbourne). The other 16 antigens have not been defined at any workshop, although 4 correspond to antigens defined by the group at Parkville (Stear et al., 1985b).

Sire family studies have demonstrated that 30 of the 31 Canberra class I antigens are inherited as autosomal co-dominant traits. They
Table 8.1  Comparison of Workshop, Canberra and Melbourne BoLA class I antigens.

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<tr>
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<td>MB10</td>
</tr>
<tr>
<td>EU27</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>EU28</td>
<td>CA12</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA5</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA13</td>
<td>MB5</td>
</tr>
<tr>
<td>-</td>
<td>CA15</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA21</td>
<td>MB8</td>
</tr>
<tr>
<td>-</td>
<td>CA22</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA24</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA28</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA31</td>
<td>MB3</td>
</tr>
<tr>
<td>-</td>
<td>CA35</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA36</td>
<td>MB11</td>
</tr>
<tr>
<td>-</td>
<td>CA38</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA39</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA40</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA42</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA45</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>CA47</td>
<td></td>
</tr>
</tbody>
</table>
appear to be coded for by a genetic system that consists of 2 mutually exclusive allelic series which may be controlled by 2 closely-linked loci (Stear et al., 1982). Antigen CA28 is very rare and has only been found in one maternal half-sibling family, where it appears to be controlled by an autosomal co-dominant gene. It is assumed to be part of the same genetic system as the other 30 antigens. At present there is insufficient data to assign all antigens to particular loci.

The relationships between pairs of the Canberra antigens are shown in Table 8.2.

Table 8.2 Subgroup relationships between Canberra BoLA class I antigens.

<table>
<thead>
<tr>
<th>Supertypic Antigen</th>
<th>Subtypic Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6</td>
<td>CA31, CA39</td>
</tr>
<tr>
<td>W20</td>
<td>CA47, CA38</td>
</tr>
<tr>
<td>EU28</td>
<td>W8</td>
</tr>
<tr>
<td>EU28</td>
<td>W12</td>
</tr>
<tr>
<td>EU28</td>
<td>W13</td>
</tr>
<tr>
<td>CA13</td>
<td>W4</td>
</tr>
</tbody>
</table>

Antigens W6, W20, EU28 and CA13 are supertypic antigens (Stear et al., 1985b). It is not known whether the relationships are due to separate determinants on the same molecule (which are shared with
other allelic molecules), distinct antigens grouped by polyspecific antisera, or the products of associated genes. Therefore, supertypic and subtypic antigens have been given different antigen numbers. In addition, antigen CA35 appears to be a subgroup of antigen W11, except for one animal which was positive for CA35 but not for W11 (Stear et al., 1985b). In this animal (a Friesian/Holstein), CA35 occurred with CA38; CA35 + CA38 is a common combination in American Simmentals and Canadian Holsteins (Dr M.J. Stear, personal communication).

In this chapter, improved definition of antigen CA21 is described. In addition, evidence is presented which shows that 2 workshop antigens, W6 and W11, do not always behave as if coded for by alleles at a single locus.

8.2 EXPERIMENTAL METHODS

The general principles used in the resolution of antigens, and in determining the relationships between them, were those described in Chapters 6 and 7. Antisera were tested by the standard class I microlymphocytotoxicity test (Section 2.12.1). One weak parous antiserum (BR285) was initially used to define antigen CA21.

Six antisera were used to define antigen W6; the mean correlation coefficient was 0.93. Three antisera were used to define antigen W11; the mean correlation coefficient was 0.91. The correlation coefficient between W6 defined in Canberra and W6 defined in several laboratories overseas was 1.0; the correlation coefficient between W11 defined in Canberra and W11 defined in several laboratories overseas was 0.92 (Stear et al., 1985b). There was no statistically significant association at a population level (using the
\( \chi^2 \) test for independence) between the presence of W6 and the presence or absence of W11 (\( n = 511 \)).

Buffy-coat leucocyte immunizations were done between the animals shown in Table 8.3, as described in Section 2.10.1. Two immunizations were directed against antigen CA21, and one immunization was directed against antigens W6 and W11 together. All 3 immunizations were haplotype-matched, as donor animals were the mothers of recipient animals. The genotypes of all 6 animals could be inferred from the phenotypes of relatives.

Table 8.3 Buffy-coat leucocyte immunizations.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>Class I</td>
</tr>
<tr>
<td>Number</td>
<td>Antigens</td>
</tr>
<tr>
<td>953</td>
<td>CA21 / W10</td>
</tr>
<tr>
<td>949</td>
<td>CA21 / W12,EU28</td>
</tr>
<tr>
<td>A10C</td>
<td>W6,W11 / CA42</td>
</tr>
</tbody>
</table>

Donor animals were the mothers of recipient animals. For recipient animals, paternally-derived haplotypes are listed first.
Leucocyte absorptions of antisera were done as described in Section 2.11.2. Absorptions were of 2 types: preparative absorptions were used to remove unwanted antibody populations from antisera and cross-absorptions were used to check whether an antiserum was monospecific. For preparative absorptions, 10 ml antiserum was incubated for 60 min with buffy-coat leucocytes prepared from 500 ml blood. For cross-absorptions, 100 µl antiserum was incubated for 60 min with lymphocytes prepared from 10 ml blood.

When an elution was done subsequent to the fractionation of an antiserum by absorption, the procedure for preparative absorptions was followed except that prior to absorption, leucocytes were fixed for 15 min in 1% formaldehyde in PBS. After absorption, antibodies were eluted by resuspending the leucocytes in 1 ml of 0.1 M citrate buffer, pH 3.5. The leucocytes were mixed with this low pH buffer on a slow rotator for 15 min at room temperature. The eluate was aspirated after centrifugation, and was dialysed extensively against PBS before use.

Antigen frequencies and gene frequencies were calculated as described in Section 2.13.

8.3 RESULTS

8.3.1 Definition of antigen CA21

The peak cytotoxic titres of antisera K36 and K41 were 64 and 512 respectively. Tentative working dilutions of 1/32 and 1/64 respectively were chosen after the antisera were screened against a reference panel of cells from 16 cattle (which included the donor animals and the range of class I antigens detected by the Canberra
antisera). The bull 850, which had previously been typed as: (EU12 / CA36, weak CA21), and 46 of his offspring were tested against antisera K36 and K41; 23 of these offspring inherited the class I haplotype which carried EU12, and 23 inherited the class I haplotype which carried CA36.

The bull 850 was negative to both these antisera. Only 3 offspring were positive, and they all appeared to inherit antigen CA21 from their mother. Table 8.4 shows some examples of the relationship between the previously defined antigen CA21 ("old" CA21), and reactivity with antisera K36 and K41. On the basis of these results, "new" antigen CA21, as defined by antisera K36 and K41, was deemed to be absent from 850. The apparent inheritance of this antigen in the 3 positive offspring is shown in Table 8.5.

Antisera K36 and K41 were screened against PBL from over 1400 cattle in the U.S.A., and appropriate preparative buffy-coat leucocyte absorptions were done. The 5 antisera subsequently used to define antigen CA21 were:

- K36 (absorbed 709) at a dilution of 1/8
- K41 (absorbed 741) " 1/8
- K41 (absorbed 709) " 1/4
- K36 (unabsorbed) " 1/32
- K41 (unabsorbed) " 1/64

The correlation coefficients between K36 (absorbed 709) and the absorbed K41 antisera are shown in Table 8.6. The definition of antigen CA21 was the same with or without the unabsorbed antisera. There was a bimodal distribution of the number of individuals reacting
Table 8.4  Family studies showing the relationship between "old" antigen CA21 and "new" antigen CA21.

<table>
<thead>
<tr>
<th>Animal</th>
<th>&quot;Old&quot; CA21</th>
<th>Reaction with K36</th>
<th>Reaction with K41</th>
<th>&quot;New&quot; CA21</th>
</tr>
</thead>
<tbody>
<tr>
<td>949</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>953</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>850 (S)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G8 (M)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K91 (O)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>850 (S)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>806 (M)</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>+ a</td>
</tr>
<tr>
<td>K88 (O)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>850 (S)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>787 (M)</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>+ a</td>
</tr>
<tr>
<td>M2 (O)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>850 (S)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>806 (M)</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>+ a</td>
</tr>
<tr>
<td>L34 (O)</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

S = sire.
M = mother.
O = offspring.
NT = not tested.
a = inferred from phenotype of offspring.
Table 8.5  Inheritance of BoLA antigen CA21.

<table>
<thead>
<tr>
<th>SIRE IDENTITY</th>
<th>POSSIBLE CLASS I GENOTYPE</th>
<th>DAM IDENTITY</th>
<th>POSSIBLE CLASS I GENOTYPE</th>
<th>CALF IDENTITY</th>
<th>POSSIBLE CLASS I GENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>850 EU12 / CA36</td>
<td></td>
<td>806 CA21 / EU28, W13</td>
<td></td>
<td>K88 EU12 / CA21</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td>787 CA21 / CA42</td>
<td></td>
<td>M2 EU12 / CA21</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td>806 CA21 / EU28, W13</td>
<td></td>
<td>L34 CA36 / CA21</td>
<td></td>
</tr>
</tbody>
</table>

For each offspring, the paternally-derived haplotype is listed first.
Table 8.6  Correlation coefficients between absorbed antisera defining class I antigen CA21.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Reaction Frequency</th>
<th>K36, absorbed 709</th>
</tr>
</thead>
<tbody>
<tr>
<td>K36, absorbed 709 1/8</td>
<td>7.3%</td>
<td>r = 0.67</td>
</tr>
<tr>
<td>K41, absorbed 741 1/8</td>
<td>13.8%</td>
<td>r = 0.62</td>
</tr>
<tr>
<td>K41, absorbed 709 1/4</td>
<td>14.1%</td>
<td></td>
</tr>
</tbody>
</table>
with a given number of antisera. "New" antigen CA21 was defined only if more than 3 of the 5 antisera reacted.

The antigen frequency and gene frequency of antigen CA21 in 4 breeds of cattle studied in the U.S.A. are listed in Table 8.7.

**Table 8.7** Antigen frequency and gene frequency of antigen CA21 in 4 breeds of cattle in the U.S.A.

<table>
<thead>
<tr>
<th>Breed</th>
<th>n</th>
<th>Antigen Frequency</th>
<th>Gene Frequency</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereford</td>
<td>57</td>
<td>0.088</td>
<td>0.045</td>
<td>0.019</td>
</tr>
<tr>
<td>Charolais</td>
<td>52</td>
<td>0.038</td>
<td>0.019</td>
<td>0.013</td>
</tr>
<tr>
<td>Simmental</td>
<td>51</td>
<td>0.118</td>
<td>0.061</td>
<td>0.024</td>
</tr>
<tr>
<td>Angus</td>
<td>86</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*n = number of cattle studied.

SE = standard error of gene frequency.

8.3.2 Joint inheritance of antigens W6 and W11

Three cows (A8, A9 and A10) from the same herd of 35 crossbred Charolais cattle were each typed as positive for W6, W11 and one other antigen (CA40, W10 and CA42 respectively). Over 3 years, the cows produced a total of 5 calves by 2 different sires (both sires were
negative for antigens W6 and W11). Two of the 5 offspring inherited both antigens W6 and W11 from their dams; the other 3 offspring inherited the third antigen possessed by their respective dams. The 2 sires, the 3 dams and the 5 offspring were each tested on 3 or more occasions, and each test gave the same result. All of the 9 W6 and W11 antisera gave positive reactions (>80% dead cells) when tested against the cattle which possessed W6 and W11, and all 9 antisera gave negative reactions (<20% dead cells) when tested against the cattle which lacked W6 and W11.

One antiserum which was used in the definition of W6 (SP52B; titre 4) and one antiserum which was used in the definition of W11 (BD880; titre 8) were each cross-absorbed with lymphocytes from 6 cattle. Three of these cattle possessed W6 and W11, one animal possessed W6 but not W11, one animal possessed W11 but not W6, and one animal was negative for W6 and W11. Each of the 6 absorbed samples of each antiserum was tested undiluted and at 1/2, 1/4, 1/8 and 1/16 against lymphocytes from each of the 6 cattle used for absorption. Antibody activity against W6 was removed only by lymphocytes from animals positive for W6, and activity against W11 was removed only by lymphocytes from animals positive for W11.

After immunization of H32, the peak-titre antiserum contained antibodies with a titre of 512 to all W6-positive cattle, and antibodies with a titre of 8 to W11-positive/ W6-negative cattle. The titre to W11 could not be determined in those cattle which possessed both W6 and W11, because of the masking effect of the high-titre anti-W6 antibodies. There was also a variety of other antibodies of unknown specificity. Preparative absorptions yielded an
antiserum, H32a, which reacted only with W6-positive cattle (p < 0.001) (18 of the 78 cattle tested were positive for W6), and an antiserum, H32d, which reacted apparently with a subgroup of W6 (only 3 of the 18 cattle positive for W6 reacted with H32d). Due to the low titre of antibodies to W11, it was necessary to first remove activity to W6 using preparative absorptions, and then absorb with and elute activity from W11-positive/W6-negative cells. By selecting an elution volume of one tenth of the volume of antiserum absorbed, the eluted antibodies were concentrated. After elution, an antiserum H32b was produced which reacted only with all W11-positive cells (p < 0.001).

8.4 DISCUSSION

The resolution of antigen CA21 was improved by the replacement of the single defining typing reagent with 2 antisera and their leucocyte-absorbed derivatives. These antisera were the result of selective, haplotype-matched immunizations. The existence of an antigen CA21 was confirmed by the results of these immunizations which were directed specifically against the apparent antigen.

The reactivity of the 2 new antisera (K36 and K41) suggested that antiserum BR285 was multispecific, and that "old" CA21 as defined by this antiserum in fact consisted of at least 2 determinants: (a) the determinant detected by antisera K36 and K41, which is now "new" CA21; and (b) another determinant, which was carried by the bull 850 and which was not detected by antisera K36 and K41, but which was detected weakly by antiserum BR285 (presumably by a population of antibodies which is absent from the working dilutions of antisera K36 and K41).
"New" antigen CA21 is defined by antisera from only 2 immunizations. Further immunizations, directed against animals identified as CA21-positive, would help confirm the definition of this antigen. Cross-absorptions also need to be done to check the apparent monospecificity of the absorbed antisera now used to define CA21 (Bodmer et al., 1969).

Although antigen and gene frequencies were calculated within breeds, these estimates will be reliable only if the animals sampled were representative of the breed. In this study, the number of animals screened for CA21 was quite small, and it was not possible to check the assumption that the samples were representative of the respective breeds. One interesting point is that antigen CA21 was not detected in Angus cattle in the U.S.A. in the present study. A study by Stear et al. (1987) also failed to detect CA21 (as defined by antiserum BR285) in Angus cattle in Australia. The absence of an antigen from some breeds means that it is necessary to use several breeds in the production of a range of typing antisera. In addition, before antisera can be assumed to be specific or non-reactive, they should be tested in several breeds (populations) as suggested by Dausset et al. (1965).

The equation used to calculate gene frequencies of CA21 assumes that the breeds sampled were in Hardy-Weinberg equilibrium for class I antigens (Mattiu et al., 1970). However, one cannot be certain that the populations sampled were in true random mating equilibrium. Therefore, comparisons between breeds will be informative only if there are large differences in the estimates of gene frequencies.
The joint inheritance of antigens W6 and W11 can be explained in at least 4 ways:

(a) poor antigen definition; the clusters of antisera defining the 2 antigens may in fact recognize the same antigen.

(b) serological artefact may result in the apparent presence of one of the antigens (e.g. cross-reactivity may exist, or antisera from the 2 different clusters may contain common contaminants).

(c) the 2 antigens may be coded for by 2 separate but linked loci.

(d) the 2 antigens may be coded for by a single allele (i.e. multiple determinants may be carried on the one molecule).

Both antigens appear to be well defined, as evidenced by the high correlation coefficients between the defining antisera, and the high correlation coefficients between the definition of the antigens in Canberra and in the overseas laboratories. There was no association between W6 and W11 at the population level. Therefore, it is unlikely that the 6 antisera used to define W6 and the 3 antisera used to define W11 detect a common determinant. Based on the results of the cross-absorption and immunization experiments, it is unlikely that poor antigen definition or serological artefact could account for the joint occurrence of the 2 antigens on the one haplotype.

These results are consistent with the possibility that W6 and W11 are coded for by 2 linked loci. These results are also consistent with the possibility that W6 and W11 are coded for by a single allele. For example, one allele may code for W6 without W11, one allele may code for W11 without W6, and a third rare allele at the same locus may code for the presence of both W6 and W11. Such relationships between
MHC alleles and class I antigens have been suggested in extensively studied species such as the mouse (Klein, 1981) and man (Schwartz et al., 1980). Population analysis may reveal simple subgroup relationships which are a consequence of multiple determinants on the one molecule. However, more complicated relationships between multiple determinants may not be detected by population analysis.

The two-locus explanation is consistent with results obtained regarding the MHC in other species (Albert & Gotze, 1977; David, 1977), and with results obtained when studying other bovine lymphocyte antigens (Stear et al., 1982). It is most likely that both loci would be class I genes, although this would not necessarily be the case.

Confirmation that these individual antigens belong to separate linked loci would require family data demonstrating recombination, or molecular data (e.g. from independent capping, lysostripping or sequential immunoprecipitation studies), or both. Further studies will be required to assign antigen CA21 and other BoLA antigens to any allelic series. These investigations should include population studies (members of an allelic series should not exist as triplets in individual animals) and family studies (members of an allelic series should not be inherited jointly on the one haplotype).

If antigens W6 and W11 are coded for by genes at 2 separate loci in these cattle, then the different titres to W6 and W11 produced by immunizing animal H32 may reflect differences in immunogenicity between the products of the 2 loci. In the HLA system, it has been suggested that the HIA-C class I locus products are less immunogenic than HLA-A and HLA-B locus products, and that the different class II locus products also differ in their immunogenicity (Ferrara et al., 1978; Ferrara, 1979).
Differences in immunogenicity between loci could explain the difficulty in detecting more than one locus coding for bovine class I MHC antigens. Spooner et al. (1978) provided genetic evidence for the existence of more than one linked locus, but subsequent testing suggested that their specificities were all controlled by a single locus (Oliver et al., 1981). Other investigators have described animals carrying 3 or more BoLA specificities, which might suggest that more than one locus exists (Harrison & Bull, 1980; Lewin & Bernoco, 1986). However, only the studies of Stear et al. (1982) have provided a combination of population evidence, family evidence and molecular evidence for the existence of more than one locus. As the number of defined polymorphisms increases, then it would be expected that the chance of resolving all existing loci will also increase.
The accumulated evidence from this study supports the hypothesis that antigens 3 and 4 belong to the class I antigen system of the HLA of cattle in our studies.

(4) The antibody activity defining these antigens remained after platelet absorption of the relevant alloantiserums. This suggests that platelet platelets do not express class I antigens for at least express class II antigens at a very low frequency, which agrees with the findings in other studies (see Wood et al., 1976; Coleman et al., 1973). Additionally, platelet platelet absorption was associated with a reduction in the anti-class II type activity of alloantiserums, but this was probably due to non-specific absorption (Chapter 9).

CHAPTER 9

GENERAL DISCUSSION

(5) The defining antigens reacted with B-sensitized lymphocyte panels over a range of dilution steps or titrations. At the dilutions used in namely about 1:100 dilutions, there was significant reactivity with B-sensitized lymphocyte panels (Chapter 4, 5). This cellular distinction agrees with that described for class II molecules in other species (see Sections 4.2.3 and 4.6.4).

(6) The pattern of preferential reactivity with associated lymphocytes was consistent with the reactivity of a restricted monoclonal antisera. These monoclonal antibodies have been shown to immunoprecipitate molecules of the size of the class II molecules from the surface of lymphocytes (Chapter 9).

(7) The antigens were inherited as autosomal codominant traits, and were inherited jointly with class 1 antigens in resolving with class 1 antigens C3 and 3 partially half-sibbing females, but could be distinguished from C3 by a recombination (Chapters 5, 7).
The accumulated evidence from this thesis supporting the hypothesis that antigens A-E belong to the class II antigen system of the MHC of cattle is as follows:

(a) the antibody activity defining these antigens remained after platelet absorption of the relevant alloantisera. This suggests that cattle platelets do not express class II antigens (or at least express these antigens at a very low density), which agrees with the findings in other species (van Rood et al., 1975; Colombani et al., 1976). Admittedly, exhaustive platelet absorption was associated with a reduction in the anti-class II type activity of alloantisera, but this was probably due to non-specific absorption (Chapter 4).

(b) the defining antisera reacted with B-enriched lymphocyte panels over a number of dilution steps on titration. At the dilutions used in defining class II antigens, there was minimal reactivity with B-depleted lymphocyte panels (Chapters 4, 6). This cellular distinction agrees with that described for class II molecules in other species (see Sections 1.4.2 and 1.6.4).

(c) the pattern of preferential reactivity with B-enriched lymphocytes was concordant with the reactivity of a number of monoclonal antibodies. These monoclonal antibodies have been shown to immunoprecipitate molecules of the size of the class II molecules from the surface of lymphocytes (Chapter 5).

(d) the antigens were inherited as autosomal co-dominant traits, and were inherited jointly with class I haplotypes in families. Class II antigen B was in close linkage with class I antigen CA30 in 2 paternal half-sibling families, but could be distinguished from CA30 by a recombination (Chapters 6, 7).
This class II antigen system appears to be the same as that described in cattle by Newman et al. (1982a). The existence of more than one BoLA class II subregion has been suggested by biochemical studies (Carter et al., 1986) and DNA hybridization studies (Andersson et al., 1986a; 1986b), and the activity of the monoclonal antibodies described in Chapter 5 supported this suggestion. Multiple class II subregions have been described in the mouse and in man (see Sections 1.4.1 and 1.6.2).

The 5 defined antigens most likely represent only a small fraction of the total polymorphism of this antigen system, given the polymorphism that exists in other species which have been studied extensively (Klein et al., 1983; Bodmer, W. et al., 1984).

The serological definition of class I antigens is simpler than that of class II antigens, as there is no need to absorb alloantisera with platelets and no need to enrich for target cells from peripheral blood lymphocytes. Consequently, the definition of BoLA class I antigens has progressed further, and there is evidence for the existence of 2 allelic series, coded for by closely-linked loci (Chapter 8).

There are 3 further areas to be considered. The first concerns ways of accelerating progress in the definition of BoLA polymorphisms. The second area concerns the possible use of naturally occurring or artificially contrived bovine haemopoietic chimeras in exploring the biological function of the MHC. The third area concerns the likelihood of using BoLA typing in the selection of breeding stock, on the basis of associations which may occur between BoLA polymorphisms and disease states.
Possible ways of improving the speed and ease of serological definition of BoIA antigens include automated reading of test trays (Naipal et al., 1984), and the use of frozen cells or propagated lymphoid cell lines of defined MHC type as target cells when screening unknown antisera (Flynn et al., 1966; Bodmer, J., 1978a). To date, serology has been the principal tool used in the definition of MHC polymorphisms in most species, but it has its limitations. Completely monospecific antisera are rare and the resolving power of antisera is not sufficient to identify some polymorphisms detectable by other methods (see Section 1.11). In addition, it is sometimes difficult to exchange reagent antisera between laboratories. In the definition of class II antigens, platelet absorption is very time-consuming and there is always the potential problem of residual anti-class I activity in antisera.

As discussed in Section 1.11, the study of MHC polymorphisms by restriction fragment length polymorphism (RFLP) analysis of DNA offers greater discriminatory power than serology. In addition, tissue can be harvested and stored indefinitely before analysis and the results are archival, thereby allowing easier comparisons between laboratories. Limited RFLP analysis of BoIA class II polymorphisms has recently been described (Andersson et al., 1986a; 1986b; Vaiman et al., 1986; Andersson & Rask, 1988). It may be that BoIA polymorphisms could now be defined more rapidly by RFLP analysis than by serological analysis, although both methods will probably be of value at least until more is known about the BoIA system.

There is probably much more polymorphism remaining to be defined in the BoIA system, given the existing and speculated polymorphism in the MHC of the mouse and man (Klein, 1981; Klein et al., 1983; Bodmer,
W. et al., 1984). More work needs to be done in defining BoLA polymorphisms before disease association studies can be carried out efficiently in cattle. Unless this is done, there is a risk that associations will be missed.

The definition of the MHC in a particular species will be advantageous for the exploitation of that species as an object of both basic and applied research. As a model for basic research, one of the notable features of cattle is the high incidence of naturally-occurring haemopoietic chimeras in cases of dizygotic twins (Owen, 1945; Owen et al., 1946). Now that the means are available to expand clones of T cells of a single specificity and to split embryos, these chimeras provide a model to test the hypothesis of thymic learning (see Section 1.9.3). An example of one experimental approach to this question is detailed below.

Embryos A and B, which are determined to be of different MHC type, are collected. They are split to obtain 2 pairs of genotypically identical embryos, A1 and A2, and B1 and B2. Embryo A1 is transferred with embryo B1 to one surrogate mother. Calves A1 and B1 will probably be haemopoietic chimeras and share each others blood cells (this can be checked easily). Embryos A2 and B2 are transferred singly to surrogate mothers.

T cells of the A genotype existing within calf B1 will be genetically identical to T cells from calf A2. The thymic learning hypothesis suggests that T cell specificity is determined by the MHC type of the host thymus and not by genes of the T cell precursors. Therefore, T cell clones of the A genotype from calf B1 should be of different specificity to T cell clones of the A genotype from calf A2. Sensitized T cell clones of the A genotype from calf B1 should be able
to be stimulated \textit{in vitro} by antigen presenting cells from calf B2, and should be able to lyse B2 target cells \textit{in vitro}, in contrast to T cell clones from calf A2.

From an applied point of view, the interest in defining the MHC of cattle is concerned mainly with the search for disease associations. Desirable polymorphisms could be selected for in breeding stock. Selection could be carried out early in an individual's life without having to wait and test the fitness of that individual later on. The very large number of offspring that a bull may sire by artificial insemination (particularly in the case of dairy bulls) creates the potential for deleterious genes to reach a high frequency in the population. It is therefore important to be aware of the presence of deleterious genes (including disease-susceptibility genes) when selecting semen donors.

N'Dama cattle of Africa show superior innate resistance to trypanosomiasis compared with \textit{Bos indicus} cattle (Murray et al., 1982). This phenomenon could be examined in the context of the MHC types of these animals. If gene transfer is ever to be undertaken in animal production as a means of modifying the genome, relevant genes must first be mapped. In addition, if subunit vaccines with a limited number of antigenic determinants become a reality, the responses seen may be more prone to MHC-associated immune response gene effects compared with the responses to conventional vaccines. Subunit vaccine efficiency might then be improved by selecting stock with particular MHC types.

In addition to the obvious economic impact of any disease associations, detailed examination of such associations could improve
our basic understanding of just how MHC molecules carry out their biological function.

It must be borne in mind, however, that genetic resistance or susceptibility to disease will also be influenced by non-MHC immunological factors, and by non-immunological factors. In addition, the existence of a very large number of MHC polymorphisms in cattle will make the search for MHC-disease associations more difficult. The total number of alleles coded for by the MHC is likely to be very large, at least in the mouse and in man (Klein, 1981; 1987), and this may also be the case in cattle. The number of polymorphisms will be even greater than the number of alleles, due to the existence of public (non-allele-specific) determinants and hybrid class II molecules. In the mouse, the serologically best characterized MHC molecule has been reported to carry at least 30 determinants (Klein, 1981). Public polymorphic parts of an MHC molecule may have the same functional importance as do private (i.e. allele-specific) polymorphic parts of the molecule, and may be just as likely to be involved in disease associations.

However, the number of frequently found MHC polymorphisms is likely to be much lower than the total number of polymorphisms. In addition, it may be that only a few factors play a limiting role in influencing whether exposure to a pathogen leads to disease, and only a few genes may be responsible for most of the variation in the outcome. To be of economic significance, a disease marker would not have to be an "all-or-none" marker for the presence or absence of the disease. If for example, the presence of a particular marker accounted for only 20% of the variation between individual animals in the occurrence of a particular disease, then such an association may
nevertheless be of economic significance. The interest would not only be in the obvious acute infectious disease situation. Lower productivity associated with the "tail" of a herd is probably partly due to relative susceptibility to chronic and/or subclinical infections.

It is possible that selection for resistance to a particular pathogen may lead to decreased protection against other pathogens. Selection for a single disease-resistance marker could also lead to the loss of other desirable genes, such as those controlling production traits, from the population. The aim, however, would generally be to increase the frequency of the disease-resistance marker in the population and at the same time maintain selection for production characters, rather than to select only for the single disease-resistance marker. Culling animals on the basis of susceptibility genes would be less likely to alter greatly the frequency of other desirable genes.

To date, there have been few confirmed associations between the MHC and infectious diseases in other species. However, if a disease association does exist in a particular species, the only way to identify and subsequently exploit the association is to study that species. In addition, the definition of the MHC of a species would be of value if the MHC is found to have a role in other physiological functions, for example in cell differentiation.
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