THE CLASS I AND CLASS II ANTIGEN SYSTEMS
IN CATTLE AND THEIR ASSOCIATION WITH DISEASE

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DOCTOR OF PHILOSOPHY

by

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STATEMENT

The experiments reported in Chapter 5 were done in collaboration with Dr. M. J. Stear of the John Curtin School of Medical Research, Department of Immunology and with Dr. R. G. Holroyd of the Department of Primary Industries, Swan's Lagoon Beef Cattle Research Station. The skin grafting reported in Chapter 6 was done by Professor B. Morris of the John Curtin School of Medical Research, Department of Immunology. All other work reported in this thesis is my own.

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ABSTRACT

Studies were undertaken to characterise further the bovine major histocompatibility complex (BoLA) with special reference towards the description of antigens and immune properties which are associated with the immune response genes (I region) of other species. The results demonstrated the existence of a B lymphocyte antigen system, in vitro mixed lymphocyte reactivity, and differential levels of resistances to tick infestation, all of which were shown to be genetically linked to or associated with the BoLA complex. This suggests the existence of an I region in cattle and demonstrates further the similarities of the BoLA complex and the major histocompatibility complexes of other species.

The bovine Class II antigen system was described using standard serological techniques. The two-colour fluorescence microlymphocytotoxicity test was used to screen 114 alloantisera for the presence of antibodies specific for B lymphocyte antigens. At least 47 antisera were shown to contain anti-B lymphocyte antibodies and 27 of these were selected for further study and absorption analyses. Absorption testing with leucocytes, platelets and erythrocytes showed that these antigens have a restricted distribution on blood cells, being present primarily on B lymphocytes. Additional absorption testing showed that several antisera contained multiple antibody specificities, suggesting a complex organisation of the B lymphocyte antigens. Typing reagents were prepared by removing antibody to Class I antigens by absorptions with leucocytes and platelets. These reagents were then used to type for B lymphocyte antigens in 10 large paternal half-sib families, the results of which demonstrated that the B lymphocyte antigens were expressed as autosomal
co-dominants, were inherited in a straightforward Mendelian manner and were linked to the BoLA complex. The distribution of these antigens on blood cells and the BoLA linkage of their genetic control suggested that these antigens were part of the Class II antigen system in cattle.

Mixed lymphocyte reactivity (MLR) was assessed by measuring the extent of cellular proliferation between allogeneic lymphocytes in an in vitro system. To test for involvement of the BoLA complex, the MLR tests were done using full-sibling cattle families which had been generated through the use of embryo transfer systems. The results showed that the responses in the MLR tests were associated with the BoLA antigen sharing status between the individuals being tested. Since the tests were done using full-sibling combinations this result suggests genetic linkage between the MLR controlling antigens and the BoLA complex.

A group of 199 cattle were experimentally infested with ticks and tested to ascertain their BoLA Class I antigen types. This group was then used to test for an association between levels of tick resistance, which are influenced by both genetically controlled and immunological components, and the BoLA complex. Three associations between BoLA Class I antigenic specificities and tick resistance levels were found. Two BoLA antigen types were associated with high resistance and one specificity with low resistance. The reasons behind these associations are not known, but several possibilities are discussed.

The antibody responses to a xenogeneic antigen and to antigens of the BoLA complex were compared. This showed that cattle respond to a xenogeneic antigen in a fashion which resembles the typical
antibody response, but that antibody responses to allogeneic antigens were different and much more variable. The responses to Class I and to Class II antigens were also compared following different immunisation procedures.

The results presented in this thesis are discussed in light of the known similarities of the major histocompatibility complexes of several species and with special reference to the associations with disease susceptibility and resistance.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 1: GENERAL INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>Terminology</td>
<td>1</td>
</tr>
<tr>
<td>I Region Definition</td>
<td>2</td>
</tr>
<tr>
<td>The MHC Association of the I Region in the Mouse</td>
<td>3</td>
</tr>
<tr>
<td>The MHC Association of the I Region in the Rat</td>
<td>4</td>
</tr>
<tr>
<td>The MHC Association of the I Region in the Guinea Pig</td>
<td>7</td>
</tr>
<tr>
<td>The Class II Antigen System; Serology, Tissue Distribution and Biochemical Characterisation</td>
<td>7</td>
</tr>
<tr>
<td>Functional Expression of Class II Antigens in the Mouse</td>
<td>14</td>
</tr>
<tr>
<td>Functional Analysis of Class II Antigens in the Rat and the Guinea Pig</td>
<td>18</td>
</tr>
<tr>
<td>The I Region Equivalent in Man</td>
<td>21</td>
</tr>
<tr>
<td>Mixed Lymphocyte Reactions (MLR) in Man</td>
<td>23</td>
</tr>
<tr>
<td>HLA-D Region Associated Antigens (HLA-DR)</td>
<td>27</td>
</tr>
<tr>
<td>The Structure and Genetic Analysis of the Bovine Population</td>
<td>38</td>
</tr>
<tr>
<td>CHAPTER 2: MATERIALS AND METHODS</td>
<td></td>
</tr>
<tr>
<td>Solutions</td>
<td>43</td>
</tr>
<tr>
<td>Experimental Animals</td>
<td>44</td>
</tr>
<tr>
<td>Collection of Blood Samples and Immunisations</td>
<td>47</td>
</tr>
<tr>
<td>Analytical and Preparative Methods</td>
<td>48</td>
</tr>
<tr>
<td>Preparation of Xenoantisera</td>
<td>49</td>
</tr>
<tr>
<td>Separation of Cells from Blood</td>
<td>52</td>
</tr>
<tr>
<td>Detection and Characterisation of Bovine Antibodies</td>
<td>53</td>
</tr>
<tr>
<td>Production of Alloantisera</td>
<td>54</td>
</tr>
<tr>
<td>Absorption of Alloantisera</td>
<td>61</td>
</tr>
<tr>
<td>Tissue Culture</td>
<td>63</td>
</tr>
<tr>
<td>Statistical Methods</td>
<td>64</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimentation with Cattle Ticks</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>CHAPTER 3: SEROLOGICAL AND GENETIC IDENTIFICATION OF A BOVINE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B LYMPHOCYTE ALLOANTIGEN SYSTEM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Experimental Methods: Identification of antisera with anti-B lymphocyte activity</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Experimental Methods: Population and family studies</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>CHAPTER 4: MIXED LYMPHOCYTE REACTIVITY IN CATTLE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Experimental Methods</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>CHAPTER 5: THE RELATIONSHIP BETWEEN THE BoLA COMPLEX AND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RESISTANCE TO THE CATTLE TICK, Boophilus microplus</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>Experimental Methods</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>CHAPTER 6: THE ALLOGENEIC ANTIBODY RESPONSE IN CATTLE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>Experiment 1: Comparison of the antibody response to allogeneic lymphocytes, xenogeneic protein and xenogeneic erythrocytes in cattle</td>
<td>140</td>
</tr>
</tbody>
</table>
CHAPTER 1

GENERAL INTRODUCTION
TERMINOLOGY

Researchers have demonstrated the existence of a Major Histocompatibility Complex (MHC) in all species of higher vertebrates so far studied (Gotze, 1977). Accompanying this is a large collection of confusing, species specific nomenclature and terminology. In an attempt to present this review in a uniform manner, the terminology used will follow that suggested by Klein (1977). This will include the description of loci or antigens by "Class" and a differential use of the terms "genes" or "loci" and genetically defined "regions". This terminology will be used for the description of systems in all inbred species and in outbred animals where applicable. The exception to this will be the HLA system in man where the well established HLA terminology will be used. In this instance, and the others where the use of species specific nomenclature is required, all terms will be described in detail with respect to their relationship to the standard nomenclature.

The term "B lymphocyte" was defined as a lymphocyte which expressed cell surface immunoglobulin. "T lymphocyte" is generally defined as a lymphocyte which does not express cell surface immunoglobulin and which forms a rosette with xenogeneic erythrocytes of the appropriate species. Definitions relating to functional properties were not used except in special cases, which are explained as necessary.
I REGION DEFINITION

The concept that the capacity of the immune system to respond to antigenic stimulation is under genetic control was first suggested in 1921 to explain differences in resistance to bacterial infection in guinea pigs (Wright and Lewis, 1921). This contention was supported by breeding experiments in which good and poor responders to diphtheria toxoid were purposely bred (Schiebel, 1943). Gorer and Schutze (1938) demonstrated similar genetic differences between strains of inbred mice. Numerous other experiments on the immune response involving both inbred and random-bred mice and guinea pigs followed (McDevitt and Benacerraf, 1969).

The first specific immune response (Ir) gene was identified in guinea pigs by using the synthetic haptenated homopolymer dinitrophenyl-poly-L-lysine (DNP-PLL) (Kantor et al. 1963; Levine et al. 1963a). The ability to respond to this synthetic antigen was shown to be inherited as a simple Mendelian dominant character. Further experimentation demonstrated that a particular strain of guinea pig (Strain 2) could respond to all PLL-hapten complexes while another strain (Strain 13) could not. The gene determining this immune response was termed the PLL Ir gene (Levine et al. 1963b). A variety of synthetic polypeptides was studied in mice and these experiments gave similar results to those obtained with guinea pigs.

The first evidence that these Ir genes were linked to the major histocompatibility complex (MHC) was obtained in 1968 from the results of cell transfer experiments. The ability of mice to respond to the multichain polymer, poly (L-tyrosine : L-glutamic acid) poly-DL-alanine : poly-L-lysine, (T,G)-A--L, or to multichain
poly (L-phenylalanine : L-glutamic acid) poly-DL-alanine : poly-L-lysine), (Phe, G)-A--L, was transferred between responder and non-responder strains by immune spleen and lymph node cells (McDevitt and Tyan, 1968). This cell transfer experiment and the results of a genetic analysis of backcrosses suggested that the Ir gene was linked in some way to the MHC. It was first thought that the linkage was not exceptionally tight as the recombination frequency of the genes appeared to be around 5%.

MHC linkage of the Ir-PLL gene was demonstrated in the guinea pig using genetic backcrossing experiments between strains 2 and 13 (Ellman et al. 1970). Subsequently, immune response genes defined by other antigens were also shown to be linked to the MHC (Bluestein et al. 1971; Green and Benacerraf, 1971; Green et al. 1970).

The mouse Ir-1 gene was actually mapped within the H-2 complex (between the K and D regions) of chromosome 17 using recombinant strains of mice in a cooperative effort between several laboratories, thus defining the "I region" (McDevitt et al. 1972). The current usage of the term I region denotes the region between the H-2K and S regions to which genes regulating the immune response are mapped (Klein et al. 1974a). This definition roughly fits the MHC of the guinea pig and rat although actual map positions for these species are not as well established (Ellman et al. 1970; Gunther and Stark, 1979).

THE MHC ASSOCIATION OF THE I REGION IN THE MOUSE

The H-2 gene complex constitutes the MHC of the mouse. This gene complex is divided into 6 major regions: K, I, S, G, D/L, and T (David, 1979; Klein, 1979)(Figure 1.1). Antigens coded by genes
Figure 1.1: A genetic map of the H-2 complex; taken from Klein (1979). The exact order of regions in the dotted line brackets is not known.
in the K and D regions are serologically defined and represent the classical transplantation antigens (David, 1977). These antigens act to restrict genetically the generation of cytotoxic T lymphocytes (Zinkernagel, 1978). The L region maps with the D region and controls the expression of serologically defined antigens, similar to those of the K and D regions. These antigens share the public specificities and have the same biochemical properties of K and D region antigens (Demant and Neauport-Sautes, 1978) but do not share any of the private specificities (Ivanyi et al. 1979). The S region contains genes which control quantitatively the levels of a serum protein Ss (Shreffler and Owen, 1963), now identified as complement component C4 (Meo et al. 1975b), and the presence of the sex-linked variant of this protein, S1p (Passmore and Shreffler, 1970). The G region controls a serologically defined antigen which is preferentially expressed on erythrocytes (David et al. 1975; Klein et al. 1975) and is apparently expressed as part of the C4 molecule (Huang and Klein, 1980). Neither the genes nor the products of the T region have been completely defined. This region codes for alloantigens (Qa1, Qa2, Qa3), all of which have their own tissue distribution and strain distribution (Flaherty, 1976; Flaherty et al. 1978; Forman and Flaherty, 1978; Klein and Chiang, 1978), as well as the Tla locus which controls the expression of a system of alloantigens (TL antigens) on the thymocytes of some strains and on some types of leukaemic cells (Old and Stockert, 1977). The I region maps between the K and S region, codes for the Class II alloantigens and is subdivided into three subregions; A, B, C, based on functional criteria (Shreffler and David, 1975).
THE MHC ASSOCIATION OF THE I REGION IN THE RAT

The MHC of the rat (RT1) is also fairly well defined (Gunther and Stark, 1979). Analysis of recombinant strains has been used to divide the gene complex into two regions, A and B. The RT1-A region determines the expression of the classical transplantation (Class I) antigens. These are controlled by two loci although the exact map order is not known (Gunther and Stark, 1979; Sporer et al. 1978). The RT1-B region controls the expression of the Class II antigens (Radka et al. 1977; Shinohara et al. 1977) as well as regulating the responsiveness to synthetic polypeptide antigens. This suggests that this region is analogous to the I region in the mouse (Davis et al. 1979; Gunther, 1979; Gunther and Stark, 1979). A third region (RT1-C) controls the expression of several antigens and is apparently analogous to the mouse T region (Stock and Gunther, 1979). The exact map of the RT1 complex is not yet known although two models have been suggested, one which resembles the mouse H-2 complex (Natori et al. 1979) and one which resembles the HLA region of man (Gunther and Stark, 1979).

THE MHC ASSOCIATION OF THE I REGION IN THE GUINEA PIG

The MHC of the guinea pig is not as well defined as its counterparts in the mouse and rat. Presently, one or possibly two Class I antigen loci have been defined serologically (Geczy et al. 1975; Sato and de Weck, 1972). The guinea pig I region was first defined by functional assays and subsequently shown to be linked to the MHC (Ellman et al. 1970). This region also appears to control the expression of Class II antigens (Geczy et al. 1975).

THE CLASS II ANTIGEN SYSTEM; SEROLOGY, TISSUE DISTRIBUTION AND BIOCHEMICAL CHARACTERISATION
Mouse

The functional properties of the I region prompted a search for serologically detectable antigens which were coded for by this region. The original experiments in mice involved the use of antisera generated by cross-immunisation between strains which were identical at the K and D regions but differed at the I region and possibly the S region (David et al. 1973; Gotze et al. 1973; Hauptfeld et al. 1973). Cells from lymph nodes, spleens and thymuses were used as the source of antigen. These antisera recognised antigens present on a subpopulation of lymphocytes from lymph nodes and to a lesser extent lymphocytes from spleen and peripheral blood. Some thymus cells also appeared to express these antigens but erythrocytes and bone marrow cells did not (David et al. 1973). The antigen-positive cells were first thought to be T lymphocytes (Gotze et al. 1973; Hauptfeld et al. 1973) but subsequent studies identified them as B lymphocytes (Hammerling et al. 1974). These antigens were termed Ia (I region associated) antigens (Shreffler et al. 1974) and later Class II antigens (Klein, 1977).

The Class II antigen system is highly polymorphic and 33 specificities have been defined in 11 inbred strains of mice (Klein et al. 1978). Studies with wild mice suggest the degree of polymorphism is actually much higher (Duncan et al. 1979; Wakeland and Klein, 1979). As found with the murine Class I antigens, public and private Class II antigen specificities exist within the strains and are present on the same gene product (Cullen et al. 1976).

The large number of recognised antigenic specificities and the differential expression of these specificities found between strains suggests that the I region is very complex, bearing out the results
of the earlier functional analyses. The use of recombinant strains of mice in conjunction with absorption and cytotoxicity tests has allowed the identification of 5 I subregions (A, B, J, E and C) (Shreffler et al. 1976) which control the expression of cell surface Class II antigens and Ir gene function (Figure 1.1). Recently, the existence of a sixth region, I-N, has been suggested to explain anomalous reactivity in the mixed lymphocyte reaction. The I-N region codes for an antigen which can be detected serologically and is thought to map between the H-2K and I-A regions (Hayes and Bach, 1980). Most of the Class II antigen specificities found on B lymphocytes map to the I-A and I-E/C subregions. Originally, specificities were mapped to the I-C subregion but most of these have since been shown to map in the closely linked I-E subregion (Cullen et al. 1980; David and Cullen, 1978). Because of this confusion the two subregions are usually considered together (I-E/C). The I-J subregion codes for antigens present on a subpopulation of T lymphocytes (Murphy et al. 1976; Shreffler et al. 1976). The I-B sub-region is defined functionally on the basis of a few Ir genes and does not appear to control the expression on any Class II antigens (Shreffler and David, 1975).

When Class II antigens were first identified there was some confusion as to whether they were expressed on B lymphocytes, T lymphocytes and/or both (David et al. 1973; Hauptfeld et al. 1973). It was shown subsequently that these antigens are expressed primarily on B lymphocytes (Hammerling et al, 1974) but they are also present on some T lymphocytes (Murphy et al. 1976; Shreffler et al, 1976). Experiments involving cross-absorption of antisera suggested that the same Class II antigen specificities were expressed on both
cell types (David et al. 1976) but titration experiments using spleen, lymph node, and thymus cells demonstrated the presence of two antibody specificities in certain antisera and it was suggested that different antibodies may recognise antigens on either B or T lymphocytes (Frelinger et al. 1974). Class II antigens are also present on blast cells, generated by different mitogens (David et al. 1976; Hauptfeld et al. 1975). Class II antigen positive macrophages have also been detected, originally by indirect immunofluorescence and cytotoxicity tests (Hammerling et al. 1975; Unanue et al. 1974). These appear to represent subpopulations of macrophages as the presence or absence of the antigen, the antigen density and the subregion controlling the expression of the antigen (I-A or I-E/C) vary between different populations of macrophages isolated by different methods and taken from different parts of the body (Cowing et al. 1978). Class II antigens have also been found on foetal liver cells and spermatozoa using absorption and cytotoxicity tests (Hammerling et al. 1975). Their presence on epidermal cells has also been suggested by absorption experiments (Frelinger et al. 1978; Hammerling et al. 1975) and by the generation of antibody following skin grafting between H-2D and K matched but I region disparate strains (Klein et al. 1974b). However, the use of electron microscopy and immunoferritin labels has suggested that the expression of Class II antigens is restricted to epidermal macrophages, the so-called Langerhans cells (Rowden et al. 1978). The expression of Class II antigens on tumours varies and probably reflects the antigen expression of the cell from which the tumour originated (Frelinger et al. 1974; Okuda et al. 1978; Schwartz et al. 1977a)
Class II antigens are generally isolated in pure form from the cell surface by dissolving the cell membranes with detergent and then precipitating the antigens with specific alloantisera. Analysis of the precipitated antigens by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has shown that Class II antigens are composed of two non-covalently linked polypeptide subunits designated α (30 - 35,000 daltons) and β (25 - 30,000 daltons) (Cullen et al. 1974; Delovitch and McDevitt, 1975; Vitetta et al. 1974). The variability in molecular weight is due to allelic differences between the strains and to the slight differences between the I-A and I-E/C subregion products (Cook et al. 1978; Uhr et al. 1979). Although most of the biochemical studies have been done with cells originating from B lymphoid tissue, the Class II antigens found on other cell types and tumours appear to be similar (Cowing et al. 1978; Frelinger et al. 1981; Frelinger et al. 1978; Okuda et al. 1978; Schwartz et al. 1977a). Analysis by trypsin digestion and limited amino acid sequencing has shown that the allelic α subunits from different strains are very similar, 84 to 97% homologous, whereas the β subunits vary much more, with only 48 to 69% homology. This suggests that it is the β subunit which is responsible for the polymorphism (Cook et al. 1978; Cook et al. 1979; Silver, 1979). Very little homology exists between the products of the I-A and I-E/C subregions (Cook et al. 1978; Uhr et al. 1979). Biochemical techniques have also been used to ascertain the molecular relationships between different antigen specificities and subregions. Sequential immunoprecipitation of membrane lysates dissolved in detergent has shown that the public and private antigenic specificities are incorporated into the same
molecule (Cullen et al. 1976). Evidence from isoelectricfocusing experiments suggests that the \( \beta \) chain of the I-E/C region antigen dimer is actually coded for by the I-A subregion with only the \( \alpha \) chain under I-E/C control (Jones et al. 1978; Uhr et al. 1979).

Class II antigen products are known to contain carbohydrate. Isolated antigenic material is bound by several lectins and this binding is destroyed by pretreatment of antigen with neuraminidase. In addition, \(^3\)H labelled sugars can be biosynthetically incorporated into these antigens. This radioactive label can be removed or reduced by treatment with several different glycosidases (Cullen et al. 1975).

Other types of I region antigens have been identified recently. These antigens are of low molecular weight, they are soluble and glycolipid in nature. They were originally found in normal mouse serum using antisera raised in rabbits (Parish et al. 1976a; Parish et al. 1976c). The identification of these antigens and the determination of their allospecificity has been accomplished by inhibition of antibody-mediated assays. Although a xenoantiserum was originally used to describe these antigens, numerous alloantisera have now been identified which also appear to recognise these new antigens (Parish et al. 1978). It has been claimed that the actual antigenic specificity resides in the carbohydrate portion of the antigen. Recognition by antisera can be blocked by the addition of one or more oligosaccharides to the test system (McKenzie et al. 1977). These antigens are actively synthesised and secreted by a subpopulation of T lymphocytes which do not express surface Class II antigens (McKenzie and Parish, 1976; Parish et al. 1976b). They are closely related to the protein Class II antigens by both physical and
serological criteria such as similar tissue distribution, distribution of allospecificity in strains and physical organisation on the cell surface (Parish et al. 1978; Parish and McKenzie, 1978a).

It has been suggested that these carbohydrate molecules may actually represent the functional I region antigens. Such a conclusion implies that there must also exist glycosyl transferase enzymes whose synthesis is controlled by I region genes. This could be the protein portion of the currently recognised glycoprotein Class II antigens (Parish et al. 1976c). However, there is little evidence to support this theory and it seems just as conceivable that these molecules are actually by-products of normal cellular metabolism. The variation in serum levels of these antigens in animals with parasitic infections or diseases known to effect lymphoid cells, lends support to this idea (Parish et al. 1979). There is other evidence which suggests that the functional component of the I region antigen which determines Class II antigen specificity is protein in character (Cullen et al. 1975).

Rat and guinea pig

The Class II antigen systems of the rat and guinea pig appear to be similar to that of the mouse with respect to tissue distribution and MHC linkage (Catto, et al. 1977; Geczy et al. 1975; Hart and Fabre, 1979; Radka et al. 1977; Schwartz et al. 1977b; Shinohara et al. 1977; Wiman et al. 1978). Biochemical analyses of these antigens have demonstrated a structure which is also similar to the H-2 Class II antigens. There are two subunits with molecular weights (MW) estimated in the ranges of 25-26,000 daltons for the α subunit and 33-35,000 daltons for the β subunit of the guinea pig (Schwartz et al. 1977b) and 28,000 daltons and 35,000 daltons for the α and β
subunits, respectively of the rat (Shinohara et al. 1977; Sporer et al. 1978).

The actual numbers of regions or loci controlling the expression of these antigens are not known. In the guinea pig 3 Class II antigen products with slightly different molecular weights can be isolated by sequential immunoprecipitation and this was originally interpreted as evidence for 3 loci (Schwartz et al. 1977b). However, these differences in molecular weights may also be due to differences in the cellular origin of the antigens. B lymphocytes and subpopulations of T lymphocytes and macrophages all express Class II antigens which differ slightly in molecular weight and in terms of their properties when subjected to isoelectric focusing even though all appear to be the same by serological tests (Schwartz et al. 1979). In the rat, at least two loci have been identified by sequential immunoprecipitation and biochemical analysis (Carpenter et al. 1979; Sporer et al. 1978) and by analysis of genetic recombinant strains (Natori et al. 1979). As with the mouse, both public and private antigenic specificities have been identified (Gotze, 1978; Lowry and Carpenter, 1980; Schwartz et al. 1977b; Shevach, 1978).

THE FUNCTIONAL EXPRESSION OF CLASS II ANTIGENS IN THE MOUSE

Histocompatibility in the mouse

Although the Class I antigens are usually thought of as the major transplantation barrier, the I region and Class II antigens have also been implicated in the rejection of allografts. Skin grafts were rejected between mouse strains which differed only at the I region and this was followed by the production of antibody against Class II antigens suggesting an active role of these antigens in the rejection process (Klein et al. 1974b). More
extensive analysis of the serology of skin graft rejection suggested that the I-A/B subregion represents a strong transplantation barrier; the I-E/C subregion is also involved but it probably represents a weak barrier (Klein et al. 1976). These ideas are supported by graft-versus-host (GVH) and cell-mediated lymphpolysis (CML) assays which have demonstrated that some I region gene products can serve as targets or recognition units. In these assays the I-A/B subregion gene products provoke the greatest response although the I-E/C subregion gene products are also recognised (Klein, 1977; Klein et al. 1976) and can provoke a great response if the correct I-E/C allelic combination is present (Clark and Hildemann, 1977). Both the CML and GVH responses can be specifically blocked by the addition of antiserum to Class II antigens, and this suggests further that it is the actual antigen on the cell surface which is recognised in the reactions (Clark and Hildemann, 1977; Klein et al. 1977).

Mixed lymphocyte reactivity (MLR) in the mouse

The in vitro allogeneic lymphocyte response is dependent on disparity between the lymphocyte-activating determinants (Lads) of the two populations of cells. The control of MLR by the H-2 complex was first demonstrated by Dutton (1966) using congenic strains of mice. Further experimentation using intra-H-2 recombinant strains localised the control to the I region (Bach et al. 1973; Meo et al. 1973) and in particular, to the I-A/B subregion, although some level of stimulation could be assigned to the I-E/C subregion (David, 1977; Dorf et al. 1975b; Okuda and David, 1978). These data suggested that the I region either controls the ability of the cell to respond or that the Class II antigens are themselves the
Lads. This latter suggestion is supported by the fact that antisera to Class II antigens of the stimulator cell population can specifically inhibit MLR (Meo et al. 1975a; Schwartz et al. 1976) while antisera to the Class I antigens or antisera to antigens of the responder cell population have no effect. The ability of a cell to respond to allogeneic stimulation may also be under I region control, and this suggests that there may be a specific Ir gene (Dorf et al. 1975b).

Class II antigens and Fc receptors in the mouse

The exact nature of the surface membrane Fc receptor and antigens of the H-2 complex has never been fully elucidated. Original reports demonstrated preferential expression of Fc receptors on B lymphocytes, suggesting that they may be associated with Class II antigens. This idea was supported by the fact that antisera to Class II antigens specifically inhibited the binding of substances to Fc receptors while antisera to Class I antigens had no effect (Dickler and Sachs, 1974). Further experimentation showed that Fc receptors were associated with Class II antigens on T lymphocytes (Dickler et al. 1976) as well as with some non-H-2 antigens on both B and T lymphocytes (Dickler et al. 1977; Dickler et al. 1975). Other studies in which different systems of analysis have been used have shown that there are Fc receptors on several different cell populations. A differential inhibition of Fc receptor activity has been demonstrated with antisera which recognise both Class I and Class II antigens (Halloran et al. 1975). These results suggest that a simple Class II antigen - Fc receptor relationship does not exist.
Class II antigens and immune responsiveness in the mouse

Recently published reviews suggest that the I region is involved in several aspects of the immune process such as antigen presentation, cell recognition, cell-cell interactions and immune regulation: the net effect of this involvement is to either enhance or suppress the immune response (Benacerraf, 1978; Munro and Taussig, 1975). The actual way in which the Class II antigens are involved is not known but they may play a part in several of these steps. The antigen presentation model suggests that the foreign antigen and the Class II antigens on macrophages bind specifically together. These complexes are then presented or released to T lymphocytes which in turn trigger either helper or suppressor functions (Benacerraf, 1978). This theory is supported by studies in which helper T lymphocyte function and subsequent antibody production were only generated in the presence of antigen-primed, syngeneic macrophages (Kapp et al., 1973; Niederhuber et al., 1979). The actual gene(s) involved have been mapped to the I-A subregion (Erb and Feldmann, 1975). The requirement for cell combinations which express the same I-A subregion antigens suggests that Class II antigens play a role as a cellular recognition unit (Erb and Feldmann, 1975).

Cellular factors which are controlled by the I region have also been implicated in the generation and control of the immune response. Helper factors generated by macrophages and T lymphocytes have been identified. The generation of these helper factors appears to be controlled by the I-A subregion. Helper activity can be removed with antisera against I-A subregion antigens. This removal of helper function is specific, requiring antisera of the proper allospecificity
suggesting that the helper factors either are associated with I-A subregion antigens or are themselves the recognised I-A antigen products (Erb and Feldmann, 1975; Taussig et al. 1975). The macrophage helper factor described by Erb and Feldmann (1975) has been shown to function in a restricted manner, interacting only with cells of the same I-A antigenic specificity. Such restriction has not been demonstrated for the T lymphocyte factor (Tada et al. 1976; Taussig et al. 1975). Suppressor T lymphocyte factors have also been identified. One factor which suppresses the secondary MLR appears to be generated by and to be under the control of the I-E/C subregion genes. The appropriate Class II antigen specificities can be detected on these factors (Mempel et al. 1973). Another T lymphocyte suppressor factor has been identified which is controlled by and carries antigens of the I-J subregion (Tada et al. 1976). The Class II antigens of T lymphocyte subpopulations and their associated factors are of different specificities than those expressed on B lymphocytes. They also appear to differ for different T lymphocyte subpopulations as the selective killing of lymphocytes with antisera directed against the appropriate Class II antigen specificity abrogates only the function of that subpopulation (Okumura et al. 1976).

FUNCTIONAL ANALYSIS OF CLASS II ANTIGENS IN THE RAT AND THE GUINEA PIG

Guinea pig

Although the I region of the guinea pig is not as well defined as that of the mouse, numerous studies have been undertaken to examine the relationship between Class II antigens and immune responsiveness (Shevach, 1978). Experiments have usually involved
measurements of the proliferation of T lymphocytes and of the plaque-forming cell response that follows in vitro primary and/or secondary immunisations with simple antigens. The effects of antisera to Class II antigens on these responses is then followed. The results of these studies have been interpreted as showing a direct association between responses controlled by Ir genes and Class II antigens. Most in vitro responses are specifically inhibited by the addition of antisera. The expression of Class II antigens on immunocompetent cells of the guinea pig is similar to that of the mouse. Most B lymphocytes express cell surface Class II antigens as do subpopulations of macrophages and T lymphocytes. The subpopulation of T lymphocytes that expresses Class II antigens is involved in the proliferative response to antigens and mitogens suggesting a functional expression of Class II antigens similar to that of the mouse. Class II antigens have not been detected on alloreactive or helper T lymphocytes subpopulations.

Some of the most informative experiments that have been done in the guinea pig have been concerned with the population of macrophages which carry the Class II antigens. It has been demonstrated that antigen-primed macrophages that are positive for Class II antigens, are required for primary T lymphocyte activation and proliferation. Destruction of these macrophages with antisera to Class II antigens plus complement stops all T lymphocyte activation to both allo-antigens, mixed lymphocyte reactivity, and soluble antigens. These results support the theory that Class II antigens and macrophages are required for antigen presentation to be an effective stimulant for lymphocyte proliferation. This macrophage-T lymphocyte interaction was first thought to be restricted by the I region but it
now appears that allogeneic macrophages can be used if the allo-
reactive T lymphocyte subpopulation is removed prior to the primary
antigen challenge. The immune T lymphocytes then show preferential
cooperation with macrophages of the same I region antigen type as
those used in the primary response, whether they are allogeneic
or syngeneic. This suggests that the I region restriction of
macrophage-T lymphocyte interactions is involved only in the gen-
eration of the secondary response.

In general, the results of experiments with guinea pigs support
the functional theory which proposes that the Class II antigen-
positive subpopulation of macrophages is important for the presenta-
tion of antigen in the initiation of the immune response.

Rat

The relationship between the Class II antigen system and
immune function in the rat is not as well defined as in the mouse
and guinea pig. However, the similarities between the species,
especially with respect to the MHC, suggest that the system probably
functions in a similar way.

As with other species, the Class II antigen system has been
mapped to the I region and is associated with MLR (Radka et al.
1977). One of the more exceptional aspects of the rat MHC is that
the association between Ir gene functions, Class II antigens and
MLR, which have been defined with inbred strains, has been shown to
hold for the wild rat population, suggesting that some type of
natural selection pressure acts on the MHC (Gunther, 1979; Shonnard
et al. 1979).

Probably the most valuable results to come out of research on the
rat MHC pertains to the role of Class II antigens in transplantation
biology. Studies involving attempts to extend the survival time of allografts have shown that the Class II antigens are directly involved. If these antigenic determinants are blocked by injecting antisera into the graft recipient then the survival time of the graft is prolonged (Catto et al. 1977; Davies, 1975; Staines et al. 1975). Although much of the early work involved skin grafting techniques applied to mice (Davies, 1976; Staines et al. 1975) more extensive experimentation was done in the rat with vascular grafts, such as heart (Davies, 1975) and kidney (Catto et al. 1977; Soulilou, 1976). The actual mechanism of this graft enhancement is not yet understood but it may well involve an interruption at the early events of recognition of the foreign graft (Davies, 1975), possibly at the level of the macrophage. This would be consistent with the theory that Class II antigens are necessary for antigen presentation. The discovery that antisera to the more common public Class II antigen specificities also enhance graft survival (Lowry and Carpenter, 1980) suggests that this method may be adaptable to clinical use in outbred species, such as man.

**THE I REGION EQUIVALENT IN MAN**

No I region has yet been identified in man. It seems likely that such a region does exist because there is indirect evidence for it and there are known similarities between the MHC of man (HLA) and the MHC's of mouse and rat (Gill et al. 1978). Most of the evidence for the existence of a human I region has accumulated in the last decade with the improvement of HLA typing procedures and the establishment of an HLA genetic map. Concurrently, the susceptibility of numerous diseases has been found to be associated with certain HLA types (Albert and Gotze, 1977; Dick, 1978). The
actual reasons for these disease associations are not known but several feasible hypotheses exist (Svejgaard et al. 1975). Firstly, it is possible that HLA antigens resemble or cross-react with antigens of pathogenic organisms, thus interfering with the recognition step by the immune system. Secondly, a particular HLA antigen may act as a receptor for a pathogen to attach to target cells thus causing an increased susceptibility to the infective agent. However, only scant evidence exists for a few disease states in man to support either of these theories. The association of the HLA antigenic specificity, B27, and ankylosing spondylitis is believed to be caused by a cross-reaction between the antigens of the HLA complex and the possible causative pathogen, Klebsiella pneumoniae (Ebringer et al. 1978). This theory is supported by the results of in vitro leucocyte proliferation tests. The leucocytes from B27 positive individuals respond at a lower level against K. pneumoniae than do leucocytes from B27 negative individuals. B27 positive individuals respond normally against other types of bacteria. The specific lack of responsiveness to K. pneumoniae may involve a lack of recognition of the bacterial antigens. This is supported by reactivity patterns of antisera raised in rabbits against K. pneumoniae which also react with human, B27 positive, lymphocytes (Seager et al. 1979). There are, however, no known examples of HLA antigens serving as pathogen receptors. One example does exist in the erythrocyte antigen system (Duffy) where the presence of the Duffy antigen is required for infection by Plasmodium knowlesi (a causative agent of human malaria) to occur (Miller et al. 1975)

Another attractive explanation for the apparent associations between certain diseases and HLA type is that MHC-linked Ir genes
may be involved. This idea is supported by the increased incidence of certain disease states within families, suggesting that some genetic basis is involved (Albert and Gotze, 1977).

Other evidence for the existence of an I region in man has come from studies involving the immune responses of humans to several antigens. HLA-associated differences in the magnitude of antibody responses have been reported following infection with measles virus (Haverkorn et al. 1975) and *Plasmodium falciparum* (Osoba et al. 1979) as well as for poliomyelitis virus and diphtheria toxoid following vaccination (Haverkorn et al. 1975). Additionally, in vitro cellular responsiveness to tetanus toxoid also appears to be controlled by the HLA complex (Sasazuki et al. 1978). It has also been reported that several types of allergic reactions and serum IgE levels may be associated with certain HLA haplotypes (Marsh and Bias, 1977; Marsh et al. 1977).

**MIXED LYMPHOCYTE REACTIONS (MLR) IN MAN**

In vitro reactions between allogeneic lymphocytes were documented in man early on (Bain et al. 1964; Hirschhorn et al. 1963) and were shown to be associated to the HLA locus (Bach et al. 1967) and to be polymorphic in nature (Bach and Amos, 1967). The observation that lymphocytes from HLA identical siblings did not stimulate in MLR tests suggested that the Class I antigens may be the actual activating determinants (Bach et al. 1969). However, it was demonstrated subsequently that the Class I antigens were not the lymphocyte activation determinants, but were closely linked to the gene(s) controlling them (Yunis and Amos, 1971).

It was the possible association of MLR with organ transplantation that provided a stimulus for research into the genetics.
of these reactions. A method of using MLR antigen homozygous typing or reference cells was established to enable MLR antigen typing to be done on a large scale (Jørgensen et al. 1973). However, the apparent high degree of polymorphism associated with MLR made it extremely difficult to locate homozygous individuals in a random set of paired tests. Class I antigen typing was used in familial groups which were limited in their HLA antigen diversity to help in the identification of these individuals. Groups with some slight degree of inbreeding such as cousin marriages (Jørgensen et al. 1973; van den Tweel et al. 1973) or families whose parents shared a detectable specificity were chosen (Dausset et al. 1973; DuPont et al. 1973; Mempel et al. 1973).

The identification of reference cells and the exchange of these cells between laboratories enabled the MLR to be defined rapidly and its relationship to the HLA region was established. The gene(s) involved in the stimulation of the major reactivity are now known to map in linkage with the Class I antigens, approximately one centimorgan from HLA-B (Mickelson et al. 1976), and to code for at least 12 allelic antigenic determinants (Bernoco et al. 1980). This region is now known as the HLA-D region. Although most observations have suggested that a single system or set of D region MLR antigens are responsible for effecting stimulation, the actual numbers of genes involved are not know. Anomalous MLR typing results within families have been interpreted as evidence supporting the existence of two D region genes which control the expression of MLR antigens (DuPont et al. 1974; DuPont et al. 1975a; DuPont et al. 1975b). Alternatively, the presence of multiple antigenic determinants on a single molecule, cross-reactivity or regulation of the reaction by an Ir
gene have also been postulated (DuPont et al. 1975a; DuPont et al. 1975b; Osoba and Falk, 1978). The existence of genes not controlled by the HLA-D region has also been established. This second set of gene(s) maps close to or within the HLA-A region and controls a weak MLR response (Bijnen et al. 1977; Fainboim and Festenstein, 1979; Suciu-Foca and Dausset, 1975).

As has been previously reviewed, the expression of MLR antigens (Lads) in the mouse, rat and guinea pig is controlled by the I region. The establishment of an MHC-linked region controlling MLR function in man adds support to the proposition that there is also an I region in man. In addition to this, several HLA associated diseases and immune functions show even stronger association with HLA-D region antigen types (Jersild et al. 1973; Keuning et al., 1976; Sasazuki et al. 1978). This evidence adds further support to the theory that the HLA-D gene region represents the I region equivalent in man.

MHC association of the HLA-D region

The HLA complex represents the MHC of man. This complex is divided into 4, closely linked regions; A, B, C and D (Figure 1.2) (Albert and Gotze, 1977; Bodmer, 1978). Antigens controlled by genes of the A, B and C regions are serologically defined. They are highly polymorphic and are thought to be equivalent to the H-2K,D and possibly to the L region antigens (Class I antigens). As with the H-2K and D/L antigens, the HLA-A,B and C antigens are considered to be classical transplantation antigens (Dausset et al. 1970). Also associated with the HLA complex are loci coding for complement components C2 (Fu et al. 1974), C4 (Rittner et al. 1975), the equivalent of Ss protein in the mouse (Meo et al. 1975b), and
Figure 1.2: A genetic map of the HLA complex; taken from Bodmer (1978b). The exact map positions of the complement components C2, C4 and BF are not known.
Chromosome 6

HLA Region

BF
C4
C2

D/DR B C A
properdin factor B(Bf) (Allen, 1974). The loci controlling these complement components map in the HLA-D region (Raum et al. 1981). The erythrocyte blood group antigen system of Chido and Rogers is also associated with the HLA complex. These antigenic determinants are present on the complement component C4 and appear to be analogous to the product of the H-2G region (O'Neill et al. 1978). As previously described, the D region controls antigens defined in MLR culture assays, and probably represents part of the human I region.

**HLA-D REGION ASSOCIATED ANTIGENS (HLA-DR)**

**Description and serology**

The HLA-DR antigen system was identified through a rather indirect series of observations. It was first noticed that serum antibody, particularly IgG, could inhibit the MLR test. These antibodies were raised by allogeneic immunisation either by kidney transplantation or as a result of pregnancy (Hattler et al. 1971; Revillard et al. 1973). Some specificity was apparent in the inhibition as antibody from a non-immune third party individual did not have the same effect and immune serum did not always inhibit the MLR in unrelated cultures. This inhibition could be demonstrated in two ways; antibody against either the stimulator cells or the responder cells could suppress reactivity. Similar results were seen with some HLA-typing sera and this suggested that HLA antigens were involved (Ceppellini et al. 1971), and that it might be possible to type MLR antigens serologically (van Leeuwen et al. 1973). The use of indirect immunofluorescence methods demonstrated that the antigens which were recognised in these tests were expressed on a subpopulation of lymphocytes, probably B lymphocytes. Absorption experiments showed
that the antigens were not expressed on platelets (van Leeuwen et al. 1973). This information led to the development of special lymphocyte-typing techniques which used enriched populations of B lymphocytes (Mann et al. 1975; van Rood et al. 1975a; Terasaki et al. 1975) or transformed B lymphoid cell lines (Bodmer et al. 1975a; Fellous et al. 1975; Winchester, et al. 1975a) and antisera absorbed with platelets to remove antibody to the HLA Class I antigens, which are expressed on platelets (van Rood et al. 1975a). The new antigen system described was HLA-linked (Bodmer et al. 1975b; Winchester et al. 1975b), closely associated with the D region (van Rood et al. 1975b; Solheim et al. 1975), and was thought to represent the HLA equivalent of the Class II or Ia antigen system of the mouse (Wernet et al. 1975).

The number of loci controlling DR antigens is not known. The lack of any complete antiserum banks for typing and the fact that recombination events could go unnoticed makes the accurate definition of the DR region much more difficult than the Class II antigen system of the mouse. To limit these difficulties, small isolated groups or communities which were inbred to varying degrees were selected as subjects. Both lymphocyte and antiserum donors (sera from parous women) were obtained from members of the Amish community of Pennsylvania and two DR antigen loci were defined (Mann et al. 1976). Other recombinant families were similarly documented (Kamoun et al. 1977; Walford et al. 1977) and together with the results of cocapping studies further evidence was obtained for the idea that at least two loci controlled DR antigens (van Rood et al. 1977). The map position of the second locus is not yet certain. It has been suggested that the second locus may map in or around the HLA-A
region, possibly in association with the second, weak MLR locus (Abelson and Mann, 1978; Johnson et al. 1977). The second locus has also been mapped to the area between the HLA-A and B regions (van Rood et al. 1977) and a DR type locus outside of the accepted HLA region has also been suggested (Park et al. 1978).

Currently, the existence of one DR antigen locus is generally accepted. This locus codes for the expression of 10 serologically defined antigen specificities (Bernoco et al. 1980). In addition, the existence of supertypic or cross-reacting "families" of antigen specificities has been established (Albert et al. 1977; Kovithavongs et al. 1978; Park et al. 1980), in line with the idea that there is a public/private organisation of the antigens.

**Tissue distribution and biochemical analysis**

The expression of HLA-DR antigens on peripheral blood lymphocytes was thought to be restricted primarily to B lymphocytes. This has been the assumption used for the development of most assays designed to identify DR antigens by cytotoxicity. These antigens are now known to have a much wider tissue distribution. Although this type of antigen is expressed on most peripheral B lymphocytes it is not expressed on all cells originating in B lymphoid tissue. Mature plasma cells do not generally express DR antigens nor do some plasma cell lymphoblastoid lines. Plasma cells without DR antigen can be isolated from normal individuals and they can be generated in vitro by treating lymphocytes, using Poke Weed Mitogen (PWM) (Halper et al. 1978). The absence of DR antigens in mature cells suggests that the antigens may function as some type of differentiation antigen.
The use of more sensitive assays and techniques of differential cell separation has shown that these antigens are present on other peripheral blood lymphocytes. A small subpopulation of DR antigen positive T lymphocytes was identified by fluorescent antibody and rosetting techniques. This population consists of 1 to 5% of the total T lymphocyte population in normal individuals (Greaves et al. 1979; Yu et al. 1980b). The percentage of these cells is increased in certain disease states, and following immunisation with antigens such as PPD and tetanus toxoid (Ko et al. 1979; Yu et al. 1980b). Their numbers can also be increased in vitro in populations of lymphocytes subjected to stimulation with allogeneic lymphocytes, MLR, or by several common mitogens (Evans et al. 1978; Greaves et al. 1979; Ko et al. 1979). It is not known whether this increase is due to clonal expansion of the small subpopulation of naturally occurring DR antigen-positive T lymphocytes or whether it is an induced expression of antigens on T lymphocytes which are normally serologically defined as negative.

Cytotoxicity tests and immunofluorescence methods in which both xenoantisera and alloantisera are used have detected DR antigens on macrophages. From 30 to 100% of the macrophages purified from peripheral blood express these antigens (Albrechtsen, 1977; Winchester et al. 1975b). The degree of fluorescent staining is highly variable, suggesting that the amount or type of antigen expressed may vary with different macrophage populations (Winchester et al. 1976). Immunofluorescent staining of single cell suspensions and tissue sections has also demonstrated the presence of DR antigen on Langerhans cells of the skin (Stingl et al. 1978).

Monoclonal antibodies which detect the species-common portion
of DR antigens have been used in conjunction with $^{125}$I and immuno-
binding assays to detect and quantitate these antigens in various
tissues. Cells from the kidney and liver were found to possess 90% and 19%, respectively, of the DR antigen found on spleen cells.
The specific location of these antigens on the cells could not be
determined. Small amounts of DR antigen were also found on heart
cells, thymus cells and bone marrow cells. Brain cells, erythro-
cytes, platelets and reticulocytes did not contain detectable quan-
tities of DR antigen (Williams et al. 1980).

The expression of HLA-DR antigens by tumour cells is generally
limited to those tumours of B lymphoid cell origin. Many individuals
affected with either acute or chronic lymphoblastic leukaemias have
circulating leukaemic cells which express DR antigen (Fu et al. 1975;
Halper et al. 1979), there are however, also negative DR antigen
types of leukaemia (Winchester et al. 1978). These cells are
readily maintained in culture and have been used for DR antigen re-
search (Barnstable et al. 1978; Bodmer et al. 1975a). Several
malignant melanoma cell lines and one epithelial carcinoma have also
been shown to express DR antigen (Carrel et al. 1979; Winchester
et al. 1978) but, in general, these types of tumours are negative
for DR antigen (Carrel et al. 1979; Winchester and Kunkel, 1979).

DR antigen was identified on human spermatozoa by absorption
studies and by immunofluorescence (Wernet, 1976). Other analyses,
however, have not substantiated this claim (Law and Bodmer, 1978).

The DR antigens have been characterised biochemically by the
same methods used for the characterisation of Class II antigens in
mice. Lymphoblastoid cell lines are generally used as a source of
DR antigens because of their availability, which is related to the
ease with which they can be grown in cultures, and the abnormally high concentration of HLA antigens expressed on their surface (Barnstable et al. 1978). Antigenic material is removed from the cell membranes by dissolving the membranes in detergent and then purified by a variety of techniques. The purification of the antigens is monitored progressively by the inhibition of specific allo-antisera (Snary et al. 1976). Analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) has shown that DR antigens have a two subunit structure; an α subunit (33–34,000 daltons) and a β subunit (28–29,000 daltons) (Snary et al. 1976; Springer et al. 1976). Peptide mapping of trypsin and pepsin digests and N-terminal amino acid sequence analysis have demonstrated a high degree of homology between α subunits but little homology was demonstrated between β subunits of different allelic products. This suggests that the β subunits may be responsible for the polymorphic differences recognised serologically (Corte et al. 1981; Walker et al. 1980). The use of these techniques has also shown that there is a remarkable degree of homology between the DR antigens of man and the I-E/C subregion antigens of the mouse, suggesting their equivalence in the respective species. Class II antigens from the I-A subregion show little similarity with DR antigens (Allison et al. 1978; Silver et al. 1979). The results of biochemical experiments also support the proposition that there are two linked DR antigen loci in man (Katagiri et al. 1979; Mann et al. 1979; Tanigaki et al. 1980), and a public/private antigen organisation similar to that which exist in the mouse (Tosi et al. 1978)

Antigen products of the DR region have been shown to contain carbohydrate by the staining of SDS–PAGE gels with periodic acid
Schiff reagent and by their binding to lectins. The major carbohydrate present on both subunits appears to be sialic acid (Snary et al. 1976; Springer et al. 1976). Additionally, "carbohydrate defined" DR antigens, similar to those of the mouse, have also been demonstrated (Sandrin et al. 1979a). Co-capping and antibody inhibition studies suggest that these carbohydrate specificities are linked to or are part of the protein DR antigen molecules (Sandrin et al. 1979b). The levels of these antigens in serum vary during the course of certain diseases and this may prove to be of some value in clinical diagnosis treatment.

**DR Antigens; Functional definition and histocompatibility**

The effects of DR antigens on the survival of allografts have not yet been well characterised. Most data available are derived from retrospective studies on frozen lymphocytes taken from individuals who had previously undergone kidney transplantation. These studies indicate that matching the donor and recipient for DR antigens can enhance the survival of grafted tissue. Up to 76% of the kidneys exchanged between DR antigen matched donor - recipient pairs survived the first year. Between 30-38% survived the same period when only one DR antigen specificity was common to both donor and recipient and this survival percentage dropped to between 18-30% between unmatched donor - recipient combinations. These results are irrespective of matching for HLA-A, B and C antigens; if these other antigens are also matched then the survival chances of the graft are increased (Albrechtsen et al. 1979; Albrechtsen et al. 1978b; Thorsby; 1979). One exciting clinical aspect of these results is that it should be easier to match organ donors and recipients for DR antigens than it has been for the other HLA antigens because of the more
restricted polymorphism of the DR antigen system (Ting and Morris, 1978). A limited study with skin grafts supports the importance of matching for DR antigens (Jonker et al. 1979).

DR antigens and MLR

Although DR antigens were first discovered through their association to MLR, the actual details of this relationship are not yet established. The existence of DR antigens was first demonstrated by the inhibition of MLR by the use of antibodies against antigens on stimulator cells (van Leeuwen et al. 1973). This suggested that DR antigens were the actual MLR activating determinants (Lads). It has since been shown that antibodies to other antigens can also inhibit MLR under certain circumstances. These antibodies include those directed against HLA-A and B antigens and to antigens not yet identified, but believed not to be part of the HLA complex. MLR can be inhibited in some of these instances by antibodies which recognise antigens on the responder cell population as well as on the stimulator cell population (Jonker et al. 1977; Jonker and van Rood, 1978). It is apparent from these experiments that the DR antigens are not the only cell surface determinants involved in MLR.

Support for the existence of an association between the serologically defined DR antigens and the D antigens defined by cellular reactions comes from the predictive value of DR antigen types on MLR tests. The DR antigen types have been used to predict such activity within families, some of which contained individuals with intra-HLA region recombinations (Albrechtsen et al. 1978a). Additionally, specificities defined by DR antisera were highly associated with D specificities, defined by homozygous typing cells, at the population level (Bodmer, 1977). However, neither of these "associations" are
without exceptions. Several cases of anomalous MLR have been reported within families (Park et al. 1977; Reinsmoen et al. 1979) and it has been suggested that the DR and D antigens may be controlled by two closely linked, but separate, loci. The report that possible recombinations occur between these loci support this theory (Decary et al. 1979; Sachs et al. 1981; Suciu-Foca et al. 1978b) but conclusive evidence has not so far been documented. In addition to this, the associations that have been found from typing results done on European and American cell donors (a predominantly caucasian sample) have not held up in other populations (Sasazuki et al. 1977; Troup et al. 1978). At present one of the most widely accepted theories is that two serologically defined loci exist, one which is identical to the MLR or D antigen loci and one which maps to the same region, but is not directly involved in MLR (Albert and Götte, 1977; Balner, 1979).

**DR antigens and Fc receptors**

Fc receptors and DR antigens are known to occur on the same cells. Both the antigen and receptor are present on B lymphocytes, on some macrophages and on a subpopulation of T lymphocytes (Wernet et al. 1975; Winchester et al. 1975b). The relationship between DR antigens and Fc receptors is not clear. Some Fc receptor activity can be blocked by DR antisera, suggesting a close physical association between the two (Arbeit et al. 1977; Rieber and Wernet, 1977). However, there also appears to be some Fc receptors which are not associated with DR antigens (Arbeit et al. 1977; Winchester et al. 1976) suggesting the presence of several different types or populations of Fc receptors, which are detectable by different techniques (Arbeit et al. 1977; Winchester et al. 1979).
The immune response and DR antigens

The suggestion that DR antigens and the cells expressing them are important in immunological reactions has come from studies with lymphocytes from individuals subjected to different types of immune challenge. The proportion of circulating T lymphocytes positive for DR antigen is usually low; about 2.6% of the total T lymphocyte population. This proportion is increased greatly during certain diseases such as rheumatoid arthritis, systemic lupus erythematosus and several bacterial infections. Additionally, this particular T lymphocyte subpopulation can be increased by immunisation with several antigens such as PPD and tetanus toxoid (Yu et al. 1980b). A similar increase in the proportion of DR antigen-positive T lymphocytes occurs after in vitro stimulation of peripheral blood lymphocytes by allogeneic cells (Evans et al. 1978; Suciu-Foca et al. 1978a) PPD and tetanus toxoid (Ko et al. 1979) and several mitogens (Albrechtsen et al. 1977a; Greaves et al. 1979; Ko et al. 1979). Mitogen responses can be inhibited by treating lymphocytes with DR antiserum and complement before culturing them (Albrechtsen et al. 1977a) This demonstrates the direct involvement of these antigen-positive cells in these responses.

The DR antigens also appear to function as recognition units which control co-operation between cells in immune functions and they may also determine genetic restriction. Macrophages modified by the addition of trinitrophenyl (TNP) to their cell-surface antigens can serve as an "altered self" antigen source for initiating the in vitro T lymphocyte proliferative response. Both primary and secondary responses require the presence of macrophages which express the same DR antigen specificities as the proliferating T lymphocyte population
(Seldin and Rich, 1978). The sharing of one DR allelic product will also work but not as well as a fully-matched combination (Seldin and Rich, 1978; Thorsby and Nousiainen, 1979). Shared DR antigen specificities are required for the generation of the in vitro response to PPD (Bergholtz and Thorsby, 1977; Bergholtz and Thorsby, 1979) while antibody to DR antigens shared by the macrophages and T lymphocytes will inhibit the response (Bergholtz and Thorsby, 1978).

Helper and suppressor functions also appear to be associated with DR antigen-positive T lymphocytes (Broder et al. 1980; Fu et al. 1978; Hirschberg and Thornsby, 1977) but it is not known whether cell to cell interactions, soluble factors, or possibly both, are involved. The existence of both helper and suppressor factors has been demonstrated (Fu et al. 1978; Larsson and Blomgren, 1979; Yu et al. 1980a) but no detailed characterisation of these materials has yet been published.

Suppressor factors can generally be found in the supernatant fluid from cell cultures responding to mitogens. These factors function in a fairly non-specific fashion and no association has been shown between these factors and DR antigens. More specific types of suppressor factors have been obtained from MLC supernatants. The function of these factors is associated with DR antigens to the extent that the production of them can be stopped by the addition of DR antisera to the proliferating cell population. The suppressor factors are not removed by treatment with DR antisera and are not thought to carry DR antigenic determinants as has been suggested in the mouse (Larsson and Blomgren, 1979; Yu et al. 1980a). Helper factors are also associated with DR antigen-positive T lymphocytes but also do not express any DR antigenic determinants. Helper function is also usually antigen specific and requires the presence of
the stimulating immunogen in the assay system (Fu et al. 1978; Yu et al. 1980a).

The existence of an I region has been demonstrated in at least two other mammals, the rhesus monkey (Dorf et al. 1975b) and the dog (Vriesendorp et al. 1977). In addition to this, the fact that MLR has been demonstrated in numerous species (Gotze, 1977) suggests a widespread existence of this type of gene region for controlling the immune response. Thus far, these systems appear to be similar to the better defined I regions of other species and will not be reviewed further.

THE STRUCTURE AND GENETIC ANALYSIS OF THE BOVINE POPULATION

The choice of the model

The best described MHC's are those of inbred laboratory animals, particularly the mouse. This situation is not unexpected since the mouse is well suited for this type of research. The short generation interval of mice, their inbred nature and the opportunity to do planned breeding experiments have been instrumental in both the development of the current H-2 map and in the functional studies. At the opposite end of the spectrum is the research involving the characterisation of the HLA system. In man the use of outbred genetic analysis and the longevity of individuals under study have allowed for rapid progress in research into the human MHC. The lack of planned breeding experiments has been overcome by systems of analysis which are done at the population level and the utilisation of the great genetic diversity present in the human population. The information gathered can then be used at the family level for more in-depth studies.

Research on the MHC's of other species has generally followed
the methods and used the tools devised for research in either the man or the mouse. The choice of the techniques employed and of the subsequent analysis is dependent on the genetic makeup of the population to be studied. The outbred large animal species such as rhesus monkey, pig and sheep required an approach similar to that used in man. Animal species with inbred lines such as guinea pig, rat and chicken could be studied with the techniques used for analysing the MHC of the mouse.

In cattle the obvious choice of a model is the HLA complex in man. However, the genetic differences in the human and cattle population mean that the system for analysing the HLA complex cannot be used without some modification. It is also important to use the peculiarities of the species under study to the best advantage. There are several specific characteristics of the bovine which must be considered. Firstly, at the population level cattle are many times kept segregated according to the breeds. These breeds are still considered to be generally outbred but the genetic variation is expected to be more limited within breeds than between breeds. By definition this means that purebred herds are likely to be inbred when compared to all other cattle. In addition, importation restrictions have meant that purebred herds in Australia have been relatively isolated and this has increased, to some extent, the level of inbreeding. This is true for several of the commonly used breeds, including Jersey (Barker, 1957), Hereford (Davey and Barker, 1963), Polled Hereford (Barker and Davey, 1960) and the Australian Illawarra Shorthorn (Herron and Pattie, 1977a; Herron and Pattie, 1977b). The limited and more recent importation of Zebu breeds of cattle has also led to a restricted genetic pool of Australian herds (Herron, 1978).
The exact influence that these breed differences might have on genetically controlled traits or marker antigens can only be speculated upon and is not within the scope of this review. However, it is obvious that these genetic limitations must be considered in the analysis of any data when studying any bovine genetic system.

Bovine immunogenetics

Immunogenetics research in cattle began with studies on the erythrocyte antigen systems and on serum protein polymorphisms. These systems probably represent the most comprehensively studied non-histocompatibility blood group systems of any species, except for the erythrocyte antigen systems in man. Currently, 11 erythrocyte antigen systems and several serum protein systems are well enough defined in cattle to be used in standard typing services (Stormont, 1978).

The highly polymorphic nature of some of these systems has led to the suggestion that one or more of them may represent the bovine MHC (Ivanyi, 1977). This idea was supported by the early work of Borovska & Demant (1967) who showed with several alloantisera which had been raised by whole blood immunisations that a significant correlation existed between lymphocytotoxic patterns and erythrocyte antigen types. Additionally, the existence of the erythrocyte and serum antigenic substance "J" has been demonstrated on the lymphocytes of cattle (Hruban and Simon, 1973). Subsequent reports have not supported these findings and it is now apparent that the common antigen systems of erythrocytes and lymphocytes are not shared (Folger and Hines, 1976; Ostrand-Rosenberg and Stormont, 1974).

Several different research groups are currently involved in attempts to define the bovine MHC. So far, only a limited number of
serologically defined antigens (Class I) have been described accurately. Most evidence suggests that all of the presently defined specificities belong to the same genetic system which may be single-locus or multi-loci in nature (Adams, 1980; Amorena and Stone, 1978; Caldwell and Cumberland, 1978; Spooner et al. 1978). The system is highly polymorphic (Caldwell, 1979) and large variations in the frequency of various antigenic specificities are found in different breeds (Caldwell et al. 1979; Oliver et al. 1981). The sharing of antigenic specificities between unrelated cattle has a profound influence on skin graft survival times and this suggests that these antigens are coded for by the bovine MHC (Adams, 1980; Amorena and Stone, 1978). In a cooperative effort involving nine laboratories at the First International Bovine Lymphocyte Antigen Workshop, eleven antigenic specificities were defined and this putative MHC system was named BoLA (Spooner et al. 1979b).

The existence of an MHC linked I region in the cow is only a speculative proposition at present (Adams et al. 1979) although the presence of an MHC which is similar to those of other species suggests that an I region may yet be defined. The demonstration of MLR in cattle also suggests the existence of such a region (Usinger et al. 1977). The variation in immune responsiveness seen in different cattle can also be taken to suggest some type of genetic control. This includes the antibody responses to several antigens, including normal allogeneic erythrocytes (Sellei and Rendel, 1968), Babesia argentina infected erythrocytes (Dimmock, 1973), Brucella abortus vaccines (Kaneene et al. 1979), KLH (Kateley and Bazzell, 1978) and human serum albumin (Lie, 1979). The differential levels of resistance to natural pathogenic and parasitic infections also
appear to have a genetic component. There are well-documented differences between breeds in susceptibility to pink-eye infections (Dodt, 1977) and the infestation with the cattle tick (*Boophilus microphus*) (Utech *et al*. 1978b). Genetically influenced resistance to other common diseases has been suggested but as yet these suggestions have not been adequately documented (Spooner *et al*. 1975).

It is the known existence of a MHC in cattle and the differential levels of resistance and susceptibility, some of which are under genetic control, which has prompted the interest in the putative bovine I region. Definition of a bovine I region by the serological description of associated antigens (Class II antigens) is probably considered as the most important and most feasible objective. In addition, knowing the exact relationships of the Class I antigens, which have been defined previously (Adams, 1980) with the other aspects of the BoLA complex, such as MLR and Class II antigens, can also be seen as essential in providing a thorough description of the bovine MHC. Finally, the actual use of BoLA antigen typing in diseased or parasite infested populations to test for associations between the BoLA complex and resistance or susceptibility levels is required. The description of such associations may well serve as the foundation upon which future attempts to select and breed for resistance in cattle will be based. The experiments described in this thesis were directed towards this goal.
CHAPTER 2

MATERIALS AND METHODS
SOLUTIONS

Physiological solutions

General Remarks: All physiological solutions were freshly made. They were sterilized by filtration through a Millipore membrane (0.22 μm filter, Millipore Corp., Bedford MA, U.S.A.) if they were to be stored for a long time. All chemicals used were of analytical reagent grade.

(i) Saline: 9.0 g of sodium chloride (NaCl) was dissolved in one litre of distilled water, making a 0.9% solution.

(ii) Phosphate buffered saline (PBS): 8.0 g of NaCl, 1.2 g disodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O) and 0.39 g sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O) were dissolved in distilled water, and the volume made up to one litre. The pH of this solution ranged from 7.2 to 7.4.

(iii) EDTA - saline: Appropriate quantities of EDTA·Na₂ were dissolved in saline and the pH adjusted to 7.4 with 2M NaOH. The solution was made to the desired volume with saline. Three EDTA - saline solutions (0.5%, 2.7% and 5.0%) were used.

(iv) Formaldehyde - saline: A 1% solution of formaldehyde was made by adding 2.5 ml of formaldehyde solution (40% w/v) to 90ml of saline. The pH was adjusted to 7.4 with solid potassium hydroxide (KOH) and made up to 100 ml with saline.

(v) Citrate - saline: A 0.1M citrate - saline solution was made by adding enough 0.1M sodium citrate (C₆H₅O₇Na₃·2H₂O) - saline solution to a 0.1M citric acid (monohydrate) - saline solution to adjust the pH of the solution to 3.
(vi) 2-mercaptoethanol (2-ME) - saline: A 0.2M solution was made by diluting 1.38 ml 2-ME to 100 ml with saline. Dilute 2-ME solutions were used immediately or stored frozen, at -25°C.

(vii) Alsever's solution: 18.66 g glucose, 8.0 g sodium citrate, 0.55 g citric acid and 4.2 g NaCl were dissolved in distilled water. The pH was adjusted to 6.1 with citric acid and the volume made up to one litre with distilled water.

(viii) Tissue culture media: Eagle's Minimum Essential Medium (MEM), F-15 and H-16 media were obtained from GIBCO (Grand Island, N.Y., U.S.A.) and RPMI-1640 medium from Commonwealth Serum Laboratories (CSL, Melbourne, Vic., Australia) in powdered form and made up as directed. Media were sterilized by membrane filtration and stored at 4°C. For tissue culture, small quantities were taken and modified by the addition of foetal calf serum (FCS, CSL) (final concentration of 10%) 2-ME (final concentration 10⁻⁴M) and antibiotics (penicillin 100 U/ml, streptomycin, 100 μg/ml and neomycin, 100 μg/ml).

Buffers for chromatography and electrophoresis

(i) Gel filtration buffer: This buffer was prepared by dissolving 0.27 g potassium dihydrogen orthophosphate (KH₂PO₄) and 1.14 g NaH₂PO₄·2H₂O in saline to a volume of one litre. The pH of the solution was about 7.3.

(ii) Anion exchange buffers: A 0.4M phosphate buffer solution pH 8.0 was made up by mixing 947 ml of stock solution 1 (71.2 g Na₂HPO₄·2H₂O made up to one litre in distilled water) with 53 ml of stock solution 2 (62.4 g NaH₂PO₄·2H₂O made up to 1 litre in distilled water). Other phosphate buffers solutions were made by appropriate dilution of the 0.4M buffer solution.
(iii) Immunoelectrophoresis buffer: Tris-barbiturate buffer, pH 8.6, was made up with pre-measured preparations obtained from LKB-Produtker AB (Sweden). Two packets were dissolved in 1 litre of distilled water.

Other solutions

(i) Ficoll - Isopaque: 6.46 g of Ficoll - 400 (Pharmacia, Uppsala, Sweden) was dissolved in 72 ml of distilled water. One vial (30 ml) of 32.8% sodium metrizoate (Nyegaard and Company, A/S. Oslo, Norway) was added to this solution. The specific gravity of the final solution was approximately 1.075. The solution was stored in the dark at 4°C and warmed to 20°C before use. The Ficoll solution was autoclaved before the addition of the sodium metrizoate when a sterile solution was required.

(ii) Erythrocyte shock lysis solution "ACE": 8.0 g of NH₄Cl, 0.1 g KH₂PO₄ and 1.0 g EDTA-Na₃ were dissolved and made up to 1 litre in distilled water.

(iii) Eosin Y Dye: 5.0 g of Eosin Yellowish (Sigma Chemical Co., St. Louis, MO., U.S.A.) was dissolved in 100 ml saline, filtered and stored at 4°C.

(iv) Buffered formaldehyde: A solution of formaldehyde (40% w/v) was adjusted to a pH between 7.2 - 7.4 with concentrated KOH solution. The solution was stored at room temperature and filtered before use.

(v) Ethidium bromide dye: 1 mg of ethidium bromide (Sigma) was dissolved in 10 ml of 5% EDTA - saline divided into 1 ml portions and stored at -25°C. Prior to use the solution was thawed and diluted 1:7 in 5% EDTA - saline to a final concentration of 12.5 μg/ml of dye.
(vi) Amido black staining and rinsing solutions: 9.0 g of amido black (Sigma) was dissolved in 1500 ml of a mixture of methyl alcohol, acetic acid, and distilled water (proportions 9:2:9). The mixture without the amido black was used as a rinsing solution for removing excess stain.

**EXPERIMENTAL ANIMALS**

**Cattle:** Most of the cattle used were maintained on the Australian National University's farm, "Spring Valley". The animals were mostly crossbreeds although some purebred animals were also available. The breeds included Charolais, Brahman, Angus, Hereford, Jersey and Holstein-Friesian. Several paternal half-sibling families were made available for experiments by the Division of Plant Industry, C.S.I.R.O., Canberra. These cattle were maintained on pastures at the C.S.I.R.O. farm at Gininderra and comprised registered Charolais, Angus, and Holstein-Friesian and their crossbreeds. Blood samples were also taken from several Illawarra Shorthorn, Red Poll and Ayrshire cattle owned by the University of Sydney. Occasionally, samples were also obtained from families of privately owned purebred herds. These included Jersey, Murray Grey and Shorthorn breeds. Samples from full sibling Charolais cattle families were made available by the Hillside Charolais Stud and the Australian Transplant Breeders Pty. Ltd., Mittagong, N.S.W.

**Rabbits:** The outbred rabbits used were maintained in the Animal Breeding Establishment (ABE), A.N.U. These were housed individually and fed a free choice diet of a commercially prepared rabbit pellets (Doust and Rabbidge, Pty. Ltd., Concord West, N.S.W., Australia) and given water ad libitum.
Goose: A single goose was obtained and housed by the A.B.E. She was fed a diet of commercially prepared poultry pellets (Doust and Rabbidge, Pty. Ltd).

COLLECTION OF BLOOD SAMPLES AND IMMUNISATIONS

Cattle: Whole blood and serum samples were obtained routinely from the jugular or tail vein. Commercially prepared, sterile, 10 and 20 ml blood collection tubes were used with or without EDTA anticoagulant (Becton - Dickinson, Rutherford, N.J., U.S.A.). Disposable 18G needles and holders were used. Where larger quantities of blood were required, stoppered serum bottles which had been evacuated previously with a hand pump or a water pump were used. A short length of polyethylene tubing (20 - 30 cm in length) with a needle at both ends was used to carry the blood from the vein to the bottle. Either a 14G or 18G needle was used. A 2.7% EDTA - saline solution was used when an anticoagulant was required in a ratio of one part EDTA - saline to 4 parts blood. Each bottle could be used to collect 80 to 110 ml of blood. All of the larger volumes of blood were taken from the jugular vein.

Serum was obtained after allowing the blood to clot overnight and then removing the clot the following morning. Erythrocytes and debris were removed by centrifugation at 1500 g for 20 min at room temperature. 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 3 to 4 hr, centrifuged and stored frozen at -25°C. Antisera were inactivated prior to freezing by heating at 56°C for 30 min. Thirty to 50 ml of blood was taken from each rabbit at one time.
Goose: Blood was taken from a wing vein using a 20G needle and a syringe containing Alsever's solution. Twenty to 30 ml of blood was taken at a time and stored at 4°C in an equal volume of Alsever's solution.

Immunisations: Cattle were immunised with the appropriate antigens in sterile saline injected either intramuscularly (IM) or intravenously (IV). No adjuvants were used. Rabbits were immunised either IM or subcutaneously (SC) with the appropriate antigens emulsified in Freund's Complete Adjuvant (FCA) (Difco Laboratories, Detroit, MI, U.S.A.).

ANALYTICAL AND PREPARATIVE METHODS

General remarks: During column chromatographic separation the effluent from the column was monitored at 280 nm (8300 Uvicord II/6520 Recorder, LKB) and fractions collected automatically (UltroRac 7000, LKB). A peristaltic pump (12000 Varioperpex, LKB) was used to control the flow of buffer. Samples were dialyzed for 16 - 24 hr in the starting buffers before chromatography.

Fractions of 5 ml and more were concentrated under pressure with nitrogen gas in a Diaflo ultrafiltration apparatus (Amicon Corp., Lexington, MA, U.S.A.). The PM10 membranes (Amicon) were used. Smaller fractions were concentrated using a Minicon Macrosolute concentration unit (Amicon).

Protein determination: The concentration of protein in the various fluids was measured by the Biuret method. Biuret reagent (Gornall et al. 1949) was made by dissolving 6.0 g NaKC$_4$H$_4$O$_6$·4H$_2$O and then 1.5 g CuSO$_4$·5H$_2$O in 300 ml of distilled water. 300ml of 2.5M NaOH was added to this solution while mixing. The solution
was then made up to a final volume of 1 litre with distilled water and stored in a plastic bottle. The protein concentration was measured using 50 µl of a sample mixed with 0.95 ml of saline and 4 ml of biuret reagent. Samples were measured against a reagent blank 30 min after mixing, at 540 nm in a Spectrophotometer (Hitachi model 100-10, Hitachi, Tokyo, Japan). Protein concentrations were estimated from a standard curve prepared with bovine serum albumin (BSA, Fraction V, Sigma).

**Gel filtration chromatography, G-200:** G-200 Sephadex gel filtration separations were done in descending glass columns (0.9 cm x 60 cm or 2 cm x 90 cm) at 4°C. Prior to packing the columns, Sephadex G-200 (Pharmacia) was allowed to swell in the filtration buffer solution for at least 3 days at 4°C. The procedure for packing was that recommended by Pharmacia. Samples of 0.5 - 1 ml were fractionated on columns 0.9 cm x 60 cm, while samples of 2 - 4 ml were fractionated on columns 2 cm x 90cm. A constant flow rate of 12 ml/hour was maintained and 5 or 10 ml volume fractions collected. Appropriate fractions were pooled, concentrated to 15 mg of protein per ml and stored at -25°C.

**Gel filtration chromatography, G-25:** G-25 Sephadex (Pharmacia) was left to swell for 24 hr at 4°C in the filtration buffer solution. A glass column of 0.9 cm x 20 cm was usually used and run at 4°C. Due to the fast flow rate and small samples sizes usually employed, samples were collected by hand with the sample size being varied as required.

**Anion - exchange chromatography:** Glass columns (2.0 cm x 90 cm) were used. DEAE - Sephadex, A50 (Pharmacia) was left to swell for 24 hr at room temperature in the starting buffer. After the
column was packed the samples (10 - 20 ml) were layered above the gel, allowed to flow into the gel bed and the elution system connected. Proteins were eluted with a continuously increasing concentration of phosphate buffer. This was achieved by joining a sealed reservoir with the starting buffer to another reservoir containing the 0.4 M buffer. The sealed reservoir served as a mixing chamber and the contents of this container were continuously mixed by a magnetic stirrer. A near linear gradient of phosphate buffer was obtained this way. Fractions of 10 ml were collected, pooled, concentrated to 15 mg protein per ml and stored at -25°C.

Agar gel immunoelectrophoresis: Slides were coated with a 1% solution of agar prepared by adding 1.0 g of Special Agar Noble (Difco) to 25 ml of Tris-barbiturate buffer and 75 ml of distilled water containing 1/10,000 parts thiomersal. The mixture was heated until the agar dissolved. The electrode chambers of the electrophoresis apparatus (LKB 6800 A) were filled with Tris-barbiturate buffer. Electrical contact between the gel plates and the electrode buffers was established with rayon wicks saturated in Tris-barbiturate buffer. The electrophoretic separation of antigen samples took place at a constant potential of 250 volts over a period of 1 hr. The antisera were allowed to react for 24 hr at room temperature in humid chambers. Slides were washed in a 1% NaCl solution for 24 hr with one solution change, and finally rinsed with distilled water. The slides were covered with strips of blotting paper and allowed to dry at room temperature. Once dry, the paper was removed and the slides washed with running tap water. The slides were stained by immersing them for one minute at room temperature in amido black staining solution, followed by four separate rinses with the same
solution without the amido black. The slides were dried, trimmed and labelled.

PREPARATION OF XENOANTISERA

**General Remarks:** The different immunoglobulin classes used in the preparation of antisera to bovine immunoglobulins were obtained from cattle immunised with killed *Brucella abortus* bacteria (CSL). The immunoglobulins were adsorbed to the bacteria and the immune complexes used as the antigens. This technique ensured the production of xenoantisera of high titres.

**Rabbit anti-bovine IgM and IgG (RabIgG α BovIg):** Bovine antibody was obtained by collecting serum from a steer 7, 14 and 21 days after an IM injection of $10^{10}$ killed *B. abortus* organisms. The samples were inactivated by heat and fractionated on G-200 Sephadex. The first exclusion peak contained the IgM and the second peak the IgG. The purity of the immunoglobulins in the column fractions was determined by immunoelectrophoresis with rabbit antisera to either bovine immunoglobulin or to whole bovine serum. The fractions containing the appropriate immunoglobulin were concentrated and the agglutination antibody titre to *B. abortus* determined with a standard bacterial agglutination assay (Grant, 1978). Immunoglobulin containing fractions were mixed with *B. abortus* in antibody excess. The sensitised *B. abortus* cells were allowed to stand for 2 hr at room temperature, washed 6 times in saline and resuspended in 1 ml of saline. This suspension was emulsified in an equal volume of FCA and injected IM into a rabbit. A second immunisation was given 2 weeks later and serum taken from the rabbit at weekly intervals. Antibody was detected and partially characterised by immunoelectrophoresis against bovine whole serum.
Conjugation of fluorescent dyes to rabbit antisera: Appropriate serum samples were fractioned on DEAE - Sephadex with a continuous phosphate buffer gradient (0.02M - 0.4M). The protein in the first two major peaks were used. Rabbit immunoglobulin was conjugated with fluorescein isothiocyanate, isomer 1 (FITC, Sigma) according to the methods of Nairn (1976). All reactions were carried out at 25°C. Four ml of rabbit immunoglobulin solution (20 mg/ml) was mixed with 2 ml of 0.2M Na₂HPO₄ (added drop wise). One mg of FITC (12.5 µg/mg protein) was dissolved in 2 ml of 0.1M Na₂HPO₄ and this was then slowly added and mixed with the protein solution. The pH was adjusted quickly to 9.5 with 0.1M Na₂HPO₄ and the final volume made up to 8 ml by the addition of saline. The reaction was allowed to proceed at 25°C for 30 min without agitation and then stopped by cooling to 4°C. Free FITC was removed by chromatography on a G-25 Sephadex column. A fluorochrome to protein ratio of 4 was considered optimal for staining the cell surface. Antisera which cross-reacted with IgM, IgG₁ and IgG₂ were generally used for cell surface staining. Peripheral blood lymphocytes were used to determine the concentration at which the FITC labelled antisera gave optimal staining.

SEPARATION OF CELLS FROM BLOOD

Bovine lymphocyte separation: Lymphocytes were separated from peripheral blood by centrifugation at 1200 g for 20 min on a Ficoll - Isopaque gradient. Four ml of blood was put onto 4 ml of the Ficoll - Isopaque. Platelets were removed with 3 washing steps using PBS and sufficient centrifugation (200 g for 8 min) to sediment the lymphocytes but not the platelets. If there was obvious erythrocyte contamination, then the second wash was done with "ACE" solution. Washed lymphocytes were resuspended in PBS, at the required concentra-
Bovine platelet separation: Platelets were separated by differential centrifugation from fresh blood. Blood was diluted with an equal volume of 0.5% EDTA - saline, and centrifuged at room temperature for 15 min at 200 g. The supernatant was removed and recentrifuged with the same procedure. The platelet rich supernatant was removed and platelets pelleted by centrifugation at 1200 g for 15 min. Platelets were then washed 6 times in the EDTA - saline and twice in PBS. Platelet samples prepared in this way were free from leucocytes, but they were usually contaminated with small numbers of erythrocytes. These erythrocytes were not readily removed by the "ACE" solution.

Buffy coat leucocyte separation: The buffy coat was removed from whole blood with a Pasteur pipette and resuspended in PBS. The cells were washed 3 times in PBS and with each wash, the buffy coat leucocytes were again removed leaving most of the erythrocytes in the pellet. No attempt was made to remove all erythrocytes.

Bovine erythrocyte separation: Erythrocytes were prepared free of leucocytes from whole blood by removal of the buffy coat and the top quarter of the erythrocyte layer. Three saline washes were used.

Goose red blood cells (GRBC) separation: GRBC were prepared from whole blood in Alsever's solution using the same techniques as described for bovine erythrocytes.

DETECTION AND CHARACTERISATION OF BOVINE ANTIBODIES

Standard microlymphocytotoxicity test: The test system was based on one previously described by Mittal et al. (1968). The tests were run in disposable 60 well Microtest trays (Nunc, Roskide, Denmark). The procedure used was the following:

(a) 1 μl of test serum was added to each well under liquid paraffin with a Hamilton dispenser (Hamilton Co.,
Reno, Nevada, U.S.A.). Trays were stored at -25°C for up to 3 months.

(b) For the actual test the trays were thawed and 1 μl of lymphocyte suspension was added to each well. The trays were then incubated for 60 min at room temperature.

(c) 5 μl of rabbit serum was added as a complement source to each well and the trays incubated for a further 90 min at room temperature.

(d) 1 μl of eosin Y dye solution was then added to each well followed in two min by 5 μl of buffered formaldehyde.

(e) When the lymphocytes had settled the test was read with an inverted phase-contrast microscope, at a magnification of 100x. Live lymphocytes appeared brilliant and normal in size, while dead cells were dark, due to the uptake of dye, and appeared slightly enlarged (Figure 2.1).

The tests were scored according to the percentage of dead lymphocytes:

<table>
<thead>
<tr>
<th>% of Dead Cells</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 10</td>
<td>-</td>
</tr>
<tr>
<td>10 - 25</td>
<td>1</td>
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<tr>
<td>25 - 50</td>
<td>2</td>
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<td>50 - 95</td>
<td>3</td>
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<td>95 - 100</td>
<td>4</td>
</tr>
</tbody>
</table>

Negative control wells containing non-reactive bovine serum (NBS, pooled sera from calves without any detectable cytotoxic activity) were included. These were used for the determination of background levels of lymphocyte death. An antiserum that reacted with more than 95% of the cell samples against which it had been tested was always included as a positive control.
Figure 2.1 (a) Negative test reaction; only a small portion of the lymphocytes have died. This is representative of the background levels of lymphocyte death usually seen.

(b) Positive test reaction; all of the lymphocytes have been killed by the antiserum.

Magnification: 710x
A two-colour fluorescence microlymphocytotoxicity test: The two-colour fluorescence cytotoxicity test was performed in the manner described by van Rood et al. (1976) with only minor modifications. This test allows the identification of B lymphocytes by the presence of a green fluorescent (FITC) "tag" on their cell surface and simultaneously the detection of cytotoxic antibody in serum by the uptake of ethidium bromide dye (red fluorescence) by dead cells. Cytotoxic antibodies specific for alloantigens carried on B lymphocytes can be detected by this test.

Washed lymphocytes were resuspended in PBS and the concentration adjusted to 16 x 10^6 ml. A 0.5 ml portion of the cell suspension was transferred to a clean glass centrifuge tube and 1 drop of FITC - RabIgG α BovIg added with gentle shaking. The suspension was then incubated at 37°C for 5 min. After this time 5 ml of PBS was added and the cells pelleted by centrifuging at 400 g for 6 min. The cells were washed 3 times in PBS and resuspended at a concentrations of 7 x 10^6/ml. All manipulations were done at room temperature.

The actual test was done according to the protocols of the standard microlymphocytotoxicity test except that 1 μl of ethidium bromide solution was added to each well in place of the eosin Y solution and no formaldehyde was added.

The test was read with a Leitz Diavert inverted microscope (Wild Leitz Pty. Ltd., North Ryde, N.S.W., Australia) with a 20x objective and 6.3x eyepiece. The microscope was equipped with a Ploempak 2.2 fluorescence vertical illuminator and a 50W ultra-high pressure mercury lamp. The use of a 12 filter block (Leitz) allowed the red and green fluorescence to be seen simultaneously.
The optimum concentration of ethidium bromide for staining was found to be 12.5 μg/ml. Higher concentrations of the dye gave a brilliant red fluorescence that tended to mask the green fluorescence of FITC, while lower concentrations of ethidium bromide gave only weak fluorescence. FITC-labelled B lymphocytes showed up as cells with brightly fluorescent green "caps"; unlabelled cells were not seen against the black background. Dead cells appear orange-red in colour due to the uptake of ethidium bromide, while dead B lymphocytes had both the green fluorescence of the FITC on their surfaces and the orange-red fluorescence of ethidium bromide in their nuclei (Figure 2.2). The test was usually read within 8 hr of staining the cells. Alternatively the trays could be left overnight at 4°C with no apparent increase in the uptake of ethidium bromide.

Measurement of antibody titres: Cytotoxic antibody titres were measured by testing doubling dilutions of sera in NBS. The endpoint of the titration was the dilution in which more than 80% of the target cells were found dead. Titres to Class I antigens were generally measured with lymphocytes from 1 to 12 selected individuals. Cattle, which were positive for the B lymphocyte antigen specificities but non-reactive when tested for Class I specificities recognised by each of the sera, were chosen from a panel and used to measure titres to the B lymphocyte antigens in unabsorbed samples of serum.

Antibody titres to Class I and B lymphocyte antigens were made roughly comparable by controlling the number of antigen-positive target cells in test wells. Lymphocyte concentration of 1.5 x 10⁶ and 7.0 x 10⁶ per ml were used to give similar numbers of cells expressing Class I and B lymphocyte antigens in each test. This
Figure 2.2 Fluorescence of lymphocytes in the two-colour micro-lymphocytotoxicity test. Dead B lymphocytes are identified by the presence of green caps on red/orange cells. Live B lymphocytes are seen as green caps or spots. Dead T lymphocytes are stained red/orange only while live T lymphocytes can not be seen.

Magnification: 1600x
assumes that all B lymphocytes antigen-positive cells are also positive for surface Ig and that 20-25% of the isolated lymphocytes are B lymphocytes (Kateley and Bazzell, 1978).

Relative contributions of IgM and IgG were determined by incubating the test sera for 60 min at 37°C in 0.2M 2-ME (final concentration 0.1M) in saline. Tests with immunoglobulins purified by G-200 Sephadex chromatography indicated that these conditions selectively destroyed IgM activity while causing only a slight depression on IgG activity. Lymphocytotoxicity tests were run without removing the 2-ME.

Incubation times: It was found that different times of incubation could be used without confounding the test. The times that were used were chosen to maximize the sensitivity of the test without causing a significant increase in the background number of dead cells.

Indirect Immunofluorescence: The possibility that non-cytotoxic antibody was present in the sera was investigated by an indirect immunofluorescent technique (Decary et al. 1975). Briefly, 1 x 10^6 lymphocytes were incubated for 60 min at room temperature with 0.1 ml of a non-cytotoxic test serum, NBS, or a known cytotoxic serum. The lymphocytes were washed three times in PBS and incubated with FITC - RabIgG α BovIg at 4°C in the presence of 0.1M NaN₃ for 30 min. After two washes in PBS-NaN₃ at 4°C the lymphocytes were fixed with 1% w/v paraformaldehyde in PBS. Lymphocytes were washed twice in PBS and mounted on microscope slides in a 75% v/v glycerol-PBS solution, pH 7.8. The lymphocytes were examined at 800x using a Leitz Orthoplan microscope fitted with a Ploemopak 2.1 fluorescence vertical illuminator, and the appropriate Leitz filter block (K). A BG-38 red suppression filter and a HBO 200 W/4 mercury lamp were installed in the light system. Lymphocytes were examined
alternately in normal light and fluorescent light. The intensity of the fluorescence was evaluated subjectively as strong, medium or weak for each serum. The percentage of fluorescent positive lymphocytes was established after counting 200 cells in different areas of the preparation. An antiserum was considered to contain non-cytotoxic antibodies if the percentage of fluorescing lymphocytes was significantly above background levels, after incubation of the cells in that serum. Background levels were determined by assessing the percentage of surface immunoglobulin-positive lymphocytes present in a sample incubated with NBS. The pattern and intensity of fluorescence were also observed in every case.

**Haemagglutination assays:** All tests were run in 96 well, round bottom, Microtest plates (Nunc), at room temperature.

Antibody against GRBC was detected by direct haemagglutination with a 1% GRBC suspension in 1% NBS - saline. Titres were measured for total and 2-ME resistant antibodies with doubling dilutions in 1% NBS - saline.

A passive haemagglutination assay was used for the detection of antibodies against ovalbumin. Ovalbumin was coupled to bovine erythrocytes with CrCl₃ using the technique of Parish and McKenzie (1978b). The test was run as described for GRBC. An anti-ovalbumin antiserum was prepared by hyperimmunising a sheep and this served as a positive control.

**PRODUCTION OF ALLOANTISERA**

**General Remarks:** Only non-parous females and steers were used for the production of alloantisera. This avoided the chance of alloreactive antibody being present from a previous pregnancy (Newman and Hines, 1980).
Lymphocyte immunisations: Cattle received 2 to 4 IV immunisations at two week intervals using 1 - 2x10⁷ freshly isolated lymphocytes in 2 ml sterile PBS. Samples of serum (20 ml) were taken before immunisation and at weekly intervals thereafter. The appropriate cytotoxicity test was used to test for antibody activity. Large volumes of blood (100 ml) were taken if antibody of the required specificities was detected.

Skin grafting: Skin grafting was performed on heifers given 2-3 ml Rompum (2% w/v xylazine, Bayer, Leverkusen, W. Germany) intravenously. Full-thickness grafts were placed dorsal to the lumbar muscles each side of the spine. This area was shaved closely and disinfected. Uniform grafts were obtained by using a circular sharpened stainless steel punch (32 mm diameter) to perform the initial incision. Four full-thickness circular grafts were removed from each heifer, 2 were taken for transfer to an allogeneic recipient and 2 were repositioned as autografts. Only 2 grafts were done at a time to minimize the delay between the removal of the graft and its reposition in the prepared graft bed.

Allografts were exchanged between selected pairs of heifers. Each heifer received 2 allografts from a selected donor. Approximately 12-15 evenly placed sutures (6-0 silk, Ethicon, Somerville, New Jersey, U.S.A.) per graft were used to prevent the graft from shifting. Antibiotic powder (Crystapen, Glaxo, Boronia, Australia) was placed on the graft which was then covered with paraffin-impregnated gauze (Allen and Hanberry Ltd., London, U.K.) Dry gauze was then placed over this and the dressings held in place by a square piece of towelling cloth sutured to the skin at the corners. Grafts were examined every second day until they were judged rejected.
or accepted on the basis of their appearance. Blood samples were taken at 7 day intervals, and the sera obtained was heat-inactivated and stored at -25°C until tested.

**ABSORPTION OF ALLOANTISERA**

*General Remarks:* Antisera were usually absorbed with appropriate cells at room temperature and care was taken to limit any non-specific absorption of the antibody by using as few cells as was required. Non-specific absorption was monitored by running negative absorption tests with cells from an animal which did not react with the test serum. There was usually a slight loss of antibody activity. An absorption test was considered positive when significant antibody activity was removed over that expected through non-specific loss. Absorbed sera were tested with the appropriate microlymphocytotoxicity test by a titration analysis.

*Platelet and lymphocyte absorptions:* Platelets and lymphocytes were isolated as described previously. They were fixed in 1% formaldehyde - saline for 15 min at room temperature and washed twice in PBS before being used. Fixation of the cells prevented the serum from clotting after absorption and made the cells more durable. Trial absorptions were carried out for one hour using 100 μl. Larger absorptions were done using up to one ml of antiserum. The number of cells required for the absorption was dependent on the titre of the serum (see Chapter 3, page 73). Following absorption of the serum the cells were removed and incubated in 0.1M citrate - saline at 4°C for 30 min to elute the antibody. The cells were recovered by centrifugation and washed 3 times in PBS. Cells treated this way could be reused for absorption on two further occasions, however, larger numbers were required to remove the same
amount of antibody activity with each absorption-elution cycle. A non-specific antibody loss of up to one titre point was usually observed with both the fresh and the recycled cells.

**Erythrocyte and buffy coat leucocyte absorptions:** Erythrocytes were used without fixation because the formaldehyde - saline caused extensive lysis of the cells. Buffy coat leucocytes were used without fixation because it was easier to isolate them freshly than to recycle them because they could be obtained with ease from small amounts of blood. Buffy coat leucocytes were used most frequently. The non-specific antibody loss of 1 - 2 titre points was the major disadvantage in using these cells.

**TISSUE CULTURE**

**Mixed lymphocyte culture:** The mixed lymphocyte reactivity (MLR) system of Emery and McCullagh (1980) was used with minor modifications. Lymphocytes were isolated from whole blood under sterile conditions and suspended in the appropriate tissue culture medium at a concentration of 5 x 10⁶/ml. Half of the sample was gamma irradiated (1500 R, ⁶⁰Co source) and the cells used as stimulator cells in the assay. Cultures were run in 96 well, flat-bottom, Microtest trays (Nunc). 0.1 ml each of the irradiated (stimulator) and normal (responder) cell suspensions was added to each well. All culture combinations were run in triplicate at 37°C in a moist atmosphere of 10% CO₂ + 7% O₂ + 83% N₂.

The extent of cellular proliferation was measured by the addition of ¹⁴C of tritiated methyl-thymidine (Amersham Pty.Ltd., Sydney, Australia) 6 hr before the cultures were harvested.

The cells were harvested by sucking the contents of each well onto glass-fibre filter paper (Whatman Inc., Clifton, N.J., U.S.A.)
with a multiple harvesting apparatus (Cambridge Technology, Cambridge, Mass., U.S.A.). The filters were dried and each disc placed in a plastic scintillation vial (Weiner Zinsser, Frankfurt, Germany) with 8.0 ml of scintillation fluid (5.0% PPO in xylene). The samples were chilled and counted in a Tricarb liquid scintillation counter (model 3320, Packard Inc., Downing Grove, Illinois, U.S.A.). Median counts per minute (CPM) from triplicate cultures were used for analysis.

**Mitogen culture**: The mitogen Concanavalin A (Con A, Sigma) was used at a concentration of 2 µg per well as a control to assess the viability of cultured lymphocytes in culture. In these cultures 5 x 10^5 untreated lymphocytes in 0.2 ml medium were used. The cultures were harvested and counted by the same procedures as used for the MLC tests.

**Indices for measuring responses**: Two scores were used for assessing MLC responses. The formulae for these scores are given below. The values and significances are discussed in the results section, Chapter 4.

(i) **Stimulation Index (SI)**:

\[
SI = \frac{(A + Bx)}{(A + Ax)}
\]

where \( (A + Bx) = \text{animal A responding to animal B (irradiated) expressed in CPM.} \)

where \( (A + Ax) = \text{animal A responding to itself (irradiated), autologous control, expressed in CPM.} \)

Total CPM are used for both the numerator and denominator (Mickey et al. 1975).
(ii) Relative Response (RR): The RR was first defined by Jørgensen, et al. (1973). To use the RR all MLR values are first corrected for background.

\[
\text{Corrected CPM} = (A + Bx) - (A + Ax)
\]

The median response value for a particular cell donor was then found and used as the standard.

\[
\text{RR} = \frac{(\text{Any response using Cells A})}{(\text{Median response using Cells A})} \times 100
\]

The value for this usually ranged from 0 to about 200.

This index was modified by Mickey et al. (1975) by using a maximum value as the standard rather than the median.

\[
\text{RR} = \frac{(\text{Any response using cells A})}{(\text{Maximum response using cells A})} \times 100
\]

This reduces the range of RR values from about 0 to 100.

The standard response for the RR is usually defined by using a pool of stimulator cells from a standardised source so that tests done on different days and run between different individuals are comparable.

The RR was used here as defined by Mickey et al. (1975) taking the maximum response seen in a family as the denominator. Only data between family members were compared.

**STATISTICAL METHODS**

**General Remarks:** Standard statistical tests and methods were used as described by Snedecor and Cockran (1967).

**Analysis of serological typing data:**

(i) Population analysis: The serological data obtained from the B lymphocyte typing sera were analysed with two sequential tests. Significant associations between two sera were established by use of Chi-square ($\chi^2$) analysis, in the form of $2 \times 2$ contingency tables.
From this, the correlation coefficient (r) was derived as a measure of the degree of association between antisera (Cavalli-Storza and Bodmer, 1971; van Rood and van Leeuwen, 1963). An r value of > 0.34 establishes that the association has a chance probability of (p < 0.001) when the data are from 100 tests. This positive value was considered as the lowest which showed a definite association between two antisera, suggesting that they were both identifying the same antigens. These values were obtained by computer, using a program from Dr. W. Bodmer, Imperial Cancer Research Fund Laboratories.

(ii) Genetic analysis: The possibility that two detectable loci were linked was tested by the method of analysis by likelihood using LOD scores (Morton, 1955). LOD score values for the matings used were obtained from tables.

EXPERIMENTATION WITH CATTLE TICKS

Tick source: The Australian cattle tick Boophilus microplus was used for experimentation. These were made available as viable larvae by the Department of Primary Industry, Beef Husbandry Branch, Queensland, Australia. All work involving these ticks was carried out at "Swan's Lagoon" Beef Cattle Research Station, Millaroo, Queensland.

Test cattle: A group of 199 mixed-sex cattle, 6 to 18 months of age was used. These cattle were crossbreeds, 3/4 Brahman and 1/4 Shorthorn. The group was made up of 120 heifers and 79 bulls. All cattle belonged to the Department of Primary Industry and were maintained at the "Swan's Lagoon" station under free range conditions common in the northern Queensland area. This meant that all cattle were naturally infested with ticks under field conditions.
Experimental tick infestation: Cattle were infested with approximately 20,000 viable tick larvae by placing a specially designed collar on the animal's neck for 4 hr. These collars held the vials containing the larvae in direct contact with the skin of the test cow and allowed all larvae the chance to attach (Utech et al. 1978a; Wharton et al. 1970). The level of tick infestation was determined by counting the number of engorged female ticks, 4.5 - 8 mm in length on one side of each test cow. Four separate counts were made on each animal, two per day, 20 and 21 days after infestation (Wharton et al. 1970).

Treatment of data: Raw data for tick counts have a strongly skewed distribution and for this reason they were transformed by using mean tick counts corrected with the following index (personal communication K. Boothby).

\[
1 - \frac{\text{Mean tick count for the individual}}{5 \times (\text{Mean tick count for the group})} \times 100
\]

This index allows individuals within a particular group to be ranked according to the number of ticks they carry when compared with the entire herd. The results are expressed as "percent resistance" and values generally range from 0% - 100%.
CHAPTER 3

SEROLOGICAL AND GENETIC IDENTIFICATION

OF A BOVINE B LYMPHOCYTE ALLOANTIGEN SYSTEM
INTRODUCTION

Class II antigens form a system of polymorphic alloantigens whose expression is controlled by the I region of the MHC. This antigen system has been defined most completely in inbred species of laboratory animals such as the mouse (David, 1976), the rat (Radka et al. 1977) and the guinea pig (Schwartz et al. 1977b). Similar antigen systems have also been defined in a few outbred species, most notably, the human (Bodmer, 1978a) and the rhesus monkey (Roger et al. 1976). The MHC of domestic animals has also been studied recently and this has led to the definition of an antigen system in the chicken (Ewert et al. 1980) and the pig (Vaiman et al. 1975) similar in terms of their distribution on blood cells, their linkage to the MHC and their relationship with mixed-lymphocyte reactivity.

Before these antigens could be typed in cattle, it was necessary to develop the appropriate methods for the isolation and typing of bovine lymphocytes. In other species B lymphocytes are usually isolated as a pure population or at least in greatly enriched populations before they are subjected to testing. The most widely used technique has been to remove T lymphocytes by allowing them to form rosettes with xenogeneic erythrocytes, and then removing the rosettes by differential sedimentation (van Rood et al. 1975a) However, other methods including those which allow a positive selection of B lymphocytes are also available (de Krester et al. 1980; Wysocki and Sato 1978). These enriched B lymphocyte populations can then be used in a standard microlymphocytotoxicity test. A microlymphocytotoxicity test involving the normal population of peripheral blood lymphocytes is also available. In this test the B lymphocytes are labelled with a FITC-
conjugated antibody directed against cell-surface immunoglobulin. The selective killing of B lymphocytes can be seen using the appropriate fluorescent filter system (van Rood et al. 1976).

Another problem lies in the detection and subsequent purification of antibody to Class II antigens from sera which contains antibody to both Class I and Class II antigens. One approach has been to absorb the sera with cells which do not express Class II antigens but do express Class I antigens. Erythrocytes and platelets have been used in the mouse (Colombani et al. 1976; Staines et al. 1976) but only platelets have proven suitable in man (van Rood et al. 1975a).

The development of typing reagents and the required techniques for the detection of a B lymphocyte alloantigen system in cattle are described in this chapter.

EXPERIMENTAL METHODS

Identification of antisera with anti-B lymphocyte activity

If it is assumed that the Class I and Class II antigens are not in a complete linkage disequilibrium then four phenotypes are expected in a random population for any Class I and Class II antigen pair:

<table>
<thead>
<tr>
<th>ANTIAGENS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal Type</td>
</tr>
<tr>
<td>(1)</td>
</tr>
<tr>
<td>(2)</td>
</tr>
<tr>
<td>(3)</td>
</tr>
<tr>
<td>(4)</td>
</tr>
</tbody>
</table>
An alloantiserum produced against lymphocytes could contain antibodies against both classes of antigens. Lymphocytes which are negative for the Class I antigen but positive for the Class II antigen (type 3) could be used to identify a serum with antibody to this Class II antigen. This means that it is possible to identify the presence of antibody to Class II antigens in serum even when lymphocytes and platelets from those animals to which the antiserum was raised are not available. With this approach, 114 alloantisera were prepared from cattle which were immunised with allogeneic lymphocytes. These antisera were screened in duplicate by the two-colour microlymphocytoxicity test against lymphocytes from 100 unrelated cattle representing 10 different breeds. A serum was classified as positive if it showed a restricted cytotoxicity against B lymphocytes from at least one animal.

Absorption analysis

Selected antisera were absorbed with erythrocytes, platelets and leucocytes from 2 - 14 positive cattle to determine which cells expressed B lymphocyte antigens and/or Class I antigens.

Preparation of typing antisera

The results of the absorption tests were taken into account in preparing the typing antisera. These antisera were prepared by removing antibody to Class I antigens and where possible the number of specificities recognized by a serum was reduced by further absorption of the anti-B lymphocyte activity.

RESULTS

Occurrence of antibody to B lymphocyte antigens

Anti-B lymphocyte activity was detected in 47 of the 114 allo-
antisera. Only antisera with antibodies to Class I antigens contained antibody to B lymphocyte antigens. Antibody titres ranged from 1 to 32 for the B lymphocyte specificities and from 1 to 64 for the Class I specificities. Based on the patterns of reactions, 27 of the B lymphocyte-positive antisera were chosen for further study. The percent reactivity of these antisera to the two types of antigens is summarized in Table 3.1.

Antigen distribution on blood cells

The distribution of both classes of antigens on blood cells was determined by absorption. Erythrocytes failed to absorb significant amounts of antibody to either the Class I or B lymphocyte antigens. Leucocytes from buffy coats and purified blood lymphocytes absorbed antibody to both types of antigens while purified platelets only absorbed antibody to Class I antigens.

The absorption capacities of the different cell types were also quantified. A cytotoxic titre of 4, to Class I antigens, was usually removed from 1 ml of serum by absorbing it with $2 \times 10^{10}$ platelets. As the titre increased, more platelets were required but this requirement was not a linear relation. A titre of 32 to 64 could be removed by absorption with $1 \times 10^{11}$ platelets. Only $2-4 \times 10^8$ lymphocytes were necessary to absorb a titre of 32 from 1 ml of antiserum. The equivalent titre was also removed by absorptions with the buffy coat leucocytes from 40 ml of blood.

Determination of specificity

The number of specificities in a serum to both the Class I and B lymphocyte antigens was determined by absorption. Class I antigen-positive, B lymphocyte antigen-negative individuals were also identified by the absorption analysis. It was hoped that cells from these
<table>
<thead>
<tr>
<th>Antiserum Identification Number</th>
<th>Reactivity to Class I Antigens (%)</th>
<th>Reactivity to B Lymphocyte Antigens (%)</th>
<th>Total anti-Lymphocyte Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP3</td>
<td>61</td>
<td>25</td>
<td>86</td>
</tr>
<tr>
<td>SP4</td>
<td>96</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>SP9</td>
<td>14</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>SP12</td>
<td>50</td>
<td>4</td>
<td>54</td>
</tr>
<tr>
<td>SP37</td>
<td>39</td>
<td>9</td>
<td>48</td>
</tr>
<tr>
<td>SP38</td>
<td>8</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>SP43</td>
<td>38</td>
<td>21</td>
<td>59</td>
</tr>
<tr>
<td>SP54</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>SP56</td>
<td>43</td>
<td>11</td>
<td>54</td>
</tr>
<tr>
<td>SP60</td>
<td>87</td>
<td>9</td>
<td>96</td>
</tr>
<tr>
<td>SP70</td>
<td>60</td>
<td>23</td>
<td>83</td>
</tr>
<tr>
<td>SP72</td>
<td>55</td>
<td>2</td>
<td>57</td>
</tr>
<tr>
<td>SP76</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>SP81</td>
<td>32</td>
<td>4</td>
<td>36</td>
</tr>
<tr>
<td>SP82</td>
<td>68</td>
<td>13</td>
<td>81</td>
</tr>
<tr>
<td>SP83</td>
<td>75</td>
<td>17</td>
<td>92</td>
</tr>
<tr>
<td>SP84</td>
<td>34</td>
<td>7</td>
<td>41</td>
</tr>
<tr>
<td>A1</td>
<td>24</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>A6</td>
<td>45</td>
<td>3</td>
<td>48</td>
</tr>
<tr>
<td>A9</td>
<td>61</td>
<td>4</td>
<td>65</td>
</tr>
<tr>
<td>A11</td>
<td>71</td>
<td>5</td>
<td>76</td>
</tr>
<tr>
<td>A13</td>
<td>81</td>
<td>10</td>
<td>91</td>
</tr>
<tr>
<td>A15</td>
<td>70</td>
<td>16</td>
<td>86</td>
</tr>
<tr>
<td>A18</td>
<td>53</td>
<td>5</td>
<td>58</td>
</tr>
<tr>
<td>A19</td>
<td>40</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>A21</td>
<td>86</td>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>A22</td>
<td>67</td>
<td>13</td>
<td>80</td>
</tr>
</tbody>
</table>
individuals could be used to absorb antibody selectively to the Class I antigens. The use of lymphocytes or buffy coat leucocytes was preferred because platelets were difficult to isolate and required large quantities of blood.

An example of the analysis of one antiserum (SP12) follows. This antiserum reacted with 54% of the test panel. The number of specificities can be estimated by counting the number of sequential absorptions required to remove all reactivity. This is a minimum estimate, as any one animal may express more than one antigenic specificity identified by a multispecific antiserum. The reaction to Class I antigens after absorptions with buffy coat leucocytes from 12 animals is shown in Table 3.2. Analysis of these results demonstrated that as many as 6 absorptions were required to remove all reactivity (Table 3.3). Although a single absorption with cells from the animal 68F was also sufficient. This implied that serum SP12 had at least 6 Class I specificities while animal 68F was positive for all 6. Once Class I activity had been removed, the anti-B lymphocyte activity was analysed with animals which were negative for Class I antigen specificities but positive for B lymphocyte antigen specificities of the original antiserum. These results identify those animals whose cells absorbed antibody to Class I antigens but left at least a partial reactivity to B lymphocyte antigens. Three cytotoxicity patterns were distinguished following absorption of SP12 (Table 3.4). For example, absorption with cells from B2 removed activity against animals 150 and B2. Absorption with cells from animal 150 removed activity against both 729 and 150. Several absorptions left activity to all 3 test animals. The presence of 3 different cytotoxicity patterns demonstrates the
TABLE 3.2  Cytotoxicity patterns for absorbed antisera (SP12) to
Class 1 antigens.

<table>
<thead>
<tr>
<th>Absorbed Antisera*</th>
<th>Test Animals**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A13 41Br 33B 53B 848 72F 16B 31 20B 51 B2 68F</td>
</tr>
<tr>
<td>Not absorbed</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>A13 - 12</td>
<td>- - - - + + + - + + +</td>
</tr>
<tr>
<td>41B - 12</td>
<td>- - - - - - - + + + +</td>
</tr>
<tr>
<td>33B - 12</td>
<td>- - - - - - - - + + +</td>
</tr>
<tr>
<td>53B - 12</td>
<td>- - - - - + + + + + +</td>
</tr>
<tr>
<td>848 - 12</td>
<td>- - + + - + + + + + +</td>
</tr>
<tr>
<td>72F - 12</td>
<td>- - - - - - - + + + +</td>
</tr>
<tr>
<td>16B - 12</td>
<td>- - - - - - - + + + +</td>
</tr>
<tr>
<td>31 - 12</td>
<td>- - - - + - + - - - +</td>
</tr>
<tr>
<td>20B - 12</td>
<td>- - - - - - - + + +</td>
</tr>
<tr>
<td>51 - 12</td>
<td>+ + + + + + - + - - +</td>
</tr>
<tr>
<td>B2 - 12</td>
<td>+ + + + - + + + - - +</td>
</tr>
<tr>
<td>68F - 12</td>
<td>- - - - - - - - - - -</td>
</tr>
</tbody>
</table>

Results expressed as (+) positive for cytotoxicity; the absorption did not remove the antibody.

(-) Negative for cytotoxicity; the absorption did remove the antibody.

* Absorbed antisera identified by the animal number of the cell donor - antiserum number.

** Only cells from those animals which were used for positive absorptions are shown.
TABLE 3.3 Absorption analysis for the determination of the number of antibody specificities present to Class 1 antigens in antiserum SP12.

<table>
<thead>
<tr>
<th>Absorbing Cells*</th>
<th>Test Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>848</td>
</tr>
<tr>
<td>Not absorbed</td>
<td>+</td>
</tr>
<tr>
<td>848 (1)</td>
<td>-</td>
</tr>
<tr>
<td>B2 (2)</td>
<td>-</td>
</tr>
<tr>
<td>41B (3)</td>
<td>-</td>
</tr>
<tr>
<td>51B (4)</td>
<td>-</td>
</tr>
<tr>
<td>72F (5)</td>
<td>-</td>
</tr>
<tr>
<td>68F (6)</td>
<td>-</td>
</tr>
</tbody>
</table>

Results expressed as (+) positive for cytotoxicity
(-) negative for cytotoxicity

* Absorptions done in this sequence are expected to remove the cytotoxic activity in this fashion. Actual data taken from Table 3.2, one antiserum was not actually absorbed 6 separate times.
TABLE 3.4  Cytotoxicity patterns for absorbed antiserum (SP12) to B lymphocyte antigens.

<table>
<thead>
<tr>
<th>Absorbed Antisera*</th>
<th>Test Animals**</th>
<th>150</th>
<th>729</th>
<th>67F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not absorbed</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>848 - 12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A13 - 12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>41B - 12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16B - 12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>53B - 12</td>
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</tr>
<tr>
<td>33B - 12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20B - 12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31 - 12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B2 - 12</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>51 - 12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>150 - 12**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>729 - 12**</td>
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</tr>
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<td>68F - 12</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>67F - 12**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results expressed as (+) positive for cytotoxicity (-) negative for cytotoxicity

* Absorbed antisera identified by the animal number of cell donor - antiserum number.
** These animals are positive for the B lymphocyte antigens recognised by SP 12 but negative for the Class I antigens.
presence of at least 3 different antibody specificities. The results for the absorption analysis of the 27 selected sera are presented in Table 3.5.

Preparation of typing antisera

The results of the absorption analysis were used to select animals for quantitative absorptions in the preparation of B lymphocyte typing reagents. The number of absorptions required for each antiserum varied but was never more than 4 as beyond this the nonspecific antibody loss became too great. In total 27 typing sera were prepared from 13 of the original alloantisera. These sera and the absorption sequences used in their preparation are listed in Table 3.6.

EXPERIMENTAL METHODS

Population and family studies

The absorbed antisera were used to type 110 cattle from paternal half-sib families and a panel of 100 unrelated cows. The data obtained from the families were used to define genetically 10 B lymphocyte antigen specificities. Confirmation of this was obtained from the absorption experiments and statistical analysis. Linkage to the Class I antigens of the bovine MHC was also studied.

RESULTS

Definition of antigens

Nine of the typing antisera were found to contain antibodies against Class I antigens and so these antisera were not used. This left 18 typing reagents derived from 11 of the original antisera. It was possible to define 10 B lymphocyte antigen specificities with
# TABLE 3.5

Number of antibody specificities detected to Class I and to B lymphocyte antigens in the 27 selected antisera.

<table>
<thead>
<tr>
<th>Antiserum Identification Number</th>
<th>Number of animals used for positive absorptions</th>
<th>Minimum number of specificities detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class I Antigens</td>
<td>B Lymphocyte Antigens</td>
</tr>
<tr>
<td>SP3</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>SP4</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>SP9</td>
<td>7</td>
<td>3</td>
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<td>SP12</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>SP37</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>SP38</td>
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<tr>
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<td>2</td>
<td>5</td>
</tr>
<tr>
<td>SP56</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>SP60</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>SP70</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>SP72</td>
<td>10</td>
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<td>SP76</td>
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<tr>
<td>SP82</td>
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<td>SP83</td>
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<td>SP84</td>
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<td>4</td>
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</table>
## Table 3.6 Characteristics of the B lymphocyte typing sera.

<table>
<thead>
<tr>
<th>Antiserum Identification Number</th>
<th>Typing Serum Identification Number</th>
<th>Absorptions Required*</th>
<th>Number of Specificities Present**</th>
<th>Typing Serum Titre Range ***</th>
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<tbody>
<tr>
<td>SP3</td>
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<td>876 - BCL</td>
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<td>1 - 2</td>
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<tr>
<td></td>
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<td>72F - BCL</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A13 - PLA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-2</td>
<td>16B - BCL</td>
<td>1</td>
<td>1 - 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72F - BCL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>A13 - PLA</td>
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</tr>
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<td></td>
<td>B-3</td>
<td>812 - BCL</td>
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<td>4 - 16</td>
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<td>73F - BCL</td>
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<td>B19 - PLA</td>
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<td>41B - PLA</td>
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</tr>
<tr>
<td>SP83 (Contd.)</td>
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<td>73F - BCL</td>
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<td>1 - 2</td>
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<td>B19 - BCL</td>
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<td>53B - BCL</td>
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</tr>
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<td>SP84</td>
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<td>4</td>
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<td></td>
<td></td>
<td>806 - BCL</td>
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<td></td>
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<td>B-27</td>
<td>A14 - PLA</td>
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<td></td>
<td>0 - PLA</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>72F - PLA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Absorptions identified as the animal number of the cell donor - cell type used:  BCL = buffy coat leucocytes  PLA = platelets

** Represents minimum number of specificities present in the typing serum.

*** Titre ranges given because there may be more than a single antibody specificity present in a serum, each with a different titre. Titres were determined with lymphocytes from 2 to 6 animals, and 1 µl of each serum per test.
these reagents (Table 3.7). This was done by assuming that a specificity, identified by either a single antiserum or a group of antisera and inherited as a unit, constituted a single antigen. One disadvantage with this type of analysis is that it can underestimate the number of antigens present. Antigens controlled by closely linked genes will not be discriminated and will be counted as one antigen. Another problem is that a specificity detected by the antisera for which there was no information available concerning its mode of inheritance was not considered. Once again this leads to an underestimate of the number of antigenic specificities present.

It was not possible to check specificities by further absorption procedures as the B lymphocyte typing sera were prepared by extensive absorption and further large scale absorptions were not practical. Results from the previous absorption tests were used when applicable.

**Chi Square ($\chi^2$) analysis and correlation coefficients**

The $\chi^2$ analysis was run on the serological data obtained from the panel of 100 unrelated cattle. The results of this analysis and the relevant correlation coefficients ($r$) are shown on Table 3.8.

**Analysis of defined specificities for B lymphocyte antigens**

**Specificity 1**

The specificity was identified by a single antiserum, B-26, which reacted with 24% of the panel. Absorption analysis with 9 animals suggested that not more than one antibody specificity was present. This specificity was carried by one Jersey bull and one Angus bull (Table 3.9). Segregation and co-dominant expression were apparent in both families. Table 3.10 summarizes all of the different matings
TABLE 3.7 Genetically defined bovine B lymphocyte antigen specificities.

<table>
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<th>Specificity Designation</th>
<th>Antisera Identification Numbers</th>
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<tr>
<td>2</td>
<td>B-6, B-11, B-12</td>
</tr>
<tr>
<td>3</td>
<td>B-24, B-25</td>
</tr>
<tr>
<td>4</td>
<td>B-25, B-27</td>
</tr>
<tr>
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<td>B-5, B-6</td>
</tr>
<tr>
<td>6</td>
<td>B-22, B-23</td>
</tr>
<tr>
<td>7</td>
<td>B-11, B-12</td>
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<td>8</td>
<td>B-13</td>
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<tr>
<td>9</td>
<td>B-23</td>
</tr>
<tr>
<td>10</td>
<td>B-5, B-15</td>
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</tbody>
</table>

Several antisera were used in more than one combination to identify specificities.
TABLE 3.8 Correlation coefficients within groups of antisera to B lymphocyte antigens.

<table>
<thead>
<tr>
<th>Group Designation</th>
<th>Number of Antisera</th>
<th>Antiserum Identification Numbers</th>
<th>Antiserum Reaction Frequency*</th>
<th>Correlation Coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2</td>
<td>B-5</td>
<td>26</td>
<td>.45</td>
</tr>
<tr>
<td></td>
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<td>B-15</td>
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</tr>
<tr>
<td>2</td>
<td>3</td>
<td>B-6</td>
<td>27</td>
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<td></td>
<td></td>
<td>B-24</td>
<td>18</td>
<td>.41</td>
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<td></td>
<td></td>
<td>B-25</td>
<td>15</td>
<td>.43 .62</td>
</tr>
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<td></td>
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<td>B-7</td>
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<td>B-22</td>
<td>15</td>
<td>.64</td>
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<td>B-23</td>
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<td>20</td>
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<td>15</td>
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<td></td>
<td>B-10</td>
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</tr>
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<td></td>
<td>B-20</td>
<td>7</td>
<td></td>
</tr>
<tr>
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<td>24</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>B-27</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

* Values expressed as percentages.
TABLE 3.9  Inheritance of bovine B lymphocyte antigens in 4 informative families.

<table>
<thead>
<tr>
<th>Sire I.D.</th>
<th>Genotype</th>
<th>Dam I.D.</th>
<th>Genotype</th>
<th>Calf I.D.</th>
<th>Genotype</th>
<th>Breed</th>
</tr>
</thead>
<tbody>
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<td>J-1</td>
<td>1/2</td>
<td>C-1</td>
<td>2/3</td>
<td>C-1A</td>
<td>2/3</td>
<td>J</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-2</td>
<td>2/3</td>
<td>C-2A</td>
<td>2/3</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-3</td>
<td>1/*</td>
<td>C-3A</td>
<td>1/*</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-4</td>
<td>1/4</td>
<td>C-4A</td>
<td>1/2</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-5</td>
<td>1/4</td>
<td>C-5A</td>
<td>1/4</td>
<td>Y</td>
</tr>
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<td>-/-</td>
<td>463</td>
<td>-/-</td>
<td>A</td>
</tr>
<tr>
<td>A90</td>
<td>6/-</td>
<td>464</td>
<td>7/-</td>
<td>A90</td>
<td>5/-</td>
<td>N</td>
</tr>
<tr>
<td>A43</td>
<td>-/-</td>
<td>465</td>
<td>7/-</td>
<td>A43</td>
<td>-/-</td>
<td>G</td>
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<tr>
<td>A119</td>
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<td>467</td>
<td>7/-</td>
<td>A52</td>
<td>3/-</td>
<td>S</td>
</tr>
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<td>A110</td>
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<td>A8</td>
<td>-/-</td>
<td>202</td>
<td>-/-</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F10</td>
<td>6/-</td>
<td>208</td>
<td>-/-</td>
<td>F</td>
</tr>
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<td></td>
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<td>205</td>
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<td></td>
<td></td>
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<td>-/-</td>
<td>201</td>
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<td>MG-2</td>
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<td>B52</td>
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<td>217</td>
<td>8/-</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>151</td>
<td>9/*</td>
<td>221</td>
<td>9/-</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>155</td>
<td>-/-</td>
<td>223</td>
<td>8/-</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>152</td>
<td>-/-</td>
<td>220</td>
<td>-/-</td>
<td>Y</td>
</tr>
</tbody>
</table>

* Homozygosity cannot be excluded.
involving this specificity. These results are consistent with this specificity having been inherited as an autosomal co-dominant.

**Specificity 2**

This specificity was present in one family and was defined by 3 antisera, B-6, B-11 and B-12. The reaction frequencies for these sera were 27%, 9% and 16% respectively. All but one of the reactions of B-11 are included in those of B-12. This result was not unexpected, as both of these reagents were derived from a single antiserum (SP43). Absorption analysis suggested that B-11 was monospecific whereas B-12 contained the B-11 specificity and at least one other. The other antiserum, B-6, did not show any association with B-11 and B-12 in the population of unrelated cattle that were tested, but it detected a specificity which was inherited together with B-11 and B-12 in this one family. There are two possible explanations for this result. Either B-6 is multispecific and shares a rare specificity with B-11 and B-12 or B-6 detects the products of a second, linked locus. Absorption analysis indicated that B-6 has at least two specificities, thus sharing of specificities cannot be excluded. The antigen or antigens recognized by these sera were inherited as a group in the Jersey family, showing an allelic or pseudo-allelic relationship with specificity 1 (Table 3.9). The matings involving this specificity are summarized in Table 3.10.

**Specificity 3**

Two associated antisera, B-24 and B-25, define specificity 3. The reaction frequencies of these antisera were 18% and 15% respectively, and the correlation coefficient was 0.62. Absorption analysis demonstrated that these antisera were both multispecific. Only two
TABLE 3.10 Mating and offspring summary for 4 specificities.

<table>
<thead>
<tr>
<th>Mating Type*</th>
<th>Offspring Type**</th>
<th>Specificity Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sire Dam Number</td>
<td>Number</td>
<td>+</td>
</tr>
<tr>
<td>+ + .3</td>
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</tr>
<tr>
<td>+ - 7</td>
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<td>+ + 2</td>
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</tr>
<tr>
<td>+ - 3</td>
<td>1</td>
<td>2</td>
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<tr>
<td>- + 0</td>
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<td>2</td>
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<td>- + 2</td>
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<td>1</td>
</tr>
<tr>
<td>- - 43</td>
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<td>+ + 1</td>
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<td>+ - 4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>- + 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>- - 45</td>
<td>0</td>
<td>45</td>
</tr>
</tbody>
</table>

* Mating type characterized by the presence and/or absence of the specificity in either or both of the parents.

** Offspring characterized as positive or negative with respect to the specificity in question.
Jersey cows possessed this specificity and it was passed on to both of their calves (Table 3.9).

Another antiserum, B-6, showed a positive association with both sera \( r=0.41, \) B-24; \( r=0.43, \) B-25) but was not inherited with specificity 3. This antiserum was not monospecific so the positive association may have been due to a shared specificity. Alternatively, this antiserum may have been identifying the product of a gene which was in linkage disequilibrium with the gene product detected by sera B-24 and B-25.

Specificity 4

This specificity was identified by sera B-25 and B-27. The reaction frequencies were 15\% and 8\% respectively. Antiserum B-25 was also used to detect specificity 3 while antiserum B-27 did not show any strong associations with other sera. Neither of these two sera was monospecific. Despite the fact that the sera were not associated in the population of the animals tested, they both detected a specificity which was inherited maternally by one calf in the Jersey family (Table 3.9). One explanation could be that both sera recognized a common specificity, although the possibility that two linked antigens from different loci were being detected cannot be dismissed.

Specificity 5

Two sera, B-5 and B-6, recognized this specificity. Their reaction frequencies were 26\% and 27\% respectively and they were significantly associated \( r=0.33, p < 0.01 \). Both sera were prepared from a single antiserum, SP12. Absorption analysis showed that the two sera were multispecific and probably shared a common specificity. Genetic data consisted of the inheritance of this specificity by one calf from
its mother in the Angus x Holstein-Friesian family (Table 3.9).

**Specificity 6**

Two associated antisera B-22 and B-23, \((r=0.64)\) identified specificity 6. B-22 appeared to be monospecific and to be totally included in B-23. Reaction frequencies were 15\% for B-22 and 27\% for B-23. This specificity was inherited by one calf from its mother in a Charolais family.

**Specificity 7**

This specificity was identified by antisera B-11 and B-12 \((r=0.54)\). Absorptions and serological analysis showed that B-11 was monospecific and almost entirely included within the multispecific antiserum B-12. This specificity was carried by the bull (117) of the Angus family and inherited by 3 of his 5 offspring (Tables 3.9 and 3.10).

**Specificity 8**

Antiserum B-13 identified specificity 8. The reaction frequency of B-13 was 20\% and it showed a positive correlation with B-14 \((r=0.52)\). Absorptions indicated that the serum was dispecific and almost inclusive over B-14. Specificity 8 was apparently the second specificity recognized by B-13 because B-14 did not react in the family studies. Specificity 8 was carried by the Murray Grey bull and was passed on to at least two of his offspring (Tables 3.9 and 3.10).

**Specificity 9**

This specificity was identified by one antiserum B-23. The reaction frequency for the dispecific antiserum B-23 was 27\%
and this serum included B-22 ($r=0.64$). Specificity 9 appeared to be the extra specificity identified by B-23. Genetic data was limited to only one cow-calf pair in the Murray Grey family (Table 3.9).

**Specificity 10**

This specificity was defined by antisera B-5 and B-15 with reaction frequencies of 33% and 26% respectively. These sera were positively correlated ($r=0.45$); and the specificity was inherited by one Charolais calf from its mother. Absorptions with cells from 14 cattle suggested that B-15 was monospecific, while B-5 was at least dispecific. However, these antisera did not form an inclusive group as would be expected if one was monospecific. Two animals were positive for B-15 only. This suggested that the antisera may be identifying two separate but linked antigens.

The 10 defined specificities were present in 41 of 110 cattle from the families. Other possible specificities were present in 11 mothers but these were not considered further because of lack of data concerning their inheritance. Fifty-eight individuals (53%) did not express any antigens detectable by the typing sera.

**Analysis of linkage between the bovine B lymphocyte and Class I antigen systems**

The possibility of linkage between the two antigen systems was studied by the LOD score method using 3 informative families (Table 3.11). An informative family was defined as one in which the bull carried as least one detectable antigen from the 2 systems and was heterozygous for both systems. Informative matings within the families were considered as either a double or a single backcross.
TABLE 3.11 Linked inheritance of the bovine Class I and B lymphocyte antigen systems.

TABLE 3.11 (a) Angus Family

<table>
<thead>
<tr>
<th>Sire Genotype</th>
<th>Dam Genotype</th>
<th>Calf Genotype</th>
<th>Mating Type*</th>
</tr>
</thead>
<tbody>
<tr>
<td>117 3/-</td>
<td>A148 -/-</td>
<td>463 -/-</td>
<td>DB</td>
</tr>
<tr>
<td>7/-</td>
<td>-/-</td>
<td>-/-</td>
<td>DB</td>
</tr>
<tr>
<td>A90 -/-</td>
<td>464 3/-</td>
<td>7/-</td>
<td>DB</td>
</tr>
<tr>
<td>6/-</td>
<td>7/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A43 3/**</td>
<td>465 3/**</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>-/-</td>
<td>7/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A119 5/-</td>
<td>466 -/-</td>
<td>DB</td>
<td></td>
</tr>
<tr>
<td>-/-</td>
<td>-/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A52 -/-</td>
<td>467 3/-</td>
<td>DB</td>
<td></td>
</tr>
<tr>
<td>3/-</td>
<td>7/-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Class I specificity 3 and the B lymphocyte specificity 7 segregate together.

Genotypes expressed as Class I antigen type over B lymphocyte antigen type.

* Mating types = DB - Double backcross, SB - Single backcross, NI - Not informative

** Homozygosity cannot be excluded.
TABLE 3.11  Linked inheritance of the bovine Class I and B lymphocyte antigen systems.

TABLE 3.11 (b)  Angus x Holstein Friesian Family

<table>
<thead>
<tr>
<th>Sire Genotype</th>
<th>Dam Genotype</th>
<th>Calf Genotype</th>
<th>Mating Type*</th>
</tr>
</thead>
<tbody>
<tr>
<td>110A 1/-</td>
<td>A8 7/1</td>
<td>202 7/-</td>
<td>SB</td>
</tr>
<tr>
<td>1/-</td>
<td></td>
<td>7/-</td>
<td>DB</td>
</tr>
<tr>
<td>F10 6/-</td>
<td>208 7/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 4/**</td>
<td>205 4/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>140 1/-</td>
<td>201 1/-</td>
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</tr>
<tr>
<td>137 1/-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Class I specificity 1 and B lymphocyte specificity 1 segregate together.
Genotypes expressed as Class I antigen type over B lymphocyte antigen type.

* Mating types = DB - Double backcross
    SB - Single backcross
    NI - Not informative

** Homozygosity cannot be excluded.
<table>
<thead>
<tr>
<th>Sire Genotype</th>
<th>Dam Genotype</th>
<th>Calf Genotype</th>
<th>Genotype</th>
<th>Mating Type*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-2 1/5,6,8 8/-</td>
<td>B52 1/2 8/**</td>
<td>211 1/5,6,8 8/**</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>147 1/** 8/-</td>
<td>217 1/5,6,8 8/-</td>
<td>SB</td>
<td></td>
<td></td>
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<tr>
<td>151 1/** 9/-</td>
<td>221 1/** 9/-</td>
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<td></td>
<td></td>
</tr>
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<td>152 1/** 9/-</td>
<td>220 1/** 9/-</td>
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</tr>
<tr>
<td>155 8/-</td>
<td>223 1/- 8/-</td>
<td>DB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Class I specificities 5, 6 and 8 segregate with B lymphocyte specificity 8, except for calf 223.

Genotypes expressed as Class I antigen type over B lymphocyte antigen type.

* Mating types - DB - Double backcross
  SB - Single backcross
  NI - Not informative

** Homozygosity cannot be excluded.
based on the bull. A double backcross consisted of a mating with a
cow which was effectively homozygous. This meant that she did not
share any detectable antigens with the bull. In a single backcross
the cow was heterozygous or homozygous and shared one antigen with
the bull but was effectively homozygous for the other antigen system.

The Jersey family was not informative because all of the off-
spring carried one common Class I antigen, presumably inherited from
the homozygous bull. The details of the other 3 families are
summarized in Table 3.11.

In the Angus family the bull (117) was positive for Class I
antigen specificity 3 and for B lymphocyte specificity 7. This
family consisted of 4 informative matings, all double backcrosses.

The second, of the informative families, was an Angus x Holstein-
Friesian crossbred family. The sire carried specificity 1 for both
antigen systems. All matings were considered to be informative. The
first mating was a single backcross whereas the other 4 were double
backcrosses.

Finally, the Murray Grey family consisted of 11 purebred cattle.
In this family the bull carried 4 Class I antigen specificities (1, 5,
6, and 8). Specificity 1 segregated away from the other 3 which
were inherited as a haplotype. One B lymphocyte antigen, specificity
8, was carried by the bull. The first mating was not informative.
However, the next 3 matings were informative and were single
backcrosses while the final mating was a double backcross.

A total of 13 informative matings were available from the 3
families. In the first two families the paternal B lymphocyte and
Class I antigens were inherited as though on one haplotype. In 3
informative matings, the B lymphocyte specificity 8 was inherited
with the Class I haplotype of 5, 6 and 8. However, in the final calf (223) the opposite result was seen. This calf inherited B lymphocyte antigen 8 with the Class I antigen specificity 1 from its sire. This meant that the B lymphocyte specificity 8 was not inherited with the Class I haplotype of 5, 6 and 8. If we assume that these two antigen systems are linked, as the rest of the data suggest, then there are two possible explanations for the inheritance observed in this family. Firstly, the calf's reported parentage might be incorrect or secondly, recombination between the two systems has occurred. Records did not indicate any reasons to suspect that the parentage was incorrectly documented, and the lack of any new unexplained antigens supported this. The second explanation seems to explain best this discrepancy in the assumed linkage.

The 13 matings suggested linkage with a possible recombinant. Whether or not this is sufficient to prove linkage can be tested statistically by an analysis of likelihood. In this case z or LOD scores were used.

The recombination frequency from these data was approximately 1/13 or 0.077. Using LOD tables, scores for recombination frequencies of 0.05 is 1.4431 and for 0.10 is 1.4665. These represent odds of 28:1 and 29:1 in favour of linkage. Although these LOD scores are not as high as is recommended to demonstrate linkage in human genetic research, they are considered to be highly suggestive of the existence of linkage between the two antigen systems.

DISCUSSION

The presence of MHC-linked genes which control the expression of Class II antigens has been well documented in numerous inbred and out-bred species. Also, the structural similarity of the MHC's between
species has suggested that a Class II antigen system could be defined in cattle.

Of the 114 alloantisera tested, 47 (41%) were found to have cytotoxic B lymphocyte activity against at least one individual out of the 100 animals that were tested. In man when a battery of 110 specific HLA typing antisera was tested against human enriched B lymphocyte populations from a random test panel, 83% were shown to contain "extra" reactivity. A battery of 400 sera from alloimmunisations were also tested against the same panel. Of these sera, 32.8% were B lymphocyte positive/T lymphocyte negative; 49.3% showed greater reactivity towards B lymphocytes than towards T lymphocytes (Terasaki et al. 1975). Some 46% of sera from parous women have antibody against B lymphocyte determinants as assessed with human B lymphoblastoid cell lines used as the target cells. These sera did not contain reactivity against known HLA antigens (Ferrone et al. 1976). The 41% incidence rate of anti-B lymphocyte reactivity in bovine alloantisera correlates well with these data. The percentage could have been higher as reactivity may have been undetected because it was "masked" in some antisera by antibodies to non-B lymphocyte specific antigens.

The specific distribution of Class II antigens on lymphoid and blood cells has received much attention because it may help explain the functional significance of these antigens. In the mouse, Class II antigens are present on mature B lymphocytes and on a subpopulation of T lymphocytes as well as on 20 - 50% of monocytes (Hammerling et al. 1974; Hammerling et al. 1975). Erythrocytes and platelets do not express Class II antigens (Colombani et al. 1976; Staines et al. 1976). A similar distribution of Class II antigens occurs in the rat (Mason and Gallico, 1978); in the guinea pig (Schwartz
et al. 1977b); in man (Greaves et al. 1979); in the rhesus monkey (Zaalberg et al. 1976); and in the pig (Vaiman et al. 1975). Based on the results of absorption procedures and the two-colour fluorescence tests these antigens are not expressed on bovine platelets or erythrocytes but exist preferentially on B lymphocytes. The possibility that they were also expressed on a subpopulation of T lymphocytes and monocytes was not investigated.

Analysis of absorption tests done on buffy coat leucocytes demonstrated the presence of numerous antibody specificities. Since each antiserum was produced against lymphocytes from only one donor this suggested the presence of a complex, possibly multigenic system which controls the expression of these antigens. In the example shown (SP12) there appeared to be 6 identifiable specificities to Class I antigens and at least 3 separate specificities to B lymphocyte antigens. There are two explanations for this, firstly more than one antigenic determinant may exist on the same molecule or secondly there might be multigenic control of antigenic expression. These 2 explanations are not necessarily exclusive of one another.

If it is assumed that different antigens are controlled by separate genes then an estimate of the minimum number of loci involved can be made. For antiserum SP12 the estimate would be 3 alleles and 2 loci for the B lymphocyte antigens. This assumes that both of the alleles for each locus are identified by the antiserum and as a consequence these are only minimum estimates. The analysis of other antisera also demonstrated the presence of multiple specificities (Table 3.5). The number of specificities present in these sera to Class I antigens suggests that there is a multigenic control for them as well.
Multilocus control of Class I antigens is well established in several species (Gotze, 1977) and suggested for cattle (Adams, 1980). As well, the multilocus nature of the mouse I region is well established (David, 1976; Shreffler et al. 1976). In outbred species, genetic recombinant families have been used to show a two locus, Class II, antigen system for both the rhesus monkey (Balner, 1976; Roger et al. 1976) and the man (Mann et al. 1976; van Rood et al. 1977). These results are supported by the results of biochemical analyses of the cell surface Class II antigenic molecules (Katagiri et al. 1979; Tanigaki et al. 1980). If the similarity found between the MHC's of different species is considered in conjunction with the absorption results then it seems plausible that two loci control the expression of bovine B lymphocyte antigens.

However attractive the two locus model may be there still exists the possibility that the different antigens may be present on the same molecule. Different antigenic determinants have been shown to occur on the same MHC molecule in both mouse and man. In the mouse these are represented by the public-private antigens of the H-2 complex. These are found for both the Class I and Class II antigens (Cullen et al. 1976; Hauptfeld and Klein, 1975; Lemonnier et al. 1975). In man a similar complex system has been postulated from results obtained from serological tests and blocking tests for the HLA-A and B antigens and for the DR antigens (Albert and Gotze, 1977; Legrand and Dausset, 1975; Park et al. 1980; Reisner et al. 1976). The results of the absorption tests indicate that the B lymphocyte antigens in cattle are part of a complex system but as yet it is not possible to determine whether this complexity is due to multiple genetic loci or multiple antigenic determinants. Biochemical
analysis and studies on the redistribution of antigens on the cell surface would be needed to answer this question.

Family studies and population data suggest that the bovine B lymphocyte antigen system is highly polymorphic. At present 10 antigenic specificities have been defined. These specificities were present in 41 of the 110 cattle from the different families. An additional 11 cattle expressed antigens recognized by the typing sera but their specificities differed from the defined group. Fifty-eight cattle did not express any detectable antigens. Because of the high percentage of blank individuals (53%) and the presence of 10 defined specificities it can be assumed the degree of polymorphism within this system will be shown to be much greater as more typing antisera become available.

These antigens are expressed and inherited in an autosomal codominant fashion. Data from the families demonstrated that inheritance was consistent with control by a single genetic system. Additionally, statistical data support the absorption analyses and suggest the presence of 2 separate but linked loci. Linkage to the Class I antigen system was also documented.

In summary, these studies have demonstrated the existence of a B lymphocyte alloantigen system in cattle. The high degree of polymorphism, the restricted distribution on blood cells and the linkage to the bovine Class I antigen system suggest that this system represents the bovine equivalent of the Class II or Ia antigens of other species.
CHAPTER 4

MIXED LYMPHOCYTE REACTIVITY IN CATTLE
INTRODUCTION

The genetic control of the lymphocyte determinants responsible for allogeneic stimulation in the mixed lymphocyte reaction (MLR) is known to reside in part of the MHC in several species (Gotze, 1977). These antigens map within the I region of the mouse H-2 complex and are associated with the HLA-DR antigen system of man (David, 1977; van Rood et al. 1975b). MLR tests have been done with bovine cells and the allogeneic reaction has many similarities with those described so far in other species. Emery and McCullagh (1980) used MLR to demonstrate identity or lack of it between cattle twins. Usinger et al. (1977) have postulated the existence of two MLR loci by assuming that one way reactions were conforming to the mathematical law of transitivity. However, no detailed genetic studies on the relationship between the bovine MLR reaction and the BoLA complex have so far been done.

Recent work in reproductive physiology led to the development of embryo transfer systems which can be used to produce full-sibling cattle families (Baker et al. 1976; Shelton, 1976). It is possible to study MLR between these family members, employing the analytical methods used in species where full-sibling families are more common (Bach and Amos, 1967; Bachvaroff et al. 1973; Bradley et al. 1973; Grosse-Wilde, 1973). The MLR in cattle was investigated as part of a study on the bovine MHC and in an attempt to define genetically the bovine I region. This chapter presents evidence that the MLR in cattle is under the control of a single genetic system which is associated with the Class I antigen system of the bovine MHC.
EXPERIMENTAL METHODS

Culture systems

Several different media and gas mixtures were compared to establish the optimal conditions for cultivating bovine lymphocytes. The preliminary work was done using mitogens to stimulate the lymphocytes. A basic MLR culture system was then established and used to test the effects of varying periods of culture and concentrations of test lymphocytes. The MLR culture system was then tested with different combinations of animals. The combinations included calf-dam pairs, half-sibling pairs and full-sibling pairs as well as unrelated animals of different age groups.

Genetic analysis

The standardised test system was used to analyse the MLR between full-sibling calves. These calves were produced through embryo transfer and all siblings were of the same age. BoLA antigen types were established with the available BoLA typing reagents. The results of the MLR tests and BoLA types were analysed within families.

RESULTS

Optimal culture conditions

The mitogen Con A was used in tests with 3 different tissue culture media and 2 different gas mixtures. The 3 media tested were Eagles MEM F-15, Eagles MEM H-16 and RPMI 1640. The 2 gas mixtures tested were 5% CO₂ in air, and 10% CO₂, 7% O₂, 83% N₂. The results of these experiments suggested that any of the media tested would be suitable for cultivating bovine lymphocytes as long as the correct gas mixture was used. The combination of MEM F-15
medium and the gas mixture of 10% CO₂, 7% O₂, 83% N₂ was chosen for all subsequent tests. This combination had been used successfully for the culture of bovine lymphocytes previously, and based on the extent of incorporation of the isotope it appeared to support cell metabolism best in this experiment.

The optimal cell concentrations were determined by varying the number of responder and stimulator lymphocytes in each test. The numbers of responder and stimulator lymphocytes varied from 5 x 10⁴ to 1.5 x 10⁵ per well, in all combinations in 0.2 ml of medium. The lymphocytes were cultivated for 120 hr. Significant proliferative responses were found only when at least 2.5 x 10⁵ responder lymphocytes were used. The best responses were usually obtained when the stimulator and responder lymphocytes were present in approximately equivalent numbers. The high degree of variability in these cultures made it impossible to select any particular concentration as being ideal. For all subsequent cultures the responder and stimulator lymphocytes were used at a concentration of 5 x 10⁵ per well because this usually gave the highest total CPM. Larger numbers of lymphocytes gave even higher total CPM but these higher concentrations were not used for regular testing because they needed large quantities of blood.

The optimal times for holding cells in culture were assessed by harvesting the cultures at 24 hr intervals from 96 to 144 hr. These tests were done with lymphocytes from 4 to 9 animals from several different groups at one time. The peak responses occurred after 96 or 120 hr in the MLR tests with most cattle, although some older cows did not respond maximally until after 144 hr in culture. The results obtained from both groups of cattle showed that it was
important to add the isotope when the lymphocytes were proliferating most rapidly. Proliferation and subsequent incorporation of the isotope declined rapidly once a sample of lymphocytes had responded maximally.

Full-Sibling MLR

A total of 29 purebred Charolais cattle were tested from 9 families with offspring of 2 - 18 months of age. Dams were available from 3 families but no sires were available. It was possible to do only a single MLR test for each family and only the offspring were used for the tests because of their similar ages. This was done so as to limit any variation in the test that might be caused by differences in the ages of lymphocyte donors. The MLR test was done on cultures of 120 hr duration. The results of these within family MLR tests are shown in Table 4.1.

The association of MLR and the bovine MHC

At least one Class I antigen specificity could be identified in all cattle but usually 2 - 4 specificities were recognized. It was not possible to determine haplotypes unambiguously because of the lack of many of the parental types. The available antisera to Class II antigens did not react with any of the cattle in this group and so no information regarding any possible associations between MLR and these antigens was available. A distinction was not made between siblings that shared some or none of the defined Class I antigens and MLR test pairs were designated only as matched or mismatched.

Background variation in the test was determined by analysis of the autologous controls. The total CPM ranged from 169 to 2456 with a mean (\(\bar{x}\)) of 909.24 and a standard deviation (s) of 707.53.
TABLE 4.1 Responses of MLR Between Full-siblings

TABLE 4.1 (a) Family Number 1, 7 Siblings

<table>
<thead>
<tr>
<th>Responder Lymphocytes</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R28</td>
</tr>
<tr>
<td>R28</td>
<td>2450</td>
</tr>
<tr>
<td>R31</td>
<td>7911</td>
</tr>
<tr>
<td>R32</td>
<td>1764</td>
</tr>
<tr>
<td>R33</td>
<td>37141</td>
</tr>
<tr>
<td>R35</td>
<td>4659</td>
</tr>
<tr>
<td>R37</td>
<td>14566</td>
</tr>
<tr>
<td>R39</td>
<td>13630</td>
</tr>
</tbody>
</table>

TABLE 4.1 (b) Family Number 2, 4 Siblings

<table>
<thead>
<tr>
<th>Responder Lymphocytes</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33</td>
</tr>
<tr>
<td>33</td>
<td>839</td>
</tr>
<tr>
<td>35</td>
<td>15219</td>
</tr>
<tr>
<td>36</td>
<td>656*</td>
</tr>
<tr>
<td>37</td>
<td>3612</td>
</tr>
</tbody>
</table>

Results are expressed as median CPM

* Indicates matching for BoLA Class I antigen types.
TABLE 4.1 Responses of MLR Between Full-siblings

TABLE 4.1 (c) Family Number 3, 4 Siblings

<table>
<thead>
<tr>
<th>Responder Lymphocytes</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34</td>
</tr>
<tr>
<td>34</td>
<td>529</td>
</tr>
<tr>
<td>39</td>
<td>1158</td>
</tr>
<tr>
<td>40</td>
<td>19042</td>
</tr>
<tr>
<td>41</td>
<td>1626</td>
</tr>
</tbody>
</table>

Results expressed as median CPM

* Indicates matching for BoLA Class I antigen types.

TABLE 4.1 (d) Family Number 4, 3 Siblings

<table>
<thead>
<tr>
<th>Responder Lymphocytes</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>27</td>
</tr>
<tr>
<td>27</td>
<td>227</td>
</tr>
<tr>
<td>29</td>
<td>856</td>
</tr>
<tr>
<td>30</td>
<td>823</td>
</tr>
</tbody>
</table>

Results expressed as median CPM

* Indicates matching for BoLA Class I antigen types.
TABLE 4.1 Responses of MLR Between Full-siblings

### (e) Family Number 5, 3 Siblings

<table>
<thead>
<tr>
<th>Responder Lymphocytes</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R26</td>
</tr>
<tr>
<td>R26</td>
<td>1222*</td>
</tr>
<tr>
<td>R29</td>
<td>1218*</td>
</tr>
<tr>
<td>R34</td>
<td>1741*</td>
</tr>
</tbody>
</table>

Results expressed as median CPM

* Indicates matching for BoLA Class I antigen types.

### (f) Family Number 6, 2 Siblings

<table>
<thead>
<tr>
<th>Responder Lymphocytes</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>44</td>
</tr>
</tbody>
</table>

Results expressed as median CPM

* Indicates matching for BoLA Class I antigen types.
TABLE 4.1 Responses of MLR Between Full-siblings

### TABLE 4.1 (g) Family Number 7, 2 Siblings

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>KVP3</td>
</tr>
<tr>
<td>KVP3</td>
<td>345</td>
</tr>
<tr>
<td>KVP4</td>
<td>12815</td>
</tr>
</tbody>
</table>

### TABLE 4.1 (h) Family Number 8, 2 Siblings

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>22</td>
</tr>
<tr>
<td>22</td>
<td>241</td>
</tr>
<tr>
<td>23</td>
<td>221</td>
</tr>
</tbody>
</table>

### TABLE 4.1 (i) Family Number 9, 2 Siblings

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>2B</td>
</tr>
<tr>
<td>2B</td>
<td>1788</td>
</tr>
<tr>
<td>4B</td>
<td>757</td>
</tr>
</tbody>
</table>

Results expressed as median CPM.
Variation within one day's tests was usually less than the total variation:

Day 1: $\bar{x} = 318.11$ CPM  
$s = 210.45$ CPM  
Includes families 4, 6, 7, 8.

Day 2: $\bar{x} = 893.80$ CPM  
$s = 739.55$ CPM  
Includes families 2, 3, 9.

Day 3: $\bar{x} = 1456.70$ CPM  
$s = 537.03$ CPM  
Includes families 1, 5.

Variation within the family groups was less than this.

Because of the more limited MLR variation within the families, it was considered best to analyse the results of each family separately. However, the small numbers of individuals in each family precluded the use of standard statistical tests. To compensate for this, all results from full-sibling MLR tests which had been done on the same day were pooled and analysed together. The results of these tests are shown in Figure 4.1. These data have been plotted so that the results from autologous reactions, as well as those from different allogeneic reaction groups (siblings matched or mismatched for BoLA Class I antigen types) are presented separately. It is obvious from this figure that there is a large overlap between the different groups with respect to the total amount of $^{3}$H methyl-thymidine incorporated by the test lymphocytes. However, it is also apparent that the higher MLR results come from MLR tests done between siblings mismatched for BoLA. The tests between BoLA-matched siblings are very much more restricted in their distribution and are generally much closer to those obtained from the autologous controls. This increase of cellular reactivity between mismatched siblings was statistically signifi-
Figure 4.1: Distribution of median CPM values from MLR tests between full-sibling cattle. Results from tests which were done on the same day were pooled; 3 test days were involved.

■ = autologous control responses
○ = responses between BoLA Class I antigen matched siblings
♦ = responses between BoLA Class I antigen mismatched siblings
cant only for the day 3 test group when only the raw data for the total CPM are used in the analysis (Mann-Whitney U Test, p < 0.001).

In order to reduce the effects of the variation between families and to enable the direct comparison of all MLR tests the data were transformed by correcting them to indices. The Relative Response (RR) as defined earlier, could only be used in 4 of the families. These results are shown in Figure 4.2. The Stimulation Index (SI) was determined for all test cattle and these results are summarized in Figure 4.3. The results of the different MLR tests were highly significant statistically once the data were transformed. A significantly higher level of response was associated with MLR tests between siblings which were not matched for their BoLA Class I antigens (Mann-Whitney U test, p < 0.001), However, it was obvious that there still existed a large portion of low responders which were mismatched pairs and this caused a large overlap between the two groups (Table 4.2).

Knowing that MLR and BoLA antigen types were associated it was possible to choose empirically "cut off" values for the RR and SI (Figure 4.4). These were used to determine whether or not a given response was statistically significant and chosen so that most of the response scores from cultures between matched animals fell below these values.

The distribution of RR values in Figure 4.2 demonstrated a good fit for most of these data. The chosen cut off value of 40 appeared to be suitable based on the 22 test results from matched animals. Only two RR values fell outside the 0 - 40 range. However, 21 of 50 cultures involving mismatched animals also fell below the chosen value. Similar results were seen for the SI and the cut off value
Figure 4.2: Distribution of Relative Response values from MLR tests run between full-sibling cattle. Values are from families 1 - 4.
Figure 4.3: Distribution of Stimulation Index values from MLR tests run between full-sibling cattle. Values from all families are shown.
TABLE 4.2 The MLR Results Obtained with Lymphocytes from Full-sibling Cattle and Their BoLA Class I Antigen.

<table>
<thead>
<tr>
<th>Class I Antigen Status</th>
<th>S.I.</th>
<th>R.R.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Matched*</td>
<td>2.5</td>
<td>1.0 - 9.5</td>
</tr>
<tr>
<td>Not Matched*</td>
<td>7.6</td>
<td>1.1 - 49.0</td>
</tr>
<tr>
<td>Matched**</td>
<td>2.3</td>
<td>1.0 - 9.5</td>
</tr>
<tr>
<td>Not Matched**</td>
<td>7.0</td>
<td>1.1 - 49.0</td>
</tr>
</tbody>
</table>

* Based on 4 families for which both the SI and RR data are available.

** All tests included; no values for RR

N.A. = Not Applicable.
Figure 4.4: Distribution of Relative Response values and Stimulation Index values for families 1 - 4. Solid symbols represent MLR tests between Class I antigen matched siblings and open symbols represent tests between mismatched siblings.

■ = family 1
● = family 2
▲ = family 3
◆ = family 4

Not all MLR results are shown to avoid a large uninterpretable cluster of symbols near the intersection of the axes.
of 3 (Figure 4.3). Twenty-two of the 26 tests between matched animals had SI values less than 3. Additionally, 28 of 60 scores from mismatched animals also fell below this value. Using both of the indices together, only two MLR responses between matched animals were above the cut-off values for both.

Analysis of the negative RR values from tests involving mismatched siblings showed that 12 negative RR values from MLR tests involving mismatched siblings were from 6 pairs of animals which were negative for response in both directions. That is to say neither of the animals in the test responded against the other. Nine of the negative results came from one-way, non-responding, test pairs. A response occurred with one individual but not with the other. For the negative SI values, 12 came from 6 two-way, non-responding pairs and the remaining 16 from one-way, non-responding pairs (Table 4.3).

The high proportion of non-responders was unexpected. To ascertain the reasons for this a test was run with 8 unrelated, pure-bred, Charolais cattle, 5 to 6 months of age. All 56 possible coculture combinations were run; additionally, lymphocytes from two Holstein-Friesian calves were used as stimulator cells. Only 20 and 21 of the 56 MLR tests were positive for the RR and SI respectively using the defined cut-off values. All of the lymphocytes from these cattle responded to the lymphocytes from the Holstein-Friesian calves. It was apparent from these results that there was a high level of non-responsiveness against individuals within the Charolais breed.

**DISCUSSION**

The *in vitro* reactivity between the lymphocytes of different
TABLE 4.3 Pairwise Analysis of MLR Responses in Families

<table>
<thead>
<tr>
<th>Relative Response</th>
<th>Class I Antigen Status</th>
<th>Matched</th>
<th>Mismatched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td></td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>One-way Positive</td>
<td></td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Two-way Positive</td>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td></td>
<td>11</td>
<td>25 = 36 pairs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stimulation Index</th>
<th>Class I Antigen Status</th>
<th>Matched</th>
<th>Mismatched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td></td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>One-way Positive</td>
<td></td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Two-way positive</td>
<td></td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td></td>
<td>13</td>
<td>30 = 43 pairs</td>
</tr>
</tbody>
</table>

Results expressed as number of pairs.
individuals and the control or association of this reactivity with the MHC has been well documented for several species. The results of the experiments in this chapter suggest that a similar situation exists for cattle.

The preliminary experiments were performed to test various parameters of the bovine MLR test. These included the choice of the tissue culture medium, gas mixture, cell concentrations and the duration of the culture period. The optimal culture conditions for bovine lymphocytes were difficult to assess but were generally found to be similar to those reported for other species (Bijnen et al. 1979; Woolnough et al. 1979).

The association between the BoLA complex and MLR was assessed using full-sibling cattle families. The high level of variability in the test results made it necessary to transform the data in some way to make them more amenable to standard statistical analysis. Two types of indices were calculated to quantitate the responses. One of these was the Stimulation Index (SI) which is the ratio between the level of isotope incorporation by unstimulated cells and the level incorporated by the test cells. The dependence of this ratio on the control responses meant that some test responses could have been misclassified when the background was exceptionally high or low. The SI is commonly used in the calculation of MLR data in spite of these problems and was used here so that response values could be compared with the data available in the literature for other species. The other index that is commonly calculated is the Relative Response (RR) (Jørgensen et al. 1973) used here as modified by Mickey et al. (1975). The RR was based on the maximum response that was measured for each individual animal's lymphocytes in the within-family MLR.
tests. Other related indices have been developed to account for possible variations in the capacity of lymphocytes from different individuals to act as stimulator cells (Mickey et al. 1975; Ryder et al. 1975). These were derived for use in HLA-D typing in which a bank of stored or frozen homozygous stimulator cells are used. All samples in the present study were fresh and the sample sizes were generally too small to make the calculation of these indices useful. Other indices based on the two-way analysis of variance (Mickey et al. 1975) and an analysis based on intra-experimental error estimates (Franks and Bradley, 1977) were not applicable because of the small sample sizes used and the large proportion of negative responses in the within-family tests.

The actual association of MLR and BoLA was demonstrated by pairwise analysis of reactivity between full-siblings. This analysis demonstrated clearly that a higher level of response was associated with the MLR tests done on cattle of different BoLA antigen types. This type of analysis was similar to that used by Bach and Amos (1967) for their early HLA and MLR studies.

This particular set of experiments provides no evidence as to which BoLA antigens may be involved in MLR. It is assumed that MLR in cattle is controlled by the same gene(s) which control the expression of the previously defined Class II antigens. However, the proof of this will require further experimentation as the lack of any recognizable Class II antigen specificities precluded analysis involving these antigens.

The results did suggest that MLR is controlled by one genetic system and this idea is consistent with MLR systems in other species. Generally, strong responses are raised by mismatching at one locus.
whereas weaker responses are raised against antigens of another locus, which is also MHC linked (Bijnen et al. 1977; Goldman et al. 1975; Gunther and Stark, 1978; Schwartz et al.; Widmer and Balner, 1978). The exception to this was the cow where the existence of two strong MLR-controlling loci has been postulated (Usinger et al. 1977). The results presented here suggest that, if two strong MLR loci do exist in cattle, then they must both be associated to each other as well as to the BoLA complex.

Knowing that MLR was associated with the BoLA complex it was possible to set threshold values for the classification of positive and negative tests using the indices. The chosen values of 40 for the RR and of 3 for the SI were similar to those chosen for MLR research in other species (Balner and Toth, 1973; van Es and Balner, 1979; Grosse-Wilde et al. 1973; Jonker and Balner, 1980; Jørgensen et al. 1973).

An unexpected result was the lack of responsiveness in a large percentage of the siblings. The sharing of antigens was assumed to be the explanation for both the one-way and two-way, non-responsiveness. In the case of two-way, non-responsiveness the animals were assumed to be totally matched for their MLR antigens. For one-way non-responsiveness one of the animals was assumed to possess a set of antigens which was a total subset of the other animal's antigens (Usinger et al. 1977). One-way and two-way non-responsiveness was seen in 26 of 36 pairs when measured by the RR and in 35 of 43 pairs by the SI. This indicated that there was a large amount of antigen-sharing within the families. The results of the lymphocyte antigen-typing using BoLA typing reagents supported this contention.

The reasons for this apparent lack of diversity are not known,
but it may be due to the effects of inbreeding, either within the families or within the Charolais breed. Pedigree analysis, which did not reveal any recent inbreeding, and the lack of responsiveness between unrelated Charolais calves together suggested that a lack of genetic diversity was a feature of the breed.

In general, the principal problem in interpreting the MLR test data was the extreme variability of the assay. This high degree of uncontrollable variation appears to be inherent in the MLR assay in all species and has led to questions concerning the validity of this assay in the analysis of genetically controlled antigens. This variation has also led to the development and use of numerous data transformations and complex statistical analyses (Franks and Bradley, 1977; Mickey et al. 1975; Piazza and Galfre, 1975; Ryder et al. 1975).

In the present study several test parameters were chosen so as to minimize the variation. Firstly, MLR tests were run between full-sibling family members thereby limiting the influences not associated with the MHC. Although other non-MHC genetic differences could have affected the tests the use of families should limit this problem as much as is possible when working with outbred species.

Secondly, high numbers of lymphocytes (5 x 10^5 per well) were used for both the responder and stimulator cell populations. This was done to maximize the amount of isotope incorporated by active lymphocytes which increases the total CPM which in turn minimizes the chances of counting errors (Franks and Bradley, 1977).

Finally, triplicate cultures were run for each test combination and the median response value of these was used for analysis. The use of median values instead of mean values corrects for discrepancies within the triplicate cultures if these discrepancies are the result
of technical errors (missing or double addition of lymphocytes or isotope to any single well) (Ryder et al. 1975).

Despite these precautions, several problems relating to the high degree of variability in the assay became apparent and the MLR test data were transformed to facilitate an accurate statistical analysis. Whether or not these data transformations affect the validity of the MLR assay is a speculative question and the answers to this tend to reflect past experiences and current requirements. In this study the MLR test was used only as an indicator to assess identity between different animals with respect to their MLR antigens. This is probably the most simple or basic use of the MLR assay. Once identity or lack of identity was established reference was made to the serologically defined BoLA antigen types. The results showed that low MLR responses were more closely associated with identity for the BoLA antigens while high responses were usually associated with tests between animals which differed in their BoLA antigen types.

In light of the association between MLR responses and BoLA antigen types and the similarities seen between the presently defined BoLA complex and the MHC's of other species, it is reasonable to assume that MLR in cattle is controlled by part of the BoLA complex which probably represents the bovine I region.
CHAPTER 5

THE RELATIONSHIP BETWEEN THE BoLA COMPLEX

AND RESISTANCE TO THE CATTLE TICK, Boophilus microplus
INTRODUCTION

The ability of individual cattle to resist infestation by the tick (*Boophilus microplus*) varies greatly. This variation in susceptibility is most noticeable between different breeds and species of cattle. In general Zebu types of cattle are more resistant than breeds of European origin. There also exists great variation in tick resistance between different individuals of the same breed (Seifert, 1971). These differences have led to the suggestion that resistance to ticks may be at least in part under genetic control. Studies done on cattle herds under conditions of natural infestation have supported this contention. Cross-breeding between Zebu and European types of cattle leads to a significant increase in the levels of tick resistance in the F₁ and F₂ generations over what would be expected for the purebred European cattle. Heritability estimates for this are as high as 82% in the F₂ generation (Seifert, 1971). Differences within breeds are not believed to be as great or influenced to the same extent by a genetic component, but there is still a clear genetic influence and heritability estimates for tick resistance range from 39% to 64% for several European types of cattle breeds (Seifert, 1971; Wharton *et al.* 1970). The results of recent studies in which cattle are infested with a known number of viable tick larvae and planned breeding experiments done with animals whose history with tick infestation is known have supported the earlier studies. In these studies resistance is assessed by determining the proportion of tick larvae which mature to adult ticks. The smaller the proportion of surviving ticks, the higher the resistance rating. These types of studies have also shown that numerous non-genetic factors are involved in tick resistance. Included are variations
with the season, the sex of the cattle, the age of the cattle and the state of pregnancy and lactation (Utech et al. 1978a). Several breeds of cattle have been tested and different levels of resistance for each breed have been documented (Utech et al. 1978b).

The actual mechanisms involved in tick resistance are only partially understood but immune mechanisms are clearly involved. Resistance to tick infestation is an acquired trait, with active mechanisms of resistance being generated only after exposure to ticks (Roberts, 1969; Hewetson, 1971; Wagland, 1978a). While both the Zebu and European breeds of cattle are susceptible to primary tick infestation, different levels of resistance develop following the primary challenge. In general, Zebu type cattle develop their resistance more quickly and reach higher levels of resistance than do the European breeds (Hewetson, 1971; Wagland, 1978a). Responses involving both humoral and the cell-mediated immune functions have been described. Brossard (1976) used indirect immunofluorescent techniques and immunoelectrophoresis to show that antibodies specific for the proteins associated with tick salivary glands appeared in cattle serum following heavy tick infestation. The levels of tick resistance and the titres of the specific antibody were positively correlated. Similar results were obtained with a passive haemagglutination assay and antigens obtained from adult ticks (Willadsen et al. 1978). The resistance conferred by antibodies can be passively transferred to non-immune individuals with plasma. Only cattle which are highly resistant can transfer this humoral resistance (Roberts and Kerr, 1976). Delayed type hypersensitivity (DTH) and allergic reactions against tick extracts have also been recorded in resistant cattle, suggesting that cellular immune functions may be important in
determining tick resistance (Schleger et al. 1976; Willadsen et al. 1978). The level of tick resistance and the magnitude of these cellular responses were highly associated.

The combination of the genetic control and involvement of the immune system in resistance to ticks suggests that an immune response (Ir) gene or genes may be directly involved. If such an Ir gene is involved then it may well be part of the bovine I region and be linked to the MHC. This chapter presents results from an experiment designed to test this hypothesis by testing for an association between levels of tick resistance and different BoLA antigen types.

EXPERIMENTAL METHODS

All laboratory and field operations were done on the premises of "Swan's Lagoon" station. A group of 199 cattle, 3/4 Brahman x 1/4 Shorthorn, 6 to 18 months of age, were chosen for the experiment. Both sexes were included in the group, 79 bulls and 120 heifers. Breeding animals were selected from the established herd. This meant that animals were not brought in from any outside sources. The consequence of this closed herd policy would be the reduction of the genetic variation within the herd.

All cattle had been previously exposed to tick infestation under field conditions. Planned tick infestations and tick counts were done as described previously. All cattle were infested on the same day.

BoLA Class I antigen types were established for all cattle using the available typing antisera and the standard microlymphocytoxicity test. Class II antigen typing was not possible because of the limitations on time, the availability of blood samples and the limited laboratory facilities on the station. Tick resistance levels
were compared with the BoLA Class I antigen types to determine if any particular BoLA antigenic specificity was associated with resistance levels. Significance of an association was ascertained by $\chi^2$ tests. The magnitudes of the associations were determined using Relative Risk analysis (Woolf, 1955).

RESULTS

The results of the planned tick infestation and the subsequent counts are shown in Figure 5.1 which shows that most of the cattle studied carried few ticks and are probably considered to be at least moderately resistant. The actual mean counts ranged from 376 to 0 and varied greatly between sexes but not between age groups. The heifers in the group that were approximately 6 months of age had a mean tick count of 33; the 18 months old heifers had a mean count of 25. The bulls carried many more ticks; mean counts of 72 and 71 for the two groups of 6 and 18 months of age respectively. Because of the differences between groups the "percent resistance index" was used to transform all data. This transformation is based on the mean tick counts within the groups and put all values in the range of approximately 0 - 100. It is possible to compare the tick counts of all cattle as a single group by using the percent resistance values. The distribution of these values is shown in Figure 5.2.

The BoLA Class I antigen types and their frequencies in the test group are shown in Table 5.1. Five other Class I antigenic specificities were totally absent from this population.

To test for the presence of any associations between the BoLA system and resistance or susceptibility to ticks the cattle were divided into high and low resistance groups based on percent resistance scores. The distribution of the BoLA Class I antigen types
Figure 5.1: The distribution of mean tick counts for the entire test group of 199 cattle.
Figure 5.2: The distribution of "percent resistance values" for the entire test group of 199 cattle.
<table>
<thead>
<tr>
<th>BoLA Specificity*</th>
<th>Number of Antigen Positive Cattle**</th>
<th>Antigen Frequency***</th>
</tr>
</thead>
<tbody>
<tr>
<td>BoLA CA-2</td>
<td>78</td>
<td>39.0</td>
</tr>
<tr>
<td>BoLA CA-3</td>
<td>36</td>
<td>18.0</td>
</tr>
<tr>
<td>BoLA CA-4</td>
<td>49</td>
<td>24.5</td>
</tr>
<tr>
<td>BoLA CA-5</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>BoLA CA-6</td>
<td>43</td>
<td>22.5</td>
</tr>
<tr>
<td>BoLA CA-7</td>
<td>10</td>
<td>5.0</td>
</tr>
<tr>
<td>BoLA CA-8</td>
<td>92</td>
<td>46.0</td>
</tr>
<tr>
<td>BoLA CA-9</td>
<td>24</td>
<td>12.0</td>
</tr>
<tr>
<td>BoLA CA-11</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>BoLA CA-12</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>BoLA CA-14</td>
<td>122</td>
<td>61.0</td>
</tr>
<tr>
<td>BoLA CA-15</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>BoLA CA-17</td>
<td>21</td>
<td>10.5</td>
</tr>
<tr>
<td>BoLA CA-19</td>
<td>15</td>
<td>7.5</td>
</tr>
<tr>
<td>BoLA CA-20</td>
<td>14</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* BoLA antigenic specificities are designated as suggested in the First International Bovine Lymphocyte Antigen (BoLA) Workshop (Spooner et al. 1979b). Definition of individual specificities is described elsewhere (Adams, 1980).

** Represents the number of cattle in the test group which have these antigenic specificities. At least one detectable BoLA specificity was carried by all cattle; 2 - 5 detectable BoLA specificities were present in most cattle.

*** Values expressed as percentages. Percentages based on 199 test cattle.

Five BoLA specificities were totally absent from the test population. These were BoLA CA-1, CA-10, CA-13, CA-16 and CA-18.
within these high and low resistance groups was then compared with that which would be expected based on a random distribution and the frequency of the antigens in the entire group of 199 cattle.

Two specificities (BoLA CA-2 and BoLA CA-4) were significantly associated with the high levels of resistance and one specificity (BoLA CA-11) was associated with low levels of resistance, Table 5.2. Specificity BoLA CA-2 was negatively associated with high tick resistance, which meant that it was present much less frequently than would be expected in the animals with high tick resistance. The lack of BoLA CA-2 was most noticeable in the group of 50 cattle with resistance of 95% and greater (0.001 < p < 0.005). A significant positive association was found for the antigenic specificity BoLA CA-4 and high resistance levels. This specificity was present in a higher frequency than was expected in cattle which were more than 90% resistant (0.005 < p < 0.01). The third association involved the lack of cattle in the resistance group of 25% and greater which were positive for the antigenic specificity BoLA CA-11 (p < 0.001).

Because of the large number of BoLA antigens involved in the typing (15 specificities) there was a chance that the disturbed antigen distribution could have been caused by chance alone. One way to correct for this bias is to multiply the "p" values by the number of specificities examined. After this correction, the negative associations with the specificities, BoLA CA-2 and BoLA CA-11 are still significant (p < 0.05) but the positive association with specificity BoLA CA-4 is no longer significant (0.075 < p < 0.15).

The standard use of Relative Risk estimates is with positive associations. The Relative Risk value for the antigenic specificity BoLA CA-4 is 2.5 for the group of cattle which are 90% or more
### TABLE 5.2 Relationships Between 3 BoLA Class I Antigens and Different Levels of Tick Resistance.

<table>
<thead>
<tr>
<th>Resistance Level</th>
<th>BoLA Antigenic Specificity</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>$\chi^2$*</th>
<th>Association Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\geq$95%</td>
<td>BoLA CA-2</td>
<td>10</td>
<td>68</td>
<td>40</td>
<td>81</td>
<td>10.32</td>
<td>Negative</td>
</tr>
<tr>
<td>$\geq$90%</td>
<td>BoLA CA-4</td>
<td>30</td>
<td>19</td>
<td>58</td>
<td>92</td>
<td>7.62</td>
<td>Positive</td>
</tr>
<tr>
<td>$\geq$25%</td>
<td>BoLA CA-11**</td>
<td>4</td>
<td>1</td>
<td>189</td>
<td>2</td>
<td>11.61</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* $\chi^2$ Values are determined by comparing the cattle above and below the designated resistance levels using $2 \times 2$ tables.

** Because of some problems with typing for this antigen the $\chi^2$ values are based only on comparisons with 196 test cattle.

A = The number of cattle positive for the antigen and present in the designated test group.

B = The number of cattle positive for the antigen and absent from the designated test group.

C = The number of cattle negative for the antigen and present in the designated test group.

D = The number of cattle negative for the antigen and absent from the designated test group.
resistant. This means that cattle with the BoLA CA-4 antigen are 2.5 times more likely to be highly resistant to ticks than cattle without the antigen. Relative Risk estimates are not as easily interpreted for the negative associations because they give values of less than one and they were not calculated for the negative associations.

DISCUSSION

It is well known that genetic and immunological parameters are included in the many components involved in resistance to tick infestation in cattle. Genetically controlled differences between breeds, and to a lesser extent between individuals within breeds, have been studied and quantitative estimates of these differences have been assessed (Seifert, 1971; Utech et al. 1978; Wharton et al. 1970). The immunological components involved in resistance include the formation of varying levels of specific antibody and the establishment of DTH against tick proteins (Brossard, 1976; Schleger et al. 1976; Willadsen et al. 1978). The involvement of both genetic and immune components led to the suggestion that the bovine MHC may be involved in tick resistance with the capacity to respond immunologically being controlled by one or more response (Ir) genes. The present study was designed to look for evidence of an association between the level of resistance to ticks and BoLA antigen types.

The results of this experiment demonstrate that an association almost certainly exists between the BoLA complex and the levels of tick resistance exhibited by different individuals within a population of cattle living in a tropical environment where ticks are prevalent. However, this observation requires confirmation in different populations.

Both negative and positive associations between BoLA antigenic
specificities and high levels of tick resistance were observed. The reasons for the 2 different associations are not known but several possibilities exist. It is normally assumed that if a particular MHC antigen is associated with disease resistance or susceptibility then it is because of an association between the MHC antigen detected and a linked Ir gene. A positive association with increased susceptibility can be taken to mean that the linked gene represents an immune defect, whereas a positive association with resistance suggests the opposite is true and that the linkage demonstrates the presence of an exceptionally competent Ir gene. In a closed population this association could be readily maintained by linkage disequilibrium. This may be the case for the positive association seen between the higher levels of tick resistance and the antigenic specificity BoLA CA-4. In this instance the BoLA CA-4 antigen is assumed to be linked to an advantageous Ir gene and the linkage is maintained by the closed nature of the cattle population which was tested.

The negative association with the antigenic specificity BoLA CA-2 and high levels of resistance may also be due to the closed nature of the particular cattle herd used and to linkage disequilibrium between the detectable antigen and an Ir gene which confers some degree of susceptibility to infestation by ticks. A situation such as this could keep cattle which were positive for the BoLA CA-2 antigenic specificity from reaching and maintaining the extremely high levels of resistance which are attainable by those cattle without this particular antigen. This type of explanation also fits the observed negative association of the antigenic specificity BoLA CA-11 with the group which was at least 25% resistant to tick infestation.

The model involving selection and linkage disequilibrium is an
attractive hypothesis for the disturbed antigen frequencies, but
certainly not the only possible explanation. The possibility also
exists that the increase or decrease in the frequency of one BoLA
antigen has caused an increase or decrease in the frequencies of
other BoLA antigens. It appears likely that this is true for the 2
antigenic specificities (BoLA CA-2 and BoLA CA-4) in the highly
resistant group of cattle. These antigens are 2 of the more common
specificities present in the test population. They are also allelic
specificities, belonging to the same segregant series (Adams, 1980).
Because of this, an increase or decrease in the frequency of either
of these antigens in a portion of the test population would alter the
frequency of the other antigen, in the opposite direction. This
suggests that the negative association of BoLA CA-2 and the positive
association of BoLA CA-4 with the high resistance group may both be
part of the same cause-and-effect relationship.

This type of relationship between antigens may also explain the
observed negative association of the antigenic specificity BoLA CA-11
with the group of cattle that exhibited resistance levels of at least
25%. An excess of a particular BoLA antigen in the highly resistant
group may have decreased the number of BoLA CA-11 antigens in this
group. An effect of this type and the fact that only 5 cattle were
positive for the BoLA CA-11 specificity suggests also that the nega­tive
association of this antigen specificity with high resistance may
be an artefact.

Analysis of the associations concerning the antigenic specifici­ties BoLA CA-2 and BoLA CA-4 suggests that the actual involvement
of these antigens in tick resistance may be very limited. The
Relative Risk value of 2.5 for the BoLA CA-4 specificity means that
animals with this antigen are 2.5 times more likely to be resistant
to infestation with ticks, at the 90% level of resistance, than animals without this antigen. Although this value is low, it is of the same order as many of the associations found between the HLA system of man and diseases (Ryder et al. 1979) and it does not necessarily mean that the BoLA complex plays only a minor role in tick resistance. If the association is due to an Ir gene in linkage disequilibrium, then a Relative Risk of 2.5 implies that the putative Ir gene plays an extremely important role in tick resistance. If the association is not due to a linked gene but reflects an intrinsic role of the antigen itself then the Relative Risk of 2.5 implies that the BoLA complex plays an important but not an overwhelming role in tick resistance. As there are many non-immunological factors affecting the level of tick resistance, both genetically and environmentally controlled (Seifert, 1971; Utech et al. 1978b; Wharton et al. 1970), an extremely high association with the BoLA complex is not expected.
CHAPTER 6

THE ALLOGENEIC ANTIBODY RESPONSE IN CATTLE
INTRODUCTION

The Class I and Class II antigens of the bovine MHC have been identified by standard serological techniques and with the use of alloantisera directed against them. These alloantisera were usually obtained from cattle inadvertently immunised during pregnancy and parturition with paternally inherited foetal antigens (Amorena and Stone, 1978; Caldwell et al. 1979). Other antisera were obtained through skin grafts and planned immunisations with leucocytes (Adams, 1980; Spooner et al. 1978). Little attention has been given to the response to alloantigens in cattle except in pregnancy and parturition. Several groups have followed the anti-lymphocyte antibody response and assumed that it was directed against the bovine Class I antigens (Adams, 1980; Newman and Hines, 1979; Newman and Hines, 1980). These studies have shown that a certain percentage (30 -60%) of first-calf heifers can be expected to raise detectable anti-lymphocyte antibody following parturition, but that very little antibody activity is seen prior to the birth of the first calf. The likelihood of each subsequent pregnancy causing antibody production seems to be similar. Antibody activity generated from a previous pregnancy may exist throughout subsequent pregnancies and this appears to have no effect on the pregnancy or the calf. The sera from multiparous cows can as a consequence be very broadly reactive. It has been recommended that samples should be taken 2 - 8 weeks after parturition from cows at their first or second calvings if these sera are to be used as typing reagents.

The titres and classes of antibody produced by alloimmunisations have also been studied although the results obtained have not been consistent. Newman and Hines (1979 and 1980) found that in first calf
heifers the highest cytotoxic titres were present 7 to 15 days after calving, and these titres ranged from 2 - 64. Column chromatography was used to separate immunoglobulin classes and IgG appeared to be the predominant immunoglobulin, although IgM was also detectable in samples from 8 of 11 heifers taken two weeks after parturition. Similar results were obtained by Adams (1980) with respect to titres (range 4 - 256) but he found no activity in the IgM class.

Two experiments were done to study further the allogeneic antibody response in cattle. Firstly, the antibody responses to allogeneic lymphocytes and to one particulate and one soluble antigen were followed. Secondly, two different immunisation schemes were compared with respect to the responses against both the Class I and the Class II antigens of the BoLA complex.

EXPERIMENT I

Comparison of the antibody response to allogeneic lymphocytes, xenogeneic protein and xenogeneic erythrocytes in cattle.

Experimental methods

Immunisations: Twenty mixed-breed heifers, 8 - 12 months of age were chosen for immunisation. Two unrelated vasectomized bulls served as the donors of lymphocytes and platelets used for the immunisations, the absorptions and the testing. A single goose was used as a donor for red blood cells (GRBC) for both the immunisations and the testing procedures. Ten of the heifers were immunised by the IV injections of $5 \times 10^8$ goose red blood cells (GRBC), 5 mg ovalbumin and $1 \times 10^7$ bovine lymphocytes all in PBS on day 0. The remaining 10 heifers were immunised on the following day with the same antigens. Ten heifers were immunised with lymphocytes from bull "A40" and 10
with lymphocytes from bull "O". GRBC and ovalbumin were used to compare the immune responses to a particulate and soluble antigen with the immune response to allogeneic lymphocytes. Blood samples were taken before immunisation and on alternate days thereafter. A second set of immunisations, with the same antigens, was given 14 days after the first challenge and blood samples were taken until day 28. Serum was prepared, heat-inactivated and stored at -25°C until tested. A total of 310 sera was collected.

**Antibody measurement:** The response to GRBC was measured by a direct haemagglutination assay and to ovalbumin by a passive haemagglutination assay with the ovalbumin coupled to bovine erythrocytes. Sensitivity of the antibody to 2-ME was used to give an approximate measure of the relative proportions of IgM and IgG antibody present in the sera.

Cytotoxic antibody directed against Class I lymphocyte antigens was detected using the standard microlymphocytotoxicity test. The possibility that cytotoxic antibody was present against only Class II antigens was investigated by the two-colour fluorescence microlymphocytotoxicity test. Alloantisera which were weak or negative in the standard microlymphocytotoxicity test were used without modification. Lymphocytotoxic antisera collected at the time when the antibody titre was at its peak were absorbed with platelets to remove antibody against the Class I antigens.

**Results**

**Antibody responses to GRBC and Ovalbumin:** All 20 heifers responded similarly to GRBC. Background titres of 2 to 8 were present in the pre-immunisation samples and these values were subtracted from
the titres measured after antigenic challenge. Antibody sensitive to 2-ME was first detected on days 4 to 8, while antibody resistant to 2-ME was detected on days 10 to 12. Peak antibody titres in the primary response ranged from 2 - 64 and occurred on days 10 to 14. The 2-ME resistant titres reached a peak on days 12 to 14. Antibody titres remained stable for 4 - 6 days following the secondary immunisation given on day 14. From day 20 onwards there was a rapid increase in antibody titres, and these titres reached a peak between days 20 - 24. The 2-ME resistant titres throughout the secondary response were similar to the total antibody titres (Table 6.1) suggesting that most of the antibody was IgG. Peak titres ranged from 16 to >2048. The primary response reflected the degree of response in the secondary, such that cattle were either low or high responders in both phases.

Antibody against ovalbumin was not detected in the sera from any of the heifers. This lack of responsiveness may indicate the need for a different route of immunisation for this soluble antigen.

**Lymphocytotoxic antibody:** Five of the heifers gave a primary lymphocytotoxic antibody response. These 5 and 5 other heifers produced lymphocytotoxic antibody following the second immunisation. The majority of the antibody was IgM as shown by its sensitivity to 2-ME (Table 6.2). The antibodies in the sera from heifers 391 and A59 which had the highest titres became more 2-ME resistant by days 26 and 28. Lymphocytotoxic antibodies against Class II alloantigens were not detected in any of the antisera.

**Non-cytotoxic antibody to lymphocyte antigens:** Only sera collected on days 22 and 23 that contained no cytotoxic antibodies
TABLE 6.1 Antibody Response to Goose Red Blood Cells

TABLE 6.1 (a) Heifer Group 1*

<table>
<thead>
<tr>
<th>Day</th>
<th>Total Antibody Titre</th>
<th>2-ME Resistant Antibody Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0, 0 - 2</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1, 1 - 4</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>4, 1 - 4</td>
<td>1, 0 - 1</td>
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<td>11</td>
<td>8, 2 - 8</td>
<td>1, 0 - 2</td>
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<td>13</td>
<td>8, 2 - 8</td>
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<td>15</td>
<td>8, 2 - 16</td>
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<td>17</td>
<td>8, 4 - 8</td>
<td>2, 0 - 8</td>
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<td>19</td>
<td>32, 16 - 64</td>
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<td>21</td>
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<td>25</td>
<td>128, 32 - 512</td>
<td>64, 32 - 256</td>
</tr>
<tr>
<td>27</td>
<td>64, 64 - 128</td>
<td>64, 16 - 128</td>
</tr>
</tbody>
</table>

Values expressed as median and interquartile range of agglutination titres.

* Serum samples were taken on day 0 and on the odd days thereafter, days 1, 3, 5, 7, ... 27.
TABLE 6.1 Antibody Response to Goose Red Blood Samples

**TABLE 6.1 (b) Heifer Group 2***

<table>
<thead>
<tr>
<th>Day</th>
<th>Total Antibody Titre</th>
<th>2-ME Resistant Antibody Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1, 0 - 2</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1, 0 - 1</td>
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<tr>
<td>8</td>
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<td>10</td>
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<td>12</td>
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<td>14</td>
<td>4, 2 - 4</td>
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<td>18</td>
<td>8, 4 - 32</td>
<td>4, 1 - 16</td>
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<td>20</td>
<td>256, 32 - &gt;2048</td>
<td>128, 16 - 512</td>
</tr>
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<td>22</td>
<td>512, 32 - &gt;2048</td>
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<td>1024, 32 - &gt;2048</td>
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<td>26</td>
<td>512, 64 - &gt;2048</td>
<td>512, 32 - 2048</td>
</tr>
<tr>
<td>28</td>
<td>512, 64 - &gt;2048</td>
<td>512, 32 - 2048</td>
</tr>
</tbody>
</table>

Values expressed as median and interquartile range of agglutination titres.

* Serum samples were taken on day 0 and on the even days thereafter, days 2, 4, 6, 8, . . . 28.
<table>
<thead>
<tr>
<th>Animal Identification Number</th>
<th>Day Antibody was First Detected</th>
<th>Peak Total Antibody Titre</th>
<th>Day of Peak Total Antibody Titre</th>
<th>Peak 2-ME Resistant Titre</th>
<th>Day of Peak 2-ME Resistant Titre</th>
<th>Lymphocyte Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>10</td>
<td>2</td>
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<td>ND</td>
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</tr>
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<td>2</td>
<td>26</td>
<td>&lt;2</td>
<td>ND</td>
<td>A40</td>
</tr>
</tbody>
</table>

* ND = Not Determined. An antibody titre of at least 2 is required to test for susceptibility to 2-ME.
were screened for non-cytotoxic antibodies. No antibody detectable by indirect immunofluorescence could be found in any of these sera based on the proportion of fluorescing lymphocytes (Table 6.3). However, lymphocytes incubated with serum from A65 showed a slightly higher percentage of fluorescence and an intensity of fluorescence similar to that observed with a known cytotoxic BoLA-typing antiserum. Although the intensity of fluorescence was estimated subjectively, the difference seen was believed to be real. The limited distribution of the fluorescence (34% of the lymphocytes) suggested that the serum may contain non-cytotoxic antibody to Class II antigens.

**EXPERIMENT 2**

Comparison of the bovine antibody responses to Class I and Class II alloantigens.

**Experimental methods**

*Lymphocyte immunisations: * Twenty-one mixed breed cattle, 4 - 14 months of age were chosen for immunisations with allogeneic lymphocytes. Recipients were of both sexes, had not been previously immunised and were nonparous. No restriction in regard to pregnancy status or age was placed on the cattle from which the lymphocytes were obtained for the immunisations. Eleven cattle served as both lymphocyte donors and as recipients of allogeneic lymphocyte immunisations. All experimental cattle were chosen from a group which had been typed for BoLA Class I and Class II antigens. Donor and recipient pairs were chosen to ensure that there was at least one detectable difference between them in both the Class I and the Class II antigen systems.

Cattle were given 4 intravenous injections, at fortnightly
<table>
<thead>
<tr>
<th>Test Serum Number</th>
<th>Percent Fluorescent Lymphocytes</th>
<th>Fluorescence Pattern*</th>
<th>Fluorescence Intensity</th>
<th>Lymphocyte Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>BoLA-Class I 98</td>
<td></td>
<td>W</td>
<td>Strong</td>
<td>0</td>
</tr>
<tr>
<td>NBS 25</td>
<td></td>
<td>PS</td>
<td>Weak</td>
<td>0</td>
</tr>
<tr>
<td>A64 - 22 29 PS</td>
<td></td>
<td>PS</td>
<td>Weak</td>
<td>0</td>
</tr>
<tr>
<td>99 - 22 29 PS</td>
<td></td>
<td>PS</td>
<td>Weak</td>
<td>0</td>
</tr>
<tr>
<td>581 - 23 22 PS</td>
<td></td>
<td>PS</td>
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<td>0</td>
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<td>363 - 23 26 PS</td>
<td></td>
<td>PS</td>
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<td>0</td>
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<tr>
<td>A65 - 23 34 W</td>
<td></td>
<td>W</td>
<td>Strong</td>
<td>0</td>
</tr>
<tr>
<td>171 - 23 31 PS</td>
<td></td>
<td>PS</td>
<td>Weak</td>
<td>0</td>
</tr>
<tr>
<td>BoLA-Class I 95</td>
<td></td>
<td>W</td>
<td>Strong</td>
<td>A40</td>
</tr>
<tr>
<td>NBS 21</td>
<td></td>
<td>PS</td>
<td>Weak</td>
<td>A40</td>
</tr>
<tr>
<td>150 - 22 16 PS</td>
<td></td>
<td>PS</td>
<td>Medium</td>
<td>A40</td>
</tr>
<tr>
<td>738 - 23 23 PS</td>
<td></td>
<td>PS</td>
<td>Weak</td>
<td>A40</td>
</tr>
<tr>
<td>191 - 23 23 PS</td>
<td></td>
<td>PS</td>
<td>Medium</td>
<td>A40</td>
</tr>
<tr>
<td>384 - 23 21 PS</td>
<td></td>
<td>PS</td>
<td>Weak</td>
<td>A40</td>
</tr>
</tbody>
</table>

The numbers of the antisera are the same as the heifer numbers - day that serum was collected. Background values were established using normal bovine serum (NBS). BoLA typing antisera were used as positive controls.

* Fluorescence Pattern = PS - Spotted surface and peripheral labelling of the cell.

W - Whole cell labelling.
intervals, of $1 - 2 \times 10^7$ live lymphocytes in sterile phosphate buffered saline (PBS), pH 7.2. Blood samples were taken immediately before immunisation and then at weekly intervals for 16 weeks.

Skin grafting: For the skin graft experiments 12 unrelated, mixed-breed heifers, 9 - 12 months of age were used. These had been matched according to their BoLA Class I antigen types so that 3 pairs shared 2 or 3 detectable specificities while the other 3 pairs were totally mismatched. At least one Class II antigenic difference existed within each pair.

Skin grafts were exchanged between reciprocal pairs of heifers and monitored as described previously. Twelve weeks after the exchange of grafts the heifers were given a series of 3 fortnightly injections of lymphocytes. Blood samples were taken immediately before the skin grafts were exchanged and at weekly intervals for 28 weeks.

The serum was separated from the cells, heat-inactivated and stored at $-25^\circ$C until tested. A total of 722 samples were collected.

Detection of Antibody: Cytotoxic antibody directed against the Class I and Class II antigens was detected with the appropriate micro-lymphocytotoxicity test. All sera were tested against a panel of 60 cattle. Individuals in this panel were selected to provide a variety of known BoLA antigen types and included all the lymphocyte and skin graft donors. This panel of cattle was used to select the individual lymphocyte sources for the determination of antibody titres to antigens of both classes.
Results

Antibody responses to Class I antigens: Seventeen of the 21 cattle immunised with lymphocytes produced detectable antibody to Class I antigens. Of the 10 animals responding to the first immunisation, two had antibody at day 7, the others at day 14. Four animals produced antibody after the second immunisation, 3 by day 21 and one by day 28. Two others required a third immunisation before antibody was detected and one required a fourth (Table 6.4). The first detectable antibody was predominantly IgM. Antibody titres increased in 14 animals as the immunisations continued and the peak titres occurred on days 14 to 49. Generally, those cattle which responded earliest reached the highest antibody titres. Sensitivity of the antibody to 2-ME decreased throughout the response. All of the antibody was 2-ME resistant by 7 to 21 days after the first antibody appeared (Figure 6.1). The antibody titres began to decrease in all animals after the immunisations at a relatively constant rate, irrespective of the magnitude of the peak responses. Consequently, the antibody titres reached undetectable levels in samples of those animals with low titres much sooner than it did in individuals with higher titres. Thus some cattle had no detectable antibody by 7 - 14 days after the final immunisation, whereas, others still had antibody in their serum at the final bleed, 10 weeks later.

The cytotoxic antibody response which followed skin grafting was remarkably similar in all 12 cattle (Table 6.5). Antibody was first detected on day 14 when the activity had already reached titres ranging from 16 - 2048. Peak activity was reached between days 14 - 21 with titres of 128 to 2048. Antibody was still detectable in all these animals when the first lymphocyte injections were given on day 84.
TABLE 6.4 Antibody Responses to Class I Antigens Following Lymphocyte Immunisations.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>First Antibody Detected</th>
<th>Peak Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day Total 2-ME Resistant Titre</td>
<td>Day Total 2-ME Resistant Titre</td>
</tr>
<tr>
<td></td>
<td>First Antibody Detected</td>
<td>Peak Antibody</td>
</tr>
<tr>
<td></td>
<td>Day Total 2-ME Resistant Titre</td>
<td>Day Total 2-ME Resistant Titre</td>
</tr>
<tr>
<td>41</td>
<td>7 2 &lt;2 1</td>
<td>14 128 32</td>
</tr>
<tr>
<td>49</td>
<td>7 128 16 1</td>
<td>28 1024 512</td>
</tr>
<tr>
<td>286</td>
<td>14 1 ND** 1</td>
<td>28 4 2</td>
</tr>
<tr>
<td>787</td>
<td>14 2 &lt;2 1</td>
<td>35 64 32</td>
</tr>
<tr>
<td>806</td>
<td>14 1 ND 1</td>
<td>49 8 4</td>
</tr>
<tr>
<td>848</td>
<td>14 1 ND 1</td>
<td>14 1 ND</td>
</tr>
<tr>
<td>A10</td>
<td>14 16 2 1</td>
<td>49 256 256</td>
</tr>
<tr>
<td>A57</td>
<td>14 2 &lt;2 1</td>
<td>42 1024 512</td>
</tr>
<tr>
<td>D31</td>
<td>14 4 &lt;2 1</td>
<td>28 8 4</td>
</tr>
<tr>
<td>G1</td>
<td>14 16 2 1</td>
<td>49 128 64</td>
</tr>
<tr>
<td>789</td>
<td>21 8 &lt;2 2</td>
<td>35 128 128</td>
</tr>
<tr>
<td>980</td>
<td>21 32 8 2</td>
<td>35 64 16</td>
</tr>
<tr>
<td>G4</td>
<td>21 2 &lt;2 2</td>
<td>49 32 32</td>
</tr>
<tr>
<td>812</td>
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<td>49 2 2</td>
</tr>
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<td>35 1 ND</td>
</tr>
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<td>M3</td>
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<td>49 8 4</td>
</tr>
<tr>
<td>G5</td>
<td>49 1 ND 4</td>
<td>49 1 ND</td>
</tr>
</tbody>
</table>

* Immunisations given on days 0, 14, 28, 42. Values represent the number of immunisations given before cytotoxic antibodies were detected in the serum.

** ND = Not Determined. An antibody titre of at least 2 is required to test for susceptibility to 2-ME.
Figure 6.1: The cytotoxic antibody responses from four representative cattle following immunisation with allogeneic lymphocytes.

(a) = animal 980
(b) = animal G1
(c) = animal 41
(d) = animal H3
• = total cytotoxic antibody titre
□ = 2-ME resistant cytotoxic antibody titre
The first immunisation was given on Day 0; subsequent immunisations are shown by ▼.
<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Number of Antigens Shared with Graft Donor</th>
<th>Day of Graft Rejection</th>
<th>First Antibody Detected</th>
<th>Peak Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day</td>
<td>Total Titre</td>
</tr>
<tr>
<td>159</td>
<td>None</td>
<td>10</td>
<td>14</td>
<td>2048</td>
</tr>
<tr>
<td>171</td>
<td>None</td>
<td>10</td>
<td>14</td>
<td>512</td>
</tr>
<tr>
<td>141</td>
<td>None</td>
<td>12</td>
<td>14</td>
<td>2048</td>
</tr>
<tr>
<td>167</td>
<td>None</td>
<td>12</td>
<td>14</td>
<td>128</td>
</tr>
<tr>
<td>144</td>
<td>None</td>
<td>10</td>
<td>14</td>
<td>1024</td>
</tr>
<tr>
<td>116</td>
<td>3</td>
<td>17</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>163</td>
<td>3</td>
<td>17</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
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<tr>
<td>71</td>
<td>2</td>
<td>17</td>
<td>14</td>
<td>256</td>
</tr>
</tbody>
</table>

During the course of the experiment one test animal died. The data are presented on 11 heifers only, since each result is dependent on the availability of a specific donor.
Most of the antibody detected on days 14 and 21 was sensitive to 2-ME treatment whereas antibody produced later on in the course of the experiment was resistant to 2-ME treatment (Figure 6.2).

The subsequent immunisation of skin-grafted cattle with lymphocytes resulted in only slight or no increases in their antibody titres. However, these immunisations did arrest the decline in antibody titre, but only for as long as they continued. The status of BoLA Class I antigen matching had a significant effect on the antibody responses. The antibody responses of cattle which were totally mismatched with the skin allograft donors reached peak levels by day 14, whereas the responses of cattle which shared some antigen specificities with the graft donors usually peaked 7 days later. The difference in total antibody titres is significant at day 14 but is not significant at any other time (Mann-Whitney U test, p < 0.004).

**Antibody responses to Class II antigens:** The cytotoxic antibody response to Class II antigens was followed with the two-colour micro-lymphocytotoxicity test. Antibody was detected in 6 of the 21 animals which had been immunised with lymphocytes. Five of these 6 animals had also produced antibody to Class I antigens. The antibody titres to Class II antigens were usually lower than the titres to Class I antigens. Antibodies to Class II antigens appeared at the same time or later in the response than those to Class I antigens (Figure 6.3). As with the response to Class I antigens, some animals required more than one immunisation before antibody could be detected. Because the antibody titres were low, ranging from 1 - 8, it was not possible to determine the sensitivity of the antibody to 2-ME (Table 6.6).
Figure 6.2: The cytotoxic antibody responses from two representative cattle following skin grafting.

(a) = animal 172
(b) = animal 141

- = total cytotoxic antibody titre
□ = 2-ME resistant cytotoxic antibody titre

Skin grafts were exchanged on Day 0; subsequent lymphocyte immunisations are shown by ▼.
Figure 6.3  Comparison of the cytotoxic antibody responses to Class I and to Class II antigens in four representative cattle following immunisation with allogeneic lymphocytes.

(a) = animal 41
(b) = animal 812
(c) = animal 787
(d) = animal A10

- = total cytotoxic antibody titre to Class I antigens
  O = total cytotoxic antibody titre to Class II antigens

The first immunisation was given on Day 0; subsequent immunisations are shown by \(\downarrow\).
TABLE 6.6 Comparison of Antibody Responses to Class I and Class II Antigens Following Lymphocyte Immunisations.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>First Antibody Detected</th>
<th>Peak Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Against Class I Antigens</td>
<td>Against Class II Antigens</td>
</tr>
<tr>
<td></td>
<td>Day</td>
<td>Titre</td>
</tr>
<tr>
<td>41</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>286</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>787</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>A10</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>812</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>G8</td>
<td>NA**</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Immunisations given on days 0, 14, 28, 42. Values represent the number of immunisations given before cytotoxic antibodies were detected in the serum.

** NA = Not Applicable. This animal did not produce a detectable response to Class I antigens.
The 15 animals that failed to produce detectable antibody to Class II antigens included 3 animals which had also failed to respond to Class I antigens. The 12 responders to Class I antigens included 9 animals whose sera reacted with cells from over 50% of the individuals of the panel. As antibody activity to Class II antigens cannot be detected in the presence of antibody to Class I antigens, this broad reactivity could easily mask any activity against Class II antigens. All of the samples collected prior to day 70 which contained activity to Class I antigens were absorbed with platelets from the lymphocyte donors. These samples were tested further for antibody to Class II antigens and two additional animals were shown to be positive following absorption. The residual peak titres in these sera varied between 1 to 4.

It was possible to detect antibody to Class II antigens in samples from 8 to 12 skin grafted animals by testing their sera against the panel. The antibody responses were similar to those seen for Class I antigens with respect to the time of appearance of the antibody and the sensitivity of the antibody to 2-ME (Table 6.7; Figure 6.4). Titres ranged from 2 to 32 at the peak of the response and decreased to undetectable levels by day 84 in 5 animals. The lymphocyte immunisations increased the antibody titres to detectable levels in 3 individuals. Three cattle had detectable antibody throughout the experiment. The highest response to the Class II antigens was seen in an animal (116) which also produced the highest response to the Class I antigens. However, this pattern of response did not hold true for the other animals. It was not possible to detect antibody to Class II antigens in 4 of the skin grafted animals. All of these had very broad reactivity patterns (82 - 100%) to Class I antigens.
### TABLE 6.7 Comparison of Antibody Responses to Class I and Class II Antigens Following Exchange of Skin Allografts.

#### TABLE 6.7 (a) First Detectable Antibody

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Against Class I Antigens</th>
<th></th>
<th>Against Class II Antigens*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>Total Titre</td>
<td>2-ME Resistant Titre</td>
<td>Day</td>
</tr>
<tr>
<td>141</td>
<td>14</td>
<td>2048</td>
<td>16</td>
<td>14</td>
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<td>167</td>
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</tr>
<tr>
<td>116</td>
<td>14</td>
<td>2</td>
<td>&lt;2</td>
<td>21</td>
</tr>
<tr>
<td>163</td>
<td>14</td>
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<td>14</td>
</tr>
<tr>
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<tr>
<td>71</td>
<td>14</td>
<td>256</td>
<td>128</td>
<td>14</td>
</tr>
</tbody>
</table>

* Antibody titres to Class II antigens were determined using target cells from a third party animal.

** ND = Not Determined. An antibody titre of at least 2 is required to test for susceptibility to 2-ME.
<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Against Class I Antigens</th>
<th>2-ME Resistant Titre</th>
<th>Against Class II Antigens*</th>
<th>2-ME Resistant Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>Total Titre</td>
<td>16</td>
<td>Day</td>
</tr>
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<td>141</td>
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<tr>
<td>71</td>
<td>14</td>
<td>256</td>
<td>128</td>
<td>21</td>
</tr>
</tbody>
</table>

* Antibody titres to Class II antigens were determined using target cells from a third party animal.
Figure 6.4: Comparison of the cytotoxic antibody responses to Class I and to Class II antigens in two representative cattle following skin grafting.

(a) = animal 98
(b) = animal 116

- = total cytotoxic antibody titre to Class I antigens
- - - = total cytotoxic antibody titre to Class II antigens
O = 2-ME resistant cytotoxic antibody titre to Class II antigens
and the antibody titres were too high for the sera to be effectively absorbed.

**Reaction Frequency of Antisera:** As sequential samples were tested against the same panel it was possible to observe changes in both the antibody titre and the reactivity patterns for most of the antibody responses.

In the lymphocyte immunisations the antibodies against the donor were detected first, and these antibodies reached the highest titre and were maintained for the longest time. Table 6.8 illustrates two examples of the changes in antibody response patterns following repeated immunisations. Sera from 11 animals showed an increase in reaction frequency as the immunisation schedule progressed. Sera from 5 others reacted with the same small proportion of animals throughout. The remaining positive animal (49) responded to the first immunisation with such a broadly reactive pattern (100% reaction frequency) that it was not possible to detect any change in specificity following subsequent immunisations (Table 6.9).

The sequential changes in the reaction patterns of the antibody produced by skin grafting was also studied. The broadest reactions patterns were seen in the serum samples taken from days 14 and 21. All animals showed a decrease in reaction frequency with time (Table 6.10). There was no obvious association between the number of antigens shared and the reaction patterns.

Due to the high titres of antibody to Class I antigens it was possible to follow the antibody response to Class II antigens in only 5 animals. Four of these had produced antibody to Class I antigens and their sera had to be absorbed with platelets. One other animal (G8) responded only to Class II antigens. -There was no evidence to
### TABLE 6.8 The Increase in Antibody Specificity Following Repeated Immunisations.

**TABLE 6.8 (a) Samples From 806**

<table>
<thead>
<tr>
<th>Day</th>
<th>Test Animals</th>
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<tbody>
<tr>
<td></td>
<td>G4*</td>
</tr>
<tr>
<td>0**</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
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<td>21</td>
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</tr>
<tr>
<td>49</td>
<td>4</td>
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<tr>
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Results expressed as titre values.

* Donor of immunising lymphocyte.

** Days on which immunisation was done.
TABLE 6.8  The Increase in Antibody Specificity Following Repeated Immunisations.

TABLE 6.8 (b)  Samples From 787

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Results expressed as titre values.
* Donor immunising lymphocyte.
** Days on which immunisation was done.
<table>
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<th>Characteristics of the Narrowest Reactivity Pattern</th>
<th>Characteristics of the Broadest Reactivity Pattern</th>
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* The percent reactivity was determined using a panel of 60 test cattle.

** No change in reactivity patterns.
### TABLE 6.10 Reaction Patterns of Cattle Grafted with Allogeneic Skin

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<tr>
<th>Animal Number</th>
<th>Brodest Pattern</th>
<th>Reactivity to Panel (%)*</th>
<th>Day</th>
<th>Reactivity to Panel (%)</th>
<th>Day</th>
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* The percent reactivity was determined using a panel of 60 test cattle.
suggest that more than one Class II specificity was being detected by any of these antisera at any time during the response.

The antibody titres to Class I antigens in the samples from the skin grafted animals were too high for the sera to be effectively absorbed.

DISCUSSION

The results of the first experiment demonstrate that cytotoxic antibodies against lymphocyte antigens are produced in cattle following the intravenous injection of allogeneic lymphocytes. The allogeneic response differs from the immune response to goose red blood cells (GRBC) a xenogeneic antigen. The response to GRBC was similar in its characteristics to a classical antibody response. As in all animals, the primary response generated low titres of antibody which initially was almost all IgM and was followed subsequently by the production of IgG. The secondary response led to much higher titres of antibody which were predominantly IgG. The immune response to living allogeneic lymphocytes differed from this normal type of response in several aspects. The response which followed the primary challenge was detectable in only 5 of the 20 animals. These 5 heifers responded subsequently to a secondary challenge. The primary responses were significantly delayed in comparison to the primary responses to GRBC. A further 5 heifers produced detectable responses only after a secondary immunisation. All responses irrespective of the number of challenges had the antibody characteristics of a primary response since IgM was the predominant immunoglobulin class generated until late in the response. There was no characteristic secondary responses in any of the heifers, even though some were challenged 2 times. It is possible that the secondary responses
were delayed, as in the case of the primary responses, and that they went undetected as blood samples were not taken after day 28, but this seems unlikely. These results are in contrast to the characteristic antibody responses in mice and rats which follow immunisation with allogeneic lymphoid tissue (Anderson et al. 1967; Klein et al. 1974c; Möller, 1965; Rolstad et al. 1974). These responses resemble the antibody responses observed in the heifers to GRBC.

In the second experiment, 18 of the 21 cattle immunised with lymphocytes produced cytotoxic antibody. Sera from 17 of these animals recognised antigens which appeared to be the Class I antigens of the bovine MHC. Seven of these also produced antibody to Class II antigens. One animal responded only to Class II antigens while 3 failed to respond at all.

In the first experiment 10 of 20 heifers (50%) produced antibody cytotoxic for lymphocytes after two immunisations while 14 animals (66%) responded to the two challenges in the second study. These results are in good agreement and the additional 16% of responding cattle is probably due to the deliberate mismatching of lymphocyte donors and immunisation recipients; animals were only assumed to differ in the first experiment and were typed retrospectively, as antisera to the BoLA antigens became available.

It seemed that the immune response to Class I antigens occurred preferentially over the response to Class II antigens. However, because many of the sera contained antibody to Class I antigens it is possible that some antibody to Class II antigens was not detected. The number of animals responding to challenge in the first experiment was limited and the positive sera had such low titres that it was possible to test for antibody to Class II antigens following absorp-
tions of the sera with platelets. In the second experiment most of
the sera had higher titres of antibody to Class I antigens so as a
preliminary step the sera were tested against a cell panel by the
same method as used to first detect anti-B lymphocyte antibody
(Chapter 3).

The probability of detecting antibody to Class II antigens with
a panel can be estimated by the formula \( p = 1 - (1-f)^n \); where \( P \) =
probability of the antigen being present in the population tested,
f = frequency of the phenotype and \( n \) = the number of animals tested.
In this case \( n \) represents those animals which were negative for the
Class I specificities recognised by the particular serum being
tested. For an antigen with \( f = 0.1 \), \( n \) must equal at least 28 in
order for the antibody to this antigen to be detected with a 95% prob­
ability. Eleven of the 17 cattle which responded to Class I antigens
had reaction frequencies which were small enough to meet this re­
quirement for \( n \). This proportion dropped to 6 out of 17 as the re­
action patterns broadened. Multispecific antisera to bovine Class
II antigens have been produced by lymphocyte immunisations (Chapter
3). If any of the sera produced here were multispecific, these sera
would be more readily detected as this would increase the value for
\( f \). The probability of detecting antibody to Class II antigens is
reduced if there is linkage disequilibrium between the Class I and
Class II antigens. In this study the possible effects of linkage
disequilibrium were minimised by selecting animals from several
breeds and of different BoLA types for the test panel. The use of
this panel and, when feasible, the absorption of the sera with plate­
lets suggests that antibody to Class II antigens would have been
detected unless it was present at a very low titre (titre \( \leq 1 \)) or
present in a serum with very broadly reacting antibody to Class I antigens. Therefore, the increased frequency of response to Class I over Class II antigens would seem to be a significant finding. The reason for this difference is not known but may reflect the amount of antigen present in the immunisation mixture. Class II antigens are expressed primarily on B lymphocytes which make up only 20 - 25% of the total peripheral blood lymphocytes in cattle. Because of this it is likely that less Class II antigen was present than Class I antigen in the immunisation procedure. The results of the second experiment suggest that any deficiency with the amount of antigen forwarded may be partially overcome by increasing the number of times the immunisation procedure was done.

The responses to the Class I and Class II antigens of lymphocytes appeared to be independent of each other. The variations in the antibody titres and in the time of appearance of the antibody in the responses to the two classes of antigens were not associated with each other and did not follow any specific pattern. Also, one animal responded only to the Class II antigens. These disassociated responses occur also in humans (Ferrone et al. 1976; Terasaki et al. 1975).

The lack of general responsiveness to immunisations with allogeneic lymphocytes was unusual. In the first experiment only 10 of the 20 cattle responded to lymphocyte antigens even though retrospective BoLA typing indicated that there were antigens on the immunising lymphocytes which were different from those of the immunised animals in all but one case. In the second experiment 3 cattle failed to respond even after 4 immunisations. Similar results have been noted in other outbred species such as man (Curtoni et al. 1972)
and dog (Epstein et al. 1968; Kasakura et al. 1964). The pattern of the antibody responses varied between animals. This variation occurred in the titres, in the time of antibody appearance, in the number of immunisations required and in the presence or absence of a response. The reasons for this variation are not known, but may reflect the genetically controlled ability of the immunised animal to respond. Genetic control of responsiveness to several alloantigen systems including the antibody response to erythrocyte antigens (Gasser, 1969), H-Y antigen (Bailey and Hoste, 1971; Gasser and Silvers, 1971), and antigens of the H-2 complex (Stimpfling and Durham, 1972) have been demonstrated in mice. It has been suggested that there is a genetic control of responsiveness to HLA antigens (Curtoni et al. 1972). In cattle there appears to be genetic differences in the responses to several erythrocyte alloantigens (Sellei and Rendel, 1969) and a genetic control to BoLA antigens could explain the variations that occur in antibody titres as well as the fact that 3 animals failed to respond at all.

The antibody responses to MHC antigens following skin-grafting have been studied in several species, generally for the purpose of raising antisera for research into aspects of immunogenetics (Amos et al. 1954; Batchelor, 1965; Cohen and Kozaki, 1969; Spooner et al. 1979a; Thorsby and Kissmeyer-Nielsen, 1968). The most noticeable aspect of the immune reaction in cattle to both the Class I and Class II antigens following skin-grafting was the uniform nature of the antibody response between different animals and between the different classes of antigens. The antibody responses to Class II antigens closely paralleled those to Class I antigens with respect to the time the first antibody appeared, the time the peak titre was reached and
the class of the antibody that was involved. The first antibody detected was of very high titre and was of limited value (as a diagnostic agent) as it was very broadly reactive. The reactivity patterns narrowed and the titres decreased over the following weeks. Sera for use in lymphocyte typing are best obtained late in the immune response, unless the high titres would be of some special advantage such as in absorption studies. This finding is similar to that seen in the dog (Cohen and Kozaki, 1969).

Although antibody to Class II antigens following skin-grafting has not been previously reported in cattle, it was not unexpected that such antibody would be generated. Skin grafts have been successfully used for the generation of antibody to Class II antigens in several species (Gunther et al. 1978; Roger et al. 1976; Staines et al. 1975; Vaiman et al. 1975). In this study the antibody to Class II antigens was of much higher titres when it was raised against skin grafts. The reasons for this are not known but may reflect the different concentrations of antigens present or differences due to the immunisation method. The response to Class II antigens is also better, quantitatively, following skin grafts in the mouse (Staines et al. 1975).

With the possible exception of one animal (A65) no non-cytotoxic antibody was produced (Table 6.3). Non-cytotoxic antibody would be expected to be of the IgG₂ or IgA class since these do not fix complement under the test conditions used. Immunoglobulins of these classes usually appear late in the immune response and in lower titres than IgG₁ and IgM (Beh and Lascelles, 1973). Because of this, non-cytotoxic antibody of the IgG₂ or IgA classes would not have been expected to be present by day 28 under these experimental conditions.
It is possible that antibody detected in serum from A65 recognised Class II antigens, but that the antibody was present in sub-lytic quantities and was not identifiable in the two-colour fluorescent microlymphocytotoxicity test. Assuming that this is true, then further immunisations with lymphocytes may have caused an increase in the titre to a detectable level. This idea is supported by the results of the second experiment where 3 of 6 animals produced detectable cytotoxic antibody to Class II antigens only after 3 or 4 immunisations (Table 6.7).

Several points are obvious in the comparison of the different antibody responses following immunisation with different antigens or with different immunisation regimes. Firstly, the response to GRBC followed the pattern of classical antibody response and demonstrated that cattle do respond in a fashion similar to that of the small laboratory species. All of the cattle responded in a similar way although antibody titres varied within the group. The antibody response to allogeneic lymphocytes did not follow this pattern and they were much more variable. There is probably more variation in the allogeneic lymphocyte challenges. With xenogeneic immunisation the common or species specific portions of the antigens are usually recognised (Ferrone et al. 1978; Solheim et al. 1979) and these would be recognised as foreign by all cattle and evoke a similar response. Within the allogeneic system different animals may be more or less mismatched than others. The current BoLA typing experience does not support this but antigens other than the Class I and Class II lymphocyte antigens which have not yet been recognised may be important.

The response following skin-grafting resembled the primary
anti-GRBC response in that the first antibody detected was IgM followed one to three weeks later by IgG. Secondary grafts were not done and further challenge was by lymphocytes immunisation. These immunisations did little to provoke the expected secondary response. These results suggested that the lymphocyte immunisations were not as efficient as skin grafts in provoking an antibody response. It is possible that a different immunisation scheme would be better suited if lymphocytes were to be used as the immunogen.

At present for applied uses, it appears that skin-grafting may provide a better method for the production of typing reagents for BoLA research. The uniform nature of the response, the high antibody titres obtained and the need to immunise only once make the procedure desirable. As the BoLA system becomes better defined it may be possible to match donors and recipients more fully and obtain more restricted responses such as has been done in man (Albrechtsen et al. 1977b) and rhesus monkey (Roger et al. 1976) for the generation of antisera to Class II antigens.
CHAPTER 7

GENERAL CONCLUSIONS
GENERAL CONCLUSIONS

The sustained, highly polymorphic nature of the mammalian MHC is of great interest and has stimulated numerous theories suggesting its possible evolutionary significance (Bodmer, 1972). The actual biological role of the MHC still remains a mystery, but its association with immune responsiveness suggests that strong natural selection forces may have acted upon or may still be acting upon this gene cluster. This selection could be the force maintaining the current polymorphic nature of the system. It is this "undefined" status of the MHC that has prompted the extensive studies of the system in so many different animal species.

The association of the HLA system with numerous disease states in man has attracted much attention (Chapter 1). Unfortunately, an I region has not yet been functionally defined in man, although few doubt its existence. Additionally, functional studies in man involving diseases and HLA system are severely restricted by ethical and moral codes. Because of this, most of the functionally related information of the I region has come from experiments in laboratory species, in particular, the mouse.

The current functional and serological definition of the H-2 system and, in particular the I region, is fairly extensive (Chapter 1). In addition, a good deal is known about several H-2 influenced disease states. These have been restricted to the study of viral induced and spontaneous tumours. Resistance, susceptibility and recovery have all been studied for several types of tumours. Different H-2 related mechanisms have been suggested in the pathogenesis of this disease, and most of the evidence suggest that the I region and therefore the Ir genes control the outcome of the disease.
process (Faraldo et al. 1979; Lilly et al. 1964; Lonai and Grumet, 1975; Meruelo et al. 1977; Whitmore and Haughton, 1975).

Immune responses involving bacterial antigens and multicellular parasitic infections have not been as easy to study. Many of the responses to these more complex antigenic stimuli are under genetic control. By analysis of genetic recombinant mice, several non-H-2 Ir-gene (like) regions have been mapped but in most instances it is apparent that several sets of genes are involved (Biozzi et al. 1979; Bradley, 1974; Bradley, 1977; Morrison et al. 1978; Rosenstreich, 1980; Tanner, 1978). Because of this it has been suggested that the MHC contained I region may not be as important to disease resistance or susceptibility as was once thought (Biozzi et al. 1979). However, recent experiments using congenic strains of mice have demonstrated that the H-2 complex is involved in the immune response to several types of multicellular parasite (Blackwell et al. 1980; Claas and Deelder, 1979; de Tolla et al. 1980; Wassom et al. 1979). It is apparent from these studies that the MHC and the associated I region are likely to be very important in various aspects of the immune response which have not yet been identified.

It is this widespread involvement of the MHC associated I region which prompted the present studies in cattle. The only attempts to date, to define a bovine I region were done functionally, with synthetic polypeptides (Adams, 1980). These studies proved to be largely uninformative and suggested the need to select different antigens and to study carefully those dose-response relationships which would best suit the generally outbred nature of cattle populations. The experiments presented in this thesis represent attempts to define the bovine I region using serological and cell culture
techniques by the same experimental designs that have been employed in basic HLA research.

The results presented in Chapter 3 clearly demonstrate the existence of a highly polymorphic blood cell antigen system. These antigens have a limited expression or distribution on blood cells, being preferentially expressed by B lymphocytes. The system is under simple Mendelian genetic control and may be linked to the bovine MHC. Results of absorption analyses demonstrated the existence of a complex antigen system and suggest that two loci may be involved. The characteristics of this antigen system suggest that it is the bovine Ia or Class II antigen system although further serological studies will be required to verify the two locus model and to determine the exact association of this system to the bovine MHC.

The ability to use these Class II antigenic specificities as a marker for disease and parasite resistance in cattle could have tremendous economic value. At present this is not feasible, primarily for technical reasons. The main problem at present is the requirement of using live lymphocytes for the typing assay. These are not easily obtained, especially when the required blood samples have to be shipped interstate or overseas. The ability to use dead cells, cellular membranes, or membrane extracts or even soluble antigens for typing assays would be a major advantage.

The use of alternative typing assays has not generated much interest, but the few published studies involving the HLA system suggest that other approaches are feasible. The first approach has been to type for soluble antigens which are present in blood serum. These antigens are detected by their ability to inhibit the standard lymphocytotoxicity test by competitive binding of typing antisera
(Reisfeld et al. 1976; Tait et al. 1981). Success with this approach has been limited because all antigen specificities are not expressed in the serum, or at least the actual antigenic molecules are not present in the same quantities (Tait et al. 1981). In addition, this type of test still requires the use of live lymphocytes, although a stored, frozen panel is generally used. Another more attractive alternative is the use of an Ouchterlony type of precipitation technique. HLA antigens, made soluble by detergents, have been successfully precipitated using xenoantisera (Welsh and Turner, 1976). The use of alloantisera would be required since discrimination between the polymorphic differences is needed. A similar type of test has been used in cattle to detect allotypic differences on the light chains of bovine immunoglobulins (Faber and Stone, 1976) and this suggests that a typing system based on this principle may serve as a viable alternative to the cytotoxicity tests used presently.

With the use of an Ouchterlony type of test and soluble antigens it would be possible to freeze leucocyte suspensions and to store the cell extracts until needed. In addition to the advantages gained by not needing live lymphocytes, a test in agar or agarose could be dried, stained and stored for later reference and records. An improved testing system such as this would allow much more accurate and complete definition of the Class II antigen system and would greatly facilitate the study of these antigens and their relationship with other BoLA loci as well as diseases and infestation by parasites.

The results presented in Chapter 4 demonstrated the association of MLR and the MHC in cattle. Although the existence of MHC-linked or controlled MLR does not prove the existence of an I region, it can be seen as supporting this idea since the MLR is known to be controlled
by the I region in mouse, rat and guinea pig. It was not possible to test for any associations between the putative Class II antigen system and MLR. The number of identifiable Class II antigen specificities was too limited for either serological analysis in the families or to attempt to specifically block MLR using antisera.

The MLR test is severely limited in value as a genetic test. The need for larger quantities of blood (> 30 ml) when large families are used, the need to isolate and culture lymphocytes under sterile conditions, the long duration of the culture period and the test variation are all disadvantages of the MLR test. The use of whole blood assays may be of some value since the lymphocyte isolation step would not be required, but the other problems would not be solved.

Although the exact relationship of serologically defined Class I and Class II antigen systems and the MLR system is not known, the similarities found between the MHC's of other species and the BoLA system suggest that the BoLA Class II antigens and MLR are probably controlled by the same gene region, the bovine I region. Because of this, it seems most likely that tests which detect Class II antigens will be more valuable in BoLA research than would be MLR tests.

The fifth chapter represents the first documented study involving tests for associations between the BoLA complex and an economically important trait. An association was found between the distribution of BoLA Class I antigen specificities and levels of tick resistance in a closed test population. The reasons for this association are not known but this preliminary finding could be of significant importance.

To test further the importance of this association, it will be
necessary to confirm this preliminary finding in a second study, possibly in a different cattle breed. If the observed association is substantiated then studies aimed at characterising the mechanisms behind tick resistance and the actual BoLA involvement should follow. The possible involvement of other antigens systems would also be important to study, especially the Class II antigen system.

In the future it may be feasible to test for the levels of resistance using tick antigen preparations in conjunction with BoLA typing. Two antigenic proteins have been isolated from tick extracts. The first of these has a molecular weight of about 60,000 daltons and is an esterase type of enzyme (Willadsen, 1976; Willadsen and Williams, 1976). The purpose that this enzyme serves for the tick is not clear but several enzymes are known to be involved with the skin lesion at the site of tick attachment and are probably present in tick saliva (Schleger and Lincoln, 1976). The isolated esterase mentioned above is a potent immunogen and causes DTH reactions in cattle which have had previous encounters with ticks (Willadsen and Williams, 1976; Willadsen et al. 1978). It is possible that this enzyme and possibly other proteins present in the saliva are the antigens against which cattle normally respond. Cellular activity involving infiltration of leucocytes and mast cells is centered around the site of tick attachment and the highest responses are associated with cattle which are highly resistant (Schleger et al. 1976). Additionally, most of the resistance in immune cattle is expressed soon after larval attachment, causing most of the larvae to detach within the first 24 hr (Roberts, 1968b). The rapid rise in immune based resistance suggests that the antigens are presented to the host immune system at the time of
attachment. It is most likely that components of the saliva serve as the antigenic stimulus for the immune reaction.

The second antigenic tick protein to be isolated is much smaller (18,500 - 24,000 daltons) and less well characterised. Its biological role is not known but it appears to be an inhibitor of proteolytic enzymes. It also causes DTH reactions in immune cattle (Willadsen and Riding, 1979; Willadsen et al. 1978).

The ability to isolate these antigenic proteins in a pure form means that it may be possible to immunise cattle with these antigens and to test the ability of each individual to respond. Immune responses could be assessed by quantitative measurement of DTH, by testing serum antibody titres or by measuring in vitro cellular proliferation, as has been done using antigens from the tick Dermacentor andersoni, which also affects cattle (Gregson, 1970). Purified antigens would also be well suited for further studies involving MHC associations and in particular for the detection and genetic characterisation of Ir genes. Selection of highly resistant cattle using either planned immunisations and follow-up assays or using MHC typing could be a great asset for choosing cattle for use in areas which require a high level of resistance to ticks.

It is hoped that these studies may lead eventually to the development of breeding schemes which are partially based on BoLA antigen types. A program of this type could be of great value in the development of tick resistant breeds or lines of cattle. The main advantage of this approach is that cattle would not need to be handled as much as is presently required in other programs, such as planned vaccinations with tick extracts (Allen and Humphreys, 1979).
Chapter 6 presents a comparison on the antibody responses of cattle to different antigens, including Class I and Class II BoLA antigens. These experiments were done as a basis for planning immunisation schemes, which will be required to produce additional typing antisera for the identification of new antigen specificities to characterise further the BoLA complex.
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