The major histocompatibility complex of cattle with particular reference to some aspects of East Coast fever

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This presentation is entirely the product of my own efforts and the work on which it is based was my own except where specifically stated in the text and in the acknowledgements section.

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Abstract

Following an introduction and review of literature pertaining to relevant aspects of major histocompatibility systems and a review of literature on the bovine system specifically, an introduction to East Coast fever (ECF) and review of literature on ECF immunology and lymphoblastoid cell line (LCL) vaccination is given.

The development and application of a technique for selection of bovine lymphocyte antigen (BoLA) - defined subpopulations within established LCLs transformed by the causative organism of ECF (*Theileria parva*) is then described. The results are discussed in the context of *T.parva*-preferred target cells in *in vitro* transformation systems, and the possible application of the technique in producing LCLs lacking BoLA expression is considered. No evidence of modulation of BoLA phenotype (workshop specificities) as a consequence of *Theileria*-induced transformation was found.

Two large scale LCL challenge experiments in cattle are reported, in which degrees of compatibility between LCLs and recipient cattle were quantified and the effect of this on the responses to LCL inoculation and subsequent stabilate challenge, assessed. Evidence is presented for the BoLA system being an important factor in determining the response to LCL inoculation and the generation of immunity to a second challenge with the homologous parasite, when cattle are inoculated with low numbers of cells initially.

Also presented are the results of a series of experiments carried out to investigate the specificity of bovine alloreactive cytotoxic cells generated *in vitro*. The results suggest that BoLA

workshop specificities, as defined by alloantisera, are restrictive in this system. The results also suggest that effects due to expression of the products of a BoLA locus other than that coding for the workshop specificities were being detected in some experiments.

The significance of these results for future studies of the bovine immune system in general, and for studies of the immune response in ECF in particular, are discussed.

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CHAPTER 1 Introduction and literature review

The work reported in this thesis concerns several aspects of the bovine lymphocyte antigen system (BoLA) which is genetically coded for by the major histocompatibility complex (MHC). Some of these aspects relate to immunisation against East Coast fever (ECF); specifically immunisation with *Theileria*-transformed lymphoblastoid cell lines (LCL).

In this review, what is currently known of the BoLA system is discussed. To place this in perspective a review of what is known of the genetics, function and structure of MHCs in general is given. The volume of literature on this subject is great and consequently an in-depth review is not attempted. Following a brief general introduction and review of the MHCs of the mouse and man, in so far as they serve to introduce the BoLA system, a summary of known MHC functions is given. Two selected aspects of the MHC are then discussed in more detail as they are felt to have particular relevance to the studies reported in the remainder of this thesis.

Following discussion of the BoLA system itself, a review of literature pertaining to LCL vaccination against ECF is presented after an introduction to the disease and a review of literature on ECF immunity.

1.1 The major histocompatibility complex

1.1.1 General

1.1.1.1 Introduction

The immunological theory of transplantation was first proposed by P.A.Gorer (1938) who demonstrated that antigenic differences between mouse strains affect transplantable tumour survival.

The MHC was originally discovered because of its role in transplant rejection when transplantations were carried out between individuals with differences within the complex. In species where investigations of the MHC are advanced it has been found to consist of a group of closely-linked genetic loci coding for cell surface antigens and it is believed to be present in similar form in all mammals and in some form possibly in all vertebrates (Snell, 1981). So far an MHC has been identified in at least 12 mammalian species (Usinger et al., 1981).

The original work of Gorer, already cited, was carried out in the mouse and it was the MHC of this species which, particularly in earlier studies, received the most attention. Progress in understanding was comparatively rapid, principally because of the availability of inbred strains.

Little & Tyzzer (1916) and Little (1941) established that resistance/susceptibility to transplants is influenced by multiple genes inherited in Mendelian fashion. Further progress on the identification of individual loci within the system came with the identification of a blood group locus in mice and the demonstration that this segregated with tumour susceptibility (Gorer, 1937). Gorer

was working with what he referred to as antigen II and because the system was known to be involved with histocompatibility (H) the mouse complex became known as H-2.

As more is known about the mouse system than any other and as information is now rapidly accrueing concerning the human equivalent system (HLA), these will be used as illustrations in an introduction to the bovine system (BoLA); the understanding of which is still at a comparatively incomplete stage.

1.1.1.2 The mouse histocompatibility complex - H-2

The work carried out on the structure of the H-2 system is reviewed in detail by Snell (1981).

The H-2 system is located on chromosome 17 and is generally divided into 5 regions, K, I, S, D, and L. The I region is further subdivided by some authorities (Klein, 1975; Demant & Neauport Santes, 1978; Klein, 1979) although the extent to which this subdivision is justified is debated (Klein *et al.*, 1981).

Klein (1977) suggested a classification of the loci which is now generally accepted. The 3 classes are,

1. Class 1 regions. These are regions K, D, and L. These products are typically serologically-defined (SD) and play a predominant role in transplantation reactions.
2. Class 2 regions. These are the I region loci. They are involved in immune responsiveness, reactivity in mixed lymphocyte culture (MLC) and graft-versus-host reactions. The antigens coded for were originally lymphocyte-defined (LD); that is by the response of lymphocytes to coculture with lymphocytes expressing different class II antigens (Dutton, 1966).

3. Class III region. This codes for or controls the level of component C4 of the complement system and is the S region of the H-2 system.

This general organisation probably pertains in a wide range of species although there may be differences in the spatial arrangement of the different loci (Bodmer, 1981). However, in most species the MHC consists of at least one locus which codes for class I antigens and closely-linked loci coding for class II antigens. These 2 regions are usually less than 2 centimorgans apart (Albert & Gotze, 1977).

The class I antigens, in the process of allograft rejection induce the formation of alloantibodies. These antibodies are used in the characterisation of the antigens in some form of microlymphocytotoxicity assay based on that described by Gorer & O'Gorman (1956). The antigens are codominantly expressed and characterised on the basis of their "private" and "public" specificities; these being respectively determinants unique to a particular SD entity and determinants which are present on more than one SD entity (Klein, 1975; Ivanyi, 1978).

The class I antigens are expressed on most but not all body cells and their density on different cell types varies (Snell et al., 1973; Klein, 1975; Gotze, 1977).

Structurally these products of the class I region are glycoproteins comprising 2 non-covalently bound polypeptides. One is β2 microglobulin (MWt, 12 kDa) which is present in association with all class I antigens. The other has a molecular weight of 45 kDa and is the variable in terms of antigenic specificity. This latter polypeptide inserts into the cell membrane at its hydrophilic terminal region (Schwartz et al., 1973; Klein, 1975;

Littman et al., 1979).

The tissue distribution of class II antigens is more limited than that of class I antigens, being principally restricted to B lymphocytes, some macrophage populations and a minority of T lymphocytes (Hammerling et al., 1974). Structurally they are distinct also, being heterodimeric glycoproteins comprising a and β subunits of molecular weights 32-35 kDa and 27-29 kDa, respectively (Cullen et al., 1974; Schwartz et al., 1977; Shackleford & Strominger, 1980).

1.1.1.3 The human histocompatibility complex - HLA

The work leading to our present understanding of the HLA system is reviewed by Dausset (1981). Because of the nature of the species under study, work has of necessity proceeded in a different way from that undertaken on the H-2 system but in many ways has more similarities with the efforts to date to unravel the bovine system.

Dausset (1958) reported that antibodies against leucocytes formed as a result of multiple blood transfusions may recognise alloantigens. Van Rood et al., (1958) and Payne & Rolfs (1958) reported that pregnancy may also generate such antibodies. Antibodies formed in these ways have been the basic tool in investigations of human class I antigens. Dausset (1958), van Rood & van Leeuwen (1963) and Payne et al., (1964) began the process of allele definition. Subsequently, international workshops have enabled analysis of sera reactivities and comparisons between laboratories resulting in a standardised approach and quite rapid progress in the characterisation of the HLA system.

In the HLA system, 4 closely-linked regions coding for histocompatibility antigens are recognised. These are HLA-A, HLA-B,

HLA-C and HLA-D/DR. These regions are situated on the short arm of chromosome 6 (Breuning et al., 1977). The first 3 regions code for class I antigens whereas the D/DR region codes for class II antigens. HLA-DR antigens are the most recently discovered and although they have largely been serologically defined they are believed to be closely related to the D-coded antigens (Bodmer, 1981). It is becoming increasingly apparent that the D/DR region is complex and that much remains to be done towards its definition (reviewed by Guy & van Heyningen, 1982). Closely linked to the HLA regions are genes coding for complement factor B and components C2 and C4.

The system shows many similarities with H-2 in that it codes for both class I and class II antigens in a closely-linked genetic group. The structures and tissue distributions are also similar (Bodmer, 1981).

The fact that there is this similarity between the systems in the 2 species so far discussed is in itself reason to suspect that the structure of other mammalian systems, including the BoLA system, will follow the basic principles established. Indeed, as will be discussed, work to date on the BoLA system has not produced any reason to believe that it will differ greatly from either H-2 or HLA.

1.1.1.4 A summary of MHC-controlled phenomena

1.1.1.4.1 Rejection of allografts.

The antigens stimulating rejection are coded for in the class I, and to a lesser extent, the class II regions (Klein *et al.*, 1981).

1.1.1.4.2 Cell-mediated lympholysis (CML).

T lymphocytes can be triggered *in vitro* to transform into cytolytic cells by coculture with lymphocytes differing in the MHC. The activated T cells then have the capability of lysing cells of the triggering MHC type.

1.1.1.4.3 Mixed lymphocyte reaction (MLR).

Resting T lymphocytes can be stimulated to proliferate by coculture with lymphocytes of different MHC type. The lymphocyte-activating determinants responsible are coded for in the I region (or its equivalent in other species), although there may be some involvment of class I antigens (Dutton, 1966; Klein *et al.*, 1981).

1.1.1.4.4 Control of the immune response.

It is clear that the MHC is implicated in the degree to which individuals are capable of responding to antigen (reviewed by Benacerraf & Germain, 1978; Zinkernagel & Doherty, 1979). The loci concerned generally map to the I region of the mouse although such loci mapping in the K region have been reported (Shearer *et al.*, 1975;

Schmitt-Verhulst & Shearer, 1975, 1976; Zaleski & Klein, 1977; Zinkernagel et al, 1978b; Maron & Cohen, 1979).

In some instances non-response with regard to antibody production is believed to be the result of suppressor T cell activity. The immune suppression genes map in the I region of the H-2 system (Klein et al., 1981).

1.1.1.4.5 Control of T-B cell collaboration.

The antibody response to most antigens requires the interaction of T helper cells with B cells, in the induction phase. It is a widely-held view that such interaction is influenced by genes mapping in the I region, although the precise nature of I region control is unclear. Thus Katz et al., (1975) suggested that a degree of I region homology between interacting T and B cells was required for optimal antibody response, a view supported by the results of Sprent & von Boehmer (1976) and Sprent (1978), whereas the results of Taussig et al., (1975) and Heber-Katz & Wilson (1975) are at variance with this.

1.1.1.4.6 Antigen presenting cell/T helper cell interaction.

T helper cells show dual recognition in their interaction with antigen presenting cells (APC); this being the stage before T helper/B cell interaction.

The T helper cell recognises the antigen presented and I region-coded MHC antigens (Erb & Feldmann, 1978; Sprent, 1978; Ishii et al., 1982).

1.1.1.4.7 Restriction of cytotoxic T lymphocyte (CTL) function.

This is discussed in detail in 1.1.1.5.

CTL are effectors in the immune response to cells rendered antigenic by infecting agents, chemical modification, minor histocompatibility antigen differences and male Y antigen. The effector CTL is restricted in the sense that it can only interact when identical MHC-coded antigens are present on both stimulator and target cell. There does not appear to be a requirement for total identity however, and in mouse systems, sharing of either K or D-coded antigens is all that is required. In this sense the effector cells show dual specificity; that is for the "new" antigenic determinant(s) and for the MHC-coded antigens of the inducing cell.

1.1.1.4.8 Control of suppressor T cell function.

I region control of suppressor T cell function is reviewed by Benacerraf (1981).

The results of recent work (Speck & Pierce, 1982) suggest MHC restriction of antibody-specific immunoregulation, mediated by T cells on B cells, although results of other workers have failed to show this (Yamauchi et al., 1981).

1.1.1.5 MHC restriction of CTL function

Mitchison (1954) speculated that cellular immune recognition of skin sensitising antigens occurs only when the antigens appear on cell membranes, thus resembling foreign transplantion antigens. Lawrence (1959) intuitively proposed that immune cells recognise parasite antigen expressed on macrophages in association with self antigen. Further evolution of the concept that foreign antigen is recognised by immune T cells in association with self antigen came with the reports of Svet-Moldavsky et al., (1967) and Holterman & Majde (1969, 1971) which suggested that rejection of virus-infected syngeneic cells or grafts resembles allograft rejection.

The concept was finally crystallised when Doherty & Zinkernagel (1974) and Zinkernagel & Doherty (1974a) reported that CTL of mice infected with lymphocytic choriomeningitis virus (LCMV) interact only with H-2 compatible virus-infected target cells and thus show the dual specificity already referred to in 1.1.1.4.7.

This phenomenon was found not to solely be a feature of virus systems when Shearer (1974) reported that it also applies to the CTL response to trinitrophenyl-haptenated lymphocytes. As reviewed by Doherty et al., (1976) and Zinkernagel and Doherty (1979) this phenomenon was also observed for a number of mouse virus infections of diverse type and was extended to other species. Furthermore, it was found to apply to the CTL response to minor histocompatibility antigens (Bevan, 1975a) and to the male Y antigen (Gordon et al., 1975) and has now been demonstrated in the human Epstein-Barr virus system (Moss et al., 1981), influenza virus system (McMichael et al., 1977; Shaw & Biddison, 1979) and for measles virus infection (Kreth et al., 1979). Restriction in the human

system was first shown with H-Y (Goulmy et al., 1977).

An important point concerning the MHC component of the dual specificity of CTL is that it is the class I antigens which are recognised (i.e., K and D-coded in mice) and further, antigens coded for by either, and not necessarily both loci are required to be shared between effector and target cells (Shreffler & David, 1975; Doherty et al., 1976). Sharing of class II antigens alone is insufficient for successful interaction.

A further important feature of MHC restriction is that it may be biased towards the MHC type of the environment in which CTL effector function developes (Bevan, 1977; von Boehmer et al., 1978; Kappler & Marrack, 1978; Sprent, 1978; Zinkernagel et al., 1978a; Waldman et al., 1979). It is demonstrated in chimaeric mice and in the classical example the chimaera is composed of F1 (P/Q) bone marrow cells inoculated into parental strain P or Q animals. CTL from such chimaeras respond preferentially to either P or Q target cells, depending on the parental host strain. The organ responsible for the bias is now known to be the thymus (Fink & Bevan, 1978; Zinkernagel et al., 1978a; Miller et al., 1979; Waldman et al., 1979) and the phenomenon is referred to as "thymic bending".

Of significance for an understanding of the relevance of MHC restriction is the fact that the cytotoxic T lymphocyte is known to be an effector in the immune response in vivo (reviewed by Doherty et al., 1976; Zinkernagel & Doherty, 1979). The implication is that the phenomenon of dual specificity is not simply an interesting observation restricted to the laboratory with no importance in immune function, a view which may well be applied to the allograft reaction, the CML response in coculture of

MHC-differing cells, and the MLR response in the same situation. Klein et al., (1981) advanced the thesis that the requirement for simultaneous recognition of antigen and MHC products is probably the real function of major histocompatibility systems. This view is shared by Benacerraf (1981).

Because of the importance of the MHC restriction of CTL, a consideration of what is understood about the mechanisms involved is justified.

With regard to the requirement for sharing of only a part of the genome (i.e., either K or D-coded specificities in mice) for successful effector-target interaction, the results of the initial work of Doherty & Zinkernagel and colleagues suggest that separate clones of T cells evolve which in the case of the H-2 system recognise only K or D, but not both simultaneously (Zinkernagel & Doherty, 1974b, 1975). This means that a mouse heterozygous at both the relevant loci could possess at least 4 clones of T cells reactive to virus-infected cells.

There has been a great deal of discussion in recent years concerning the molecular mechanisms involved in MHC restriction. One view is that the driving factor in the generation and the effector activity of the immune T cell is a response to altered self. This so-called "altered-self" hypothesis does indeed acknowledge much of the experimental evidence. Shortly after the demonstration of dual recognition, Doherty et al., (1976) favoured the altered-self hypothesis partly on the grounds that it accomodated what was known concerning the recognition of alloantigens. This aspect of MHC restriction is elaborated on by Benacerraf (1981), whose hypothesis is that alloreactivity is a result of the differentiation of T cells in the thymus that are strongly reactive with variants of self MHC

antigens because allogeneic MHC antigens are in fact seen by the immune system as self variants. Matzinger (1981) takes this further in accomodating alloreactivity in the altered-self hypothesis.

Further supportive evidence for the altered-self model is that mutations within the H-2 system can affect interaction of T cells with virus-infected targets and at the same time leave unaltered the interaction with TNP-modified target cells (Doherty et al., 1976).

A basic assumption inherent in this view is that the T cell recognises neither foreign antigen nor self MHC-coded products, but an antigenic entity formed by a molecular complex of the two. This is discussed in detail by Matzinger (1981). In this respect, as pointed out by Doherty et al., (1976), the altered-self hypothesis is immunologically more conventional than the alternative view that the two specificities recognised are recognised as separate, distinct entities (the dual recognition model).

A second basic assumption is that T cells are triggered to mature or differentiate only when they bind to MHC-coded molecules on the surface of a specialised antigen-presenting cell. T cells binding to free antigens will not be activated, but those binding to antigen in some association with MHC molecules will respond. As with other theories concerning the initiation of T cell activity, some form of tolerisation to prevent activation against self MHC alone, and in combination with other self antigens, must occur. The altered-self/single receptor view of CTL induction and effector activity is consistent with many of the findings of studies undertaken since it was first proposed as a possible explanation.

Thus,

1. MHC restriction is not absolute, i.e., T cells capable of recognising self combinations with foreign antigen can (although to a much more limited degree) recognise the same antigen on a non-self background (Peavey & Pierce, 1975; Fischer Lindahl & Wilson, 1977; Teh et al., 1978a & b). This is explained by similarities, as well as differences, between different MHC molecules allowing a degree of cross-reactivity.

2. Neither free MHC-coded products nor free antigen block T cell activity (Shearer, 1974; Plata & Levy, 1974; Todd et al., 1975; Basten et al., 1975). Further, to be bound by T cells, MHC molecules and antigen must be together on the same membrane (Bevan, 1975b; Swierkosz et al., 1977; Watt & Gooding, 1980).

3. Alloreactivity is easily accomodated within the altered-self/single receptor hypothesis if it is accepted that there is no reason why a T cell reacting with a cell of foreign MHC type cannot be induced to differentiate in just the same way as when interacting with self cells. On the other hand, the dual recognition model has to invoke a special case phenomenon for alloreactivity because the separate receptor for self MHC in this model would not be effective. Benacerraf (1981) however, argues for cross-reactivity on this point but this again is to invoke a concept which is not required in the interaction model of antigen/MHC singularity. Further, the latter readily explains the extremely strong antigenic nature of MHC antigens which may result in activation of up to 10% of T cells (Dutton, 1966; Fischer Lindahl & Wilson, 1977), again without new assumptions. Thus an individual presented with foreign

cells will see not only the foreign MHC antigens but many other antigens in association with the MHC. Therefore response is in fact against a large number of antigenic determinants and the response is consequently very great. The important point here is that many of the antigens of non-MHC type, which are responded to in association with foreign MHC, need not be different from their equivalents in the responding animal, but their interaction with foreign MHC makes them recognisable to the immune system. The recognition however, is only on this foreign background and hence no anti-self response is involved.

An extension of this is that if alloreactive cells only recognise MHC antigens in association, as interaction antigens, with other cell surface molecules, purified MHC-coded molecules of the challenge type should not block alloreactivity. This is found to be the case (Stulting et al., 1975; Todd et al., 1975). It should be emphasised that if such a view is accepted the assumption is made that alloreactivity is not essentially different from reactivity against self altered by foreign antigen and does not therefore require to be viewed as a special case.

4. Immune response genes can also be accomodated in the model with 3 possible explanations,

a: Any given antigen may be able to form a successful interaction antigen with some MHC-coded molecules but not with others (Benacerraf & Germain, 1978).

b: For any given interaction antigen there may be some determinants for which there is no T cell receptor gene (Schwartz, 1978; Benacerraf & Germain, 1978).

c: An interaction antigen may cross-react with a self antigen

against which, as a result of tolerance, there can be no response (Schwartz, 1978).

The phenomenon of thymic bending poses problems for the altered-self hypothesis in that it suggests preselection of MHC specificity prior to foreign antigen recognition in the context of the MHC. Matzinger (1981) however proposes that thymic bending is a result of suppressor activity on the side of the less-preferred MHC type and is therefore a result of negative selection as opposed to the positive selection inherent in the dual receptor model.

It should be emphasised however that other theories to explain the molecular basis of MHC restriction (Janeway et al., 1976; Klein, 1977; Reinherz et al., 1983; Siliciano et al., 1983) cannot be refuted on the basis of information acquired to date, and at the present time the mechanism involved is not known. The altered-self/interaction model is emphasised in this review because it has the attraction of simplicity and conceptual appeal and serves well to illustrate the important points involved in consideration of the phenomenon of restriction.

1.1.1.6 Polymorphism and linkage disequilibrium

The major histocompatibility systems studied in detail have proved to be highly polymorphic; the HLA system being a good example of this. This system is by far the most polymorphic system known in man (Bodmer, 1972). In man, the overall average frequency of heterozygotes across all polymorphic loci in the genome is of the order of 15% (Harris, 1975). This is much lower than that for the A, B, C and D/DR regions of the HLA system. The HLA A, B, C and D/DR regions, on the basis of the number of codominant alleles so far discovered, has several million haplotype or genotype combinations

possible (Dausset, 1981). As Dausset points out, if one adds to these combinations, variability (polymorphism) within the complement-coding region and within other closely-linked loci (which probably remain to be discovered) virtually every member of an outbred population will have a uniquely constructed MHC.

It is clear that throughout evolution this complexity has been preserved in a wide range of species and consequently it must be assumed that it is of vital importance to survival. Dausset (1981) advances the theory that the MHC is basically a self-recognition system derived from primitive genes coding for cell surface molecules (a view shared by others, as already discussed). Diversity within the system would become a necessity with the development of organised multicellular organisms, the tissues of which must co-exist and cooperate and the cells of which cannot merge with the cells of an organism of the same species. Recent work leading to the discovery of a MHC in protochordates provides support for this view (Scofield et al., 1982).

Maintenance of polymorphism within the system would be aided by any selective advantage bestowed on heterozygotes (D'Amaro, 1978; Bodmer & Bodmer, 1978). Some studies have suggested heterozygote advantage on the basis of a higher degree of heterozygosity at the A and B loci in old as compared to young individuals (Wood & Yunis, 1972; Gerkins et al., 1974; Macurova et al., 1975). The findings of another study are however at variance with this (Bender et al., 1976).

Linkage disequilibrium is another fundamental feature of the MHC. In the human species for example, the 4 genetic regions are closely-linked but are sufficiently distant for relatively frequent recombinations to occur; the frequencies being 0.8% between A and B

and 1% between B and D/DR (Dausset, 1981). However certain haplotypes (combinations of alleles on a single chromosome) occur more frequently than would be expected to occur by chance. Dausset describes it as "preferential genetic association between alleles of several loci of the same complex".

Linkage disequilibrium is measured on the basis of the difference between observed haplotype frequency (the frequency with which alleles are actually found to be associated on the same chromosome) and the product of the individual gene frequencies (Bodmer et al., 1966).

Dausset (1981), in his consideration of linkage disequilibrium within the HLA system, asks whether it is a relic of ancestral combinations when populations were isolated some thousands of years ago, or is it a reflection of selective advantage attributable to certain haplotypes in certain environmental situations? These two possibilities are not mutually exclusive, although most workers in the field would attribute greater significance to a selection influence on both the polymorphism and linkage disequilibrium which are such basic features of major histocompatibility systems (Fisher, 1958; Cepellini, 1968; Bodmer, 1972; Bodmer et al., 1973; Bodmer & Bodmer, 1978). Relevant in this regard is that in the role of the MHC in the immune response, complementation of genes at at least 2 loci appears to occur and this would appear to operate most efficiently when the genes are on the same chromosome (Dorf et al., 1975; Munro & Taussig, 1975; Dorf et al., 1976; Benacerraf & Germain, 1978). This could in part explain the linkage disequilibrium observed.

Indirect evidence for the importance of selective advantage in the evolution of the system has been provided by population

studies in which gene frequency variations between different polymorphisms have been compared (Bodmer et al., 1973; Cann et al., 1973; Piazza et al., 1973).

The overriding significance of this concept, and one which is basic to the understanding of the function of the system as it is viewed at the present time, is that the system itself is of indispensable importance to the organism, whether the organism is a primitive tunicate or a primate. Beginning possibly as a mechanism for maintaining individual organism identity, it apparently has taken on other functions in the area of cooperation between cells within the individual which have benefited from extreme variation, from the point of view of population as well as individual survival. As a result, the selective adaptation of the system has resulted in complexity and almost bewildering diversity. An understanding of the reason for this is vital to, and an integral part of, an understanding of the biological role of this portion of the genome.

Additionally, the diversity within the system does make it of considerable interest to workers not solely involved in the unravelling of the basic genetics. It may be used to characterise populations and discern origins and histories. Moreover, it must be of interest to all involved in the search for genetic markers and for a number of reasons, some of which have already been discussed, especially to those concerned with markers of disease resistance/susceptibiltity.

1.1.2 The bovine MHC.

Knowledge of the bovine MHC is still in its infancy, although studies on both the class I and class II antigens is gaining momentum in laboratories in Europe, North America, Australia and Africa.

Borovska & Demant (1967) reported a correlation between lymphocytotoxic activity and the S antigen system on bovine erythrocytes using antisera produced by whole blood immunisation. Later, Hruban & Simon (1973) showed that the J antigen could be associated with bovine lymphocytes.

McGary & Stone (1970), Ostrand-Rosenberg & Stormont (1974) and Folger & Hines (1976) failed to demonstrate a relationship between antilymphocyte activity and antierythrocyte specificity in typing reagents, thus suggesting that distinct leucocyte antigens were a reality.

Schmid & Cwik (1972) reported the production of 28 lymphocytotoxic sera by iso and heteroimmunisation which they believed recognised a leucocyte-specific antigen system and Iha et al., (1973), using alloantisera raised against whole blood and isolated lymphocytes, reported activity in such sera against HLA specificities.

Following this, a number of groups independently pursued a definition of the BoLA system. Caldwell et al., (1977) and Caldwell (1979) reported early attempts at serological definition. Amorena & Stone (1978) defined 11 antigens and suggested that they were the products of alleles at a single locus and importantly they reported prolongation of graft survival in donor/recipient combinations sharing these antigens. Spooner et al., (1978) reported evidence for a MHC in cattle, again as the result of the use of antilymphocyte

sera. In common with other groups they demonstrated simple Mendelian inheritance of the specificities which they were able to detect in their study population.

Spooner et al., (1978) also reported their methodology in detail which demonstrates a clear difference from that employed in HLA typing, particularly with regard to the production of antilymphocyte sera. Whereas serological HLA typing to date has relied on the use of sera obtained from multiparous donors, most groups engaged in BoLA research have to a large degree worked with sera raised by alloimmunisation, whether with lymphocytes or skin. Further, as pointed out by Spooner et al., absorption of such sera with lymphocytes does not present great difficulties because of the ease with which absorbing cells can be obtained in large numbers from cattle donors.

Alloimmunisation results in the production of antisera which generally are of higher titre than parous sera and which, by a process of selective absorptions and/or careful selection of titre level for antilymphocyte testing, can be made operationally monospecific (Spooner et al., 1980).

Further progress in the characterisation of the class 1 system has come as the result of an international collaborative effort. To date, 2 international comparison tests have been undertaken (Spooner et al., 1979; Anon, 1982). The first such test was undertaken in Edinburgh in which 249 alloantisera to bovine lymphocytes contributed by 9 laboratories were compared for reactivity on a panel of lymphocytes from 130 cattle of 21 breeds. The test used was the microlymphocytotoxicity test as described by Spooner et al., (1978). Eleven serum clusters were defined and given BoLA designations. In the second test, cells rather than sera were

exchanged and in all, 362 alloantisera originating in 9 laboratories were tested against a panel of 144 lymphocyte samples from 7 laboratories. Ten of the 11 specificities defined in the first comparison test were confirmed and an additional 6 specificities were designated, 2 of which appear to be subgroups of w6 as defined in both tests.

It is interesting to note that despite this intense effort there is as yet no firm evidence to suggest that class I specificities coded-for by alleles at more than 1 locus are being detected. A further indication that there is still a considerable amount of work to be done in characterising the system is that in the second international comparison test, even on the basis of a single locus model, only 66% of the alleles were identified as accepted specificities. One must assume that those so far discovered are either the most frequent in the cattle types studied and/or the most antigenic specificities.

A limited amount of work has so far been reported on breed frequencies of BoLA specificities, but it is clear that there may be significant differences between breeds. Caldwell et al., (1979) reported breed differences in the frequencies of 6 antigens. In this study, 675 cattle were tested for lymphocyte reactivity against a panel of typing antisera. The cattle were of 5 Bos taurus breeds and a Bos indicus/Bos taurus cross (Brahman/Hereford). Although the authors did not claim that their results were representative of the breeds, there were strong indications of significant differences in frequencies between the breeds studied. Furthermore, some breeds failed to evidence certain specificities. Interestingly, the highest frequency of the null allele was observed in the B.indicus/B.taurus crossbred group.

Oliver et al., (1981) reported a further study of BoLA breed frequencies, and the first family studies of BoLA specificities. This concentrated on 13 of the internationally-agreed specificities and a regionally-defined specificity in 6 breeds of cattle. Again, significant differences were found between breeds; examples being the frequency of w9 which was not detected in 3 breeds but was found to represent a gene frequency of 22% in the Herefords studied with the next highest frequency of 2.4% in the Ayrshire group. Specificity w6 produced a gene frequency of 46% in the Jerseys with the next highest frequency of 16.4% in the Friesians. It was again evident from this study that a number of alleles are still to be detected. The frequency of the null allele ranged from 74% in 18 Charolais bulls to 10% in a group of Friesian females. In this regard it is perhaps significant that the Friesian breed was the largest source of antilymphocyte sera.

Relatively little work has been reported concerning putative class II antigens of cattle. Usinger et al., (1977) and Curie Cohen et al., (1978) suggested that 2, and possibly 4, determinants may be involved in mixed lymphocyte reactivity. A further report (Usinger et al., 1981) suggests close linkage of this region with the BoLA A locus controlling class I antigens. In this respect therefore, the bovine system would appear to be essentially similar to others so far studied.

An attempt to define class II antigens serologically has also been reported (Newman et al., 1982). In this work, cytotoxicity of alloantisera for B lymphocytes was studied by a 2-colour flourescence technique whereby B cells were identified by the presence of immunoglobulin on the cell surface with an anti-bovine Ig/flourescein conjugate and cytotoxicity was assessed by the uptake

of ethidium bromide by dead cells. Using family studies to support and extend cluster analysis of typing reagents, evidence was presented for the identification of B lymphocyte-associated specificities, expressed and inherited in an autosomal codominant fashion. The null allele frequency in this study (53%) suggests a high degree of polymorphism.

There was no clear evidence of multilocus control of the specificities defined.

The restricted distribution of these antigens on blood cells, and the probable linkage with class I, demonstrated together with the degree of polymorphism suspected, are consistent with the fact that class II specificities were being detected.

What is presently known of the BoLA system suggests a close resemblance with the equivalent systems in other species. Additional evidence for similarity is furnished by the biochemical studies of Hoang-Xuan et al., (1982a) on class I products and Hoang-Xuan et al., (1982b) on class II products, which show similarities of structure with equivalent products in other species.

1.2 East Coast fever

1.2.1 Introduction

ECF is a disease of bovids occurring in East, Central and Southern Africa. The first description of the causative organisms can be ascribed to several workers in the latter part of the last century and the early 1900's (reviewed by Neitz, 1957). Prominent among later workers in their description of the parasites are Neitz (1957) and Barnett (1977).

ECF can be regarded as a syndrome wherein 3 types of

disease can be distinguished on clinical, epidemiological and parasitological grounds. The causative protozoan organisms belong to the genus Theileria.

The classification of the family Theileriidae according to Levine (1980) is;

subkingdom	Protozoa
phylum	Apicomplexa
class	Sporozoea
subclass	Piroplasmia
order	Piroplasmida

The 3 disease manifestations within the ECF syndrome have in the past been ascribed to infection with 3 different forms of Theileria. A trinomial nomenclature has been suggested (Young, 1981) to distinguish the causative organisms. These and the diseases with which they are associated are;

Theileria parva parva	classical ECF
Theileria parva lawrencei	Corridor disease
Theileria parva bovis	Rhodesian malignant theileriosis

As suggested by Uilenberg (1981), this categorisation should probably at the present time be considered as only a convenience. It seems more likely that ECF in its broadest sense is the manifestation of infection by any one of a variety of strains of T.parva, which in effect are a spectrum of types producing a variety of disease syndromes.

The T.parva group can be considered to encompass parasites of cattle (Bos indicus and Bos taurus), the African buffalo (Syncerus caffer) and domestic buffalo (Bubalus bubalis). Classical ECF can be regarded as the typical disease resulting from the maintenance of the parasite in B.indicus and B.taurus and the vector in the field with

no involvment of the buffalo. Corridor disease results from transfer of the parasite into cattle from a buffalo-maintained reservoir. Rhodesian malignant theileriosis covers disease syndromes due to infection with types of the causative organism falling between those responsible for classical ECF and those responsible for Corridor disease (Uilenberg, 1981). There have been a number of reviews of the symptomatology, epizootiology and pathology of ECF (Neitz, 1957; Barnett, 1968, 1977). In brief, field transmission of the protozoan parasite between mammalian hosts occurs via the tick vector Rhipicephalus appendiculatus (the Brown Ear Tick). The vector is a 3 host tick and transmission is transtadial. Transovarial transmission has not been demonstrated. The disease is restricted to those areas where R.appendiculatus occurs and within these areas is a major constraint on the cattle industry. Morbidity and mortality rates approach 100% in susceptible cattle exposed in ECF areas when no form of disease control is practised (Cunningham, 1977). Neitz (1957) stated that mortality rates in calves and adult stock in South Africa were more than 95%, but cites Lanham (1957) who gives a mortality rate of 78%. Although not stated, it must be presumed that these figures refer to fully susceptible populations.

The pathogenesis of the disease is covered by the reviewers already cited. Briefly, the first evidence of the parasite in bovine host cells is the appearance of an intracellular macroschizont in cells of the lymphoid series, which in natural tick infections is first seen in the parotid lymph nodes 4-6 days after tick attachment. As the disease progresses there is a rise in body temperature, usually occurring several days after first appearance of macroschizonts and 10-17 days after tick attachment. Macroschizont-containing lymphoid cells are then found in increasing

numbers in the vascular system, parenchymatous organs, and have a widespread lymph node distribution.

Several days after the appearance of macroschizonts and pyrexia, microschizonts are found in lymphoid cells and it is believed that at some point, macroschizont production switches to the microschizont production phase, but the factors which stimulate this change are presently unknown. The microschizont eventually disentegrates to produce micromerozoites which invade erythrocytes to produce the piroplasms typical of theileriosis. These are infective for the tick and represent the culmination of the mammalian part of the parasite cycle. Death may occur 2-4 weeks following tick attachment.

It is believed that the macroschizont development stage is the principal pathological stage and it is the macroschizont-bearing lymphoid cell which has been adapted for _in vitro_ study (Hulliger _et al._, 1964; Malmquist _et al._, 1970). Hulliger _et al._, (1964) also made the observation that the macroschizont divides in synchrony with the host cell, so that each daughter cell bears a macroschizont following division. This important observation has significance for the understanding of the immunology of the disease.

Terminally in the disease process severe leucopenia occurs and post-mortem findings are consistent with widespread lymphoid involvement with enlarged, moist and often haemorrhagic lymph nodes, which on histological examination appear exhausted and show lymphocytolysis and degeneration (De Martini & Moulton, 1973).

It is evident that in cattle, the disease is extremely virulent. Moreover, Barnett & Bailey (1957) determined that a fatal disease reaction can, and usually does, ensue as the result of the

feeding of a single infected tick on naive animals.

In general terms therefore, ECF in cattle can be considered as primarily a disease of the lymphoid system, characterised by the intracellular proliferation of parasites of the species T.parva, the result of which, in fully susceptible animals is the breakdown of that system. Animals undergoing a disease episode may mount an immune response, but in those animals suffering fatal infections the development of this response is late in relation to parasite development (Jarrett et al., 1966; Pearson et al., 1979; Morrison et al., 1981).

1.2.2 Immunity to East Coast fever

1.2.2.1 Humoral immune mechanisms

As a result of the apparent failure of most, though not all, workers to reveal a therapeutic effect of recovery serum (reviewed by Wilde, 1967) and the universal failure to detect colostral transfer of immunity, the possibility that humoral factors play a part in protection against the disease has for some time been largely discounted. Further, to date it has not proved possible using serological techniques to demonstrate "new" antigens on the surface of infected cells cultured in vitro (Duffus et al, 1978; Creemers, 1982). However it may be premature to completely rule out a humoral factor in ECF immunity. Antibodies to the parasite are indeed produced in challenged animals but so far these have been found to be restricted to the intracellular schizont and piroplasm stages. The development of this response is well characterised (Burridge & Kimber, 1972; Burridge et al., 1973a & b; Duffus & Wagner, 1974; Wagner et al., 1975). The demonstration of such

activity in sera by the indirect fluorescent antibody test (IFAT) (Burridge & Kimber, 1972) is the standard method for assessing immunity to ECF and previous exposure in epidemiological studies.

Such antibody activity however, may not play a role in protective immunity for several reasons. First, immunity to ECF outlasts detectable antibody to a considerable degree (Burridge et al., 1973b). Second, Wagner et al., (1975) in a study of the antibody response in cattle to a severe stabilate challenge found that animals succumbing to the challenge developed antibody responses which were qualitatively and quantitatively indistinguishable from the responses in those animals surviving the infection. Moreover, in a comparison with these results and those obtained previously following a less severe challenge (Duffus & Wagner, 1974) it is apparent that those animals suffering the more severe challenge developed a greater antibody response than those undergoing more mild disease responses. As these workers pointed out, this was probably a reflection of the greater antigenic stimulus occurring in the heavy parasitoses.

Wagner et al., (1974) reported on the development of the antibody response following the inoculation into cattle of isolated piroplasm and schizont antigen. Significantly, despite the fact that all the cattle involved developed specific antibody to these antigens, and to the same degree as cattle recovering from clinical disease episodes, the animals receiving the isolated antigens all succumbed to stabilate challenge subsequently. Emery et al., (1981a) also found no correlation between the antibody response to schizont material and the level of resistance to challenge after the inoculation of various putative immunogens.

Wagner & Duffus (1974) reported a different approach which

involved a study of the development of antilymphocyte activity (ALA) in sera of cattle subjected to inoculations of uninfected and T.parva-transformed lymphoid cells. T.parva parva and T.parva lawrencei-transformed LCLs were used in this work. ALA was generated in recipients of all 3 types of cell with slight differences in the kinetics of the response. ALA was measured by the migration-inhibition properties of post-inoculation serum samples measured against infected lymphoblasts and uninfected lymphocytes. Cattle receiving Theileria-transformed cells developed ALA earlier, and ultimately to a greater degree, than cattle receiving uninfected lymphocytes. Wagner & Duffus (1974) hypothesised as a result that transformation resulted in increased antigenicity. It did not appear from the results that ALA developed by individuals bore any relationship to resistance to subsequent challenge. A further point for comparison with the results of later work is that no ALA was detected in this system in the sera of animals undergoing patent ECF reactions induced by the inoculation of infected tick material, or in animals recovered from such infections.

Should there be a humoral factor in resistance to ECF it seems most likely that it would operate on the newly-inoculated sporozoite or early transforming cell rather than on an established intracellular parasite per se. This area has not been fully investigated to date. However, preliminary results of work on the effects of immune serum on the in vitro infectivity for lymphoid cells of T.annulata (causative agent of tropical theileriosis) sporozoite preparations suggest that there is a neutralisation effect (Gray & Brown, 1981). Neutralisation of sporozoites assayed by in vivo infectivity has also now been demonstrated in the T.parva system (Musoke et al., 1982). If such a system were to operate in vivo

directly on the sporozoite, recent electronmicrograph studies of the sporozoite/target cell interaction (Stagg *et al.*, 1981; Fawcett *et al.*, 1982) would suggest that it must be capable of producing an effect at the time that the sporozoites are introduced into the animal in view of the fact that sporozoites *in vitro* are capable of finding and invading host cells within minutes of being added to the cell preparation. It is not inconceivable that such a humoral mechanism could operate *in vivo* and because of this, work continues on inactivated-sporozoite vaccines, which hitherto have been unrewarding.

1.2.2.2 Cell-mediated immune mechanisms

It has for some time been suspected that cell-mediated immune mechanisms operate in ECF resistance and a number of workers have proposed that cell-mediated immunity (CMI) is the principal, or even sole factor in conferring resistance to the disease.

Aspects of CMI as measured by leucocyte-migration inhibition tests were studied by Muhammed *et al.*, (1974). In this work, leucocyte migration was measured and the effect on this of exposure to *T.parva* antigens assessed. Both schizont and piroplasm antigens were used and cattle donating the PBL for the tests were either ECF naive, recovered from tick tissue-derived infection, or previously inoculated with killed *T.parva* antigen (schizont) in combination with adjuvant. Migration inhibition of PBL from naive animals was not induced by exposure to antigen in 4 animals tested. Of 5 animals recovered from tick-derived infections, all showed sensitivity to either or both antigens used in the test and in 2 cases for up to 8 months after initial infection. Nine of 12 cattle exposed to killed parasite antigen were sensitised as assessed by the

test when tested up to 28 days after inoculation. Of particular interest are the results of tests on cells of 5 cattle inoculated with 10^6 LCL cells. PBL of 3 of these cattle were sensitive to schizont antigen in the migration inhibition test.

Care needs to be exercised in the interpretation of these results because of the possible complication of sensitisation to cellular components rather than parasite material *per se* in the cattle studied, with the exception of those cattle recovered from a tick-derived infection. However it was the latter group which showed the best and most prolonged sensitivity in the migration inhibition test, which adds weight to the fact that this test was genuinely measuring sensitivity to parasite antigen.

The immunity of the cattle studied, as assessed by resistance to subsequent challenge was only reportedly studied in the tick-derived infection group and these were resistant to challenge. It would seem unlikely that the short-lived responses in the other groups would indicate immunity because, as will be discussed in a subsequent section, inoculation of killed parasite material is not generally regarded as an effective means of inducing immunity to ECF.

Muhammed (1975) made a further report on migration inhibition after exposure to *T.parva* antigens. In this work the assay used assessed the migration-inhibiting properties of PBL from ECF-immune animals on *T.parva*-transformed lymphoblasts. The PBL of only 1 of 10 naive animals were capable of eliciting migration inhibition. This was also true of 7 of 8 PBL samples from animals which had received a 10^7 LCL cell inoculation followed by stabilate challenge (2 of these died at 3-4 weeks following challenge). The PBL of 2 of 4 animals successfully immunised against homologous challenge by stabilate combined with tetracycline administration,

inhibited lymphoblast migration. The result of the work on the tissue-culture material immunised animals again could be complicated by the fact that they had, prior to the test, experienced both cell and parasite antigen and in this respect a control in the form of a measurement of migration inhibition activity of PBL from animals which had experienced non-infected allogeneic leucocytes would have been informative.

If the tissue culture recipients are ignored in these results, 2 of 4 immune cattle had a migration inhibition capability compared with 1 of 10 susceptible cattle. Of the tissue culture inoculated/stabilate challenge animals, 2 of these died of ECF a few days after the PBL were shown to have the greatest migration inhibition activity of all the cattle studied.

Summarising these results, it would seem that the migration inhibition measured was not proven to be a good indication of immune status, conforming with the results of the 2 other migration inhibition assays already discussed.

The results of Emery et al., (1981a) are consistent with this overall view. In this case the development of a lymphocyte transformation response to ultrasonicated infected cell line material was not correlated with resistance to challenge.

Real progress in the development of knowledge of CMI in ECF came with the results of Pearson et al., (1979). In this study, the antigenic nature of the surface of T.parva-transformed cells was studied in vitro using the mixed-lymphocyte reaction (MLR) and cell-mediated cytotoxicity (CMC) assays. They found that transformed cells stimulated radio-labelled nucleotide uptake in autologous PBL from immune and naive animals and with the same kinetics in each case. Further, normal PBL were equally stimulated in the MLR by

autologous and allogeneic transformed cells. Interestingly, in the allogeneic system, transformed cells had a greater stimulating effect than the equivalent non-transformed PBL. This suggested that the antigenicity of the cell surface is in some way increased by T.parva-induced transformation which is consistent with the earlier results of Wagner & Duffus (1974) in which the ability of allogeneic cells to induce a humoral response was reported.

Pearson et al., (1979) concluded from their results that the stimulation induced by T.parva-transformed cells was not simply a response to differences in "normal" histocompatibility antigens. If "normal" refers to MHC-coded antigens as they would appear on non-transformed cells there is every reason to agree with this conclusion as this does not exclude the possibility that the increased antigenicity of the transformed cell is due to alterations in histocompatibility antigens. That such alterations may occur, and indeed be solely responsible for the antigenic changes occurring in transformation cannot, with present knowledge, be excluded.

A number of control experiments were included in this work which strengthened the view that stimulation in MLR was due to Theileria-induced changes, and such stimulation was only evident when intact and viable cells were used as stimulators. Free schizonts were not stimulatory.

When cell-mediated cytotoxicity was studied, a difference was found between the cells of immune and non-immune donors. The PBL of 3 immune donors, after stimulation in vitro by coculture with autologous transformed cells, were cytotoxic for autologous T.parva-transformed targets. A lower level of killing of allogeneic transformed targets was also reported for these 3 immune donors. Cells derived from 2 non-immune donors did not effect cytolysis.

Pearson et al., (1979) suggested that the MLR was a reflection of the generation of helper or cytotoxic cell precursors taking place on initial exposure to antigen and that the secondary CMC was the result of this when the cells of immune animals were used. However in this regard, it is perhaps significant that the MLRs did not correlate with donor immunity. Further, the restriction of cytotoxicity to in vitro systems derived from immune rather than non-immune donors has not been repeatable in other hands (Emery & Kar, 1983).

Eugie & Emery (1981) studied the development of a cytotoxic cell population in the PBL of immunised animals. Immunisation was achieved using the stabilate plus tetracycline regime (Radley et al., 1975b) followed by either a stabilate challenge or the inoculation of sporozoites in association with autologous PBL. At intervals following this the direct cytotoxic capability of PBL was monitored. The targets were autologous and allogeneic T.parva-infected cells, derived from in vitro transformations, and xenogeneic cells not infected with Theileria parva.

When immune animals were challenged with stabilate, macroschizont-bearing cells could not be detected histologically in the draining lymph nodes nor in efferent lymph. Further, these lymph node cells (LNC) were unable to stimulate an autologous MLR in cryopreserved PBL. This contrasts with the situation found in the second week of a lethal primary infection (Emery & Morrison, 1980).

That the autologous MLR is dependent on a degree of parasite-associated transformation, was confirmed by the fact that some animals challenged with sporozoites in association with cells did show a transient intracellular parasitosis in lymph efferent from the inoculation site draining lymph node, and blastogenesis (degree

of parasite-associated transformation) in LNC, and these elicited a MLR. The association between parasitosis and *in vitro* stimulation in MLR was also noted by Emery & Morrison (1980).

In the study reported by Eugie & Emery (1981) both forms of challenge induced cytotoxic activity against autologous infected cells in post-inoculation PBL taken from 10 days post-challenge. This development of cytotoxic activity was less delayed following subsequent challenge of immunised animals.

Cytotoxic activity was also induced by challenge with autologous infected cells, and was also restricted to autologous targets.

In their system, Eugie & Emery (1981) found that cytotoxic activity of post-challenge PBL waned after peaking at about 14 days after a primary challenge and was undetectable by approximately 21 days post-challenge.

These workers also reported some differences in their results in a comparison with those of Pearson *et al.*, (1979). At no time in their immunised animals were they able to detect allogeneic cytotoxic capability in PBL. The evidence for genetic restriction was reinforced by an experiment on cotwins in which it was found that cotwin cells were killed as effectively as autologous cells. Pearson *et al.*, (1979) reported effective cytotoxicity in the autologous *in vitro* generation system which appeared to be well-developed, but also reported a "low level" of allogeneic killing.

In summary, genetic restriction was found in both systems studied, which appeared absolute in one case, and was marked in the other. This contrasts with the allogeneic and xenogeneic killing seen in lethal primary infections (Eugie & Emery, 1981;

Eugie et al., 1981). Eugie & Emery (1981) also reported that cytotoxic capability was seen post-challenge in immunised cattle in PBL which had not been prestimulated in mixed lymphocyte culture (MLR). Pearson et al., (1979) studied secondary CMC.

In a subsequent report Pearson et al., (1982) presented results of further studies on the ability of Theileria-transformed cells to induce MLR and CMC responses in PBL harvested from immune and non-immune cattle. This work largely confirmed their previous findings, in that cytotoxic cells were only generated in MLR in PBL from immune cattle. The report goes further however in a number of respects. First, it confirms that in addition to a specific, self-restricted cytotoxic cell response there may be a non-restricted response following in vitro stimulation with either autologous or allogeneic infected cells; this again being in contrast to the findings of Eugie & Emery (1981) where the in vivo generation system was studied. Cold-target blocking in conjunction with autoradiography/immune fluorescence characterisation suggested that the genetically-restricted and non-restricted components are mediated by different effector populations. The results of Emery & Kar (1983) are consistent with this and further, suggest that the principal difference between the in vivo and in vitro generation systems is the component of non-restricted effector cells generated in the in vitro system.

Interestingly, the results reported by Pearson et al., (1982) suggest that cell lines differ in their efficiency as in vitro stimulators in both autologous and allogeneic situations.

Pearson et al., (1979, 1982) and Eugie & Emery (1981) associated the CMC response with immunity and suggested that the

cytotoxic cells developed are a principal effector mechanism *in vivo*. Eugie & Emery (1981) interpreted the kinetics of response to mean that unless an animal is under challenge, these cells and/or their precursors reside in the solid lymphoid organs and are mobilised and appear in the peripheral circulation on challenge, with repeated challenge making the activation/mobilisation process more rapid.

Emery *et al.*, (1981b) reported results of studies on the *in vivo*-generated effector cell which suggest that it is of T cell type.

Very good evidence that cell-mediated immune mechanisms are involved in immunity to ECF was supplied by Emery (1981). In this work resistance to challenge was passively-transferred between cotwins with thoracic duct lymphocytes. Further, Emery *et al.*, (1981a) were able to correlate the degree of lytic activity of PBL against autologous infected cell lines, following allogeneic cell line immunisation, with resistance conferred to subsequent sporozoite challenge.

Eugie *et al.*, (1981) reported initial results of a study of the specificity of *in vitro* CMC with regard to parasite isolate. Prior to immunisation of 3 cattle with *T.parva parva* (Muguga), 4 LCLs were established *in vitro* infected with 4 different parasite isolates, from PBL of each of the 3 cattle. One of the isolates was *T.parva parva* (Muguga), another was cross-immunising with this *in vivo*, and the remaining 2 were not completely cross-immunising with the Muguga isolate. Following the immunisation with *T.parva parva* (Muguga), PBL were assessed for lytic acivity against the 4 respective autologous lines. Good lytic activity was observed against *T.parva parva* (Muguga)-infected lines and against the lines infected with the *in vivo* cross-immunising strain. PBL from 1 of the 3 cattle had no lytic activity against the 2 lines infected with the

other strains, PBL from a second animal showed lytic activity against 1 of these other lines, and PBL from the third, showed a degree of activity against both of these other lines. Where lysis of the lines infected with either of the 2 non-cross-immunising strains was observed, it was at a lower level than against the line infected with the *in vivo* immunising strain and the strain which cross-immunises with this.

These limited results could prove to be very significant if it can be shown that CMC does indeed reflect *in vivo* resistance to challenge. This would certainly strengthen the view that T cell-mediated cytotoxicity is a major protective mechanism. Second, it would show that there are significant differences between individuals in the degree to which the immune system can respond qualitatively and quantitatively to *T.parva*.

1.2.3 Immunisation against ECF with Theileria-transformed lymphoblastoid cell lines

The potential of infected cells in a vaccine capacity was considerably increased following the development of 2 important techniques. First, the *in vitro* culture of infected bovine lymphoid cells taken from patently-infected animals (Hulliger *et al.*, 1964; Malmquist *et al.*, 1970; Malmquist & Brown, 1974).
Hulliger *et al.*, (1964) and Brown *et al.*, (1978a) also reported on the mode of multiplication of the parasite *in vitro* and the fact that the division of the parasite schizont occurs in synchrony with a division of the host cell. Such infected cells are transformed by the parasite and in suitable conditions of laboratory maintainance, the cell lines produced are a constant source of parasite material.

The second major step in the development of *T.parva* *in*

vitro culture came with the work of Brown *et al*., (1973) who were able to establish a technique for the *in vitro* infection of bovine lymphoid tissues using tick-derived sporozoite preparations to infect and transform cells. This technique is now routinely used in a number of laboratories and has greatly facilitated studies of the cell/parasite relationship, whilst at the same time making possible the production in the laboratory of the schizont stage of parasite isolates of varying origin and making them available for various immunological studies.

From the time that the LCLs became available they were an obvious subject for study with the object of establishing an ECF vaccination system. Indeed they held great promise, with lines which could be grown in suspension cultures yielding 1-3 x 10^6 cells/ml (of which approximately 95% contain macroschizonts), a log 10 growth rate of 3 days and potential 4 L harvests twice weekly (Cunningham, 1977).

Early vaccination trials were carried out by Brown *et al*., (1964, cited by Wilde, 1967). It was shown in this work that the intracellular schizont stage of the parasite could be established in recipient animals by inoculation of infected LCLs. In addition, as a result of studies undertaken by this group, Wilde suggested that cattle may be able to mount an immune response to infected LCL cells *per se*, and it was subsequently shown that cattle will, under certain circumstances, generate an antilymphocyte response following inoculation of *T.parva*-infected lymphoblastoid cells (Wagner & Duffus, 1974), as previously discussed.

Wilde and coworkers also investigated the efficacy of inactivated LCLs administered with and without 3 different adjuvants (Wilde, 1967). They did not demonstrate the development of immunity

to either tick-transmitted parasites or homologous tissue culture strains following the application of these procedures.

Since this work, there has been little published on this aspect of ECF although the later work has clarified some of the points concerning the potential of LCLs as ECF vaccines and has certainly produced a better understanding of the infected LCL/recipient animal interaction. Moreover, the problems to be faced in the production of a field vaccine based on LCLs are now more clearly defined.

Brown et al., (1971) reported that cattle could be infected by inoculating 10^7 or more cells infected with T.parva. In this study, it was found that the parasite completed its mammalian cycle. It was also found that high doses of cells (10^9) could produce fatal infections and that cattle surviving various dose levels between 10^5 and 10^9 could be resistant to subsequent challenge.

Significant advances in the study of the LCL vaccination system were reported by Brown et al., (1978a). It was confirmed that the parasite can transfer from LCL cells to recipient cells following inoculation. On transfer to recipient cells both parasite and cells continue to multiply. This was established by karyotypic analysis of inoculated cells and of cells subsequently recovered from infected animals. It should be emphasised here that it has been generally accepted to the present time that the parasite must establish in the cells of the recipient animal before immunity can be induced (Emery et al., 1981a).

Brown et al., (1978a) also reported the effect of continued passage in vitro of infected LCLs. They established that infectivity, and as they inferred, immunogenicity, varied between cell lines infected with different isolates of T.parva and between

different passages of the same line. Two cell lines and 2 isolates of T.parva parva were studied over a period of 3 years and 300 passages. Both parasites were attenuated by continued passage. Inocula of 10^8 and 10^9 cells were used. With increasing passage fewer animals were killed as a result of cell inoculation and in the case of one line at passage 319, all animals survived LCL inoculation and subsequent stabilate challenge. The second cell line was effectively attenuated between passages 49 and 205 to the point where a 10^8 cell inoculum did not prove fatal in any of the recipients and 87% of these were subsequently immune to challenge with the homologous parasite isolate in stabilate form. Further passage of the second, less virulent line, reduced its immunising efficiency in that only 50% of recipients of 10^8 cells at passage 282 were subsequently resistant to challenge.

This work was extended to test the immunity produced with the less virulent line by exposing cattle immunised at an optimal level in a tick vector-infested paddock in which 3 sequential groups of susceptible control cattle all died. The cell line-immunised animals proved resistant to this challenge. Brown et al., (1978a) concluded that "....immunisation against homologous tick-derived challenge is possible using defined quanta of selected passages of specific cell lines.".

The work done to this point, although showing the potential of LCLs in a vaccine capacity, also revealed a number of problems still to be overcome. Prominent amongst these was the number of LCL cells required to engender immunity in recipient animals. The work of Brocklesby et al., (1965), Jarrett et al., (1966), Pirie et al., (1970) and Brown et al., (1971, 1978a), in particular, had shown that in order to establish the parasite reliably in the

majority of recipients, of the order of 10^8 to 10^{10} cells were required. These findings contrast with the results of those workers exploring and developing the T.annulata system in which Pipano & Tsur (1966), Sergent et al., (1972) and Hooshmand-Rad (1973) had obtained reliable immunogenesis with 10^4 to 10^6 cells. Barnett (1977) summarised the difference between the 2 systems, at which time as he pointed out, artificial immunisation against T.annulata had been practised for 40 years using firstly strains isolated and attenuated by serial passage in calves and latterly, LCLs. He stated that inocula as low as 2×10^6 infected tissue culture cells enjoyed a good measure of success in immunisation of even highly-susceptible exotic stock against tropical theileriosis. At this time LCL vaccination against tropical theileriosis was already an established entity and a practical field proposition and is currently successfully applied in a number of tropical theileriosis areas (Pipano, 1981).

This situation, if it ever can be achieved with ECF, would still seem to be some way off. The principal problem to be overcome at the present time is the requirement for large numbers of cells in the T.parva system, which necessitates the use of 100-1000 ml of tissue culture suspension for each animal vaccinated. It has been known for some time that the inoculation of LCLs could produce a response to these cells per se in the recipient. This was first suggested by Wilde (1967) in the context of infected cell inoculation. Subsequently, Wagner & Duffus (1974) reported that anti-lymphocyte activity (ALA), as measured by migration-inhibition and a fluorescent antiglobulin test, was induced in cattle receiving T.parva parva and T.parva lawrencei-transformed lines.

It is clear from this work that as already stated, the ALAs

measured in response to infected-LCL inoculation did not correlate with immunity to subsequent challenge. However it is possibly significant that the antigens on the LCLs inducing the ALA responses are unknown. Importantly, in most, if not all cases it appears that the cells used in the 2 tests for determining ALA could not have been derived from the same source as the inoculated material. It is perhaps also pertinent that this fact raises questions about the meaning of the comparisons between ALA responses to parasitised and non-parasitised cells previously discussed (1.2.2.1).

Wagner & Duffus (1974) in discussing their results, drew attention to the possible importance of the histocompatibility system in the LCL/recipient interaction and subsequent work also indicated that this was indeed an area requiring study. Of particular relevance in this context are the results of work carried out by Brown *et al.*, (1978b). These workers utilised an *in vitro* tick-feeding technique to produce bovine blood and its various fractions containing infective material. They studied the effect of inoculating this material into a variety of cattle of *B.taurus* type. The parasite used was *T.parva parva* (Muguga). In 2 initial experiments in which heparinised-blood tick feed pools were inoculated into the blood donors (autologous system), and into unrelated cattle (allogeneic system), all 11 cattle receiving their own post-tick feed blood developed ECF. Of 9 allogeneic recipients, none reacted and all subsequently proved susceptible to challenge. In a further experiment, a tick feed blood pool was inoculated into the donor, its monozygotic twin and 2 unrelated cattle. The donor underwent a fatal ECF reaction and its twin underwent a severe reaction. Neither of the 2 allogeneic recipients reacted and both proved susceptible on challenge.

In an attempt to define the transmitting factor in the feed blood, composite feed substrates were used comprising plasma and erythrocytes from one donor and leucocytes from another. Five pairs of cattle were used. All 5 leucocyte donors underwent patent ECF reactions following inoculation of the tick feed substrate and only 1 of the plasma/erythrocyte donors responded with a detectable reaction. In the latter category however at least 1, and possibly 2 of the animals not showing obvious initial reactions, survived challenge and so may have undergone subpatent infections following inoculation of feed substrate.

The results of these experiments suggested two things. First, that there is some form of self-restricion in the LCL/recipient interaction. Such a phenomenon is not observed where high numbers of infected LCL cells are inoculated into allogeneic recipients and one must suppose that the parasite/cell dose in the tick feeds used in this work (Brown et al., 1978b) was below such a "universal take" level. Second, there was good evidence from the results that the blood component involved is the leucocyte fraction. It is now known that the tick-derived sporozoite becomes associated with PBL within minutes of first contact (at least in vitro) (Stagg et al., 1981; Fawcett et al., 1982), which is entirely consistent with the hypothesis proposed by Brown et al., (1978b) that in an allogeneic system the infected blood fraction is treated as an allograft and there may be allogeneic inhibition of further development of the parasite/allogeneic recipient relationship. This conforms with the early proposals of Wilde (1967) concerning possible homograft rejection as a consideration in infected cell inoculation systems.

Further evidence for self-restriction in LCL immunisation

is reported by Morrison et al., (1981). In this case it was shown that in contrast to the number of cells required to effect immunisation in most allogeneic LCL/recipient combinations (for which BoLA typing was not available) resistance to subsequent challenge can be achieved by the inoculation of as few as 10^2 LCL cells into autologous recipients (cell line donors).

Evidence for "similar type" preference was reported by Dolan et al., (1982). In this work the response was monitored of B.taurus and B.indicus cattle to the inoculation of LCLs transformed by T.parva parva (Muguga). The transformed lines originated from lymphoid cells of both B.taurus and B.indicus animals. Cattle received inocula of 10^9 cells of the respective type. Of significance was the finding that both cattle types were more susceptible to infection with cells derived from their own cattle type. Combining the results of 2 experiments, of 13 B.indicus cattle (Boran) receiving 10^9 cells of 1 of 2 taurine lines, parasite development was seen in 7, a temperature response in 2 and only 1 animal succumbed to infection. All survivors were resistant to subsequent challenge. Of 5 indicus cattle receiving 10^9 indicus-type LCL cells, all 5 evidenced a disease response although all recovered. Similarly, 5 taurines receiving 10^9 taurine LCL cells all developed parasitosis. Four of these developed a temperature response and 2 died. The 5 taurines receiving indicus cells all developed a patent disease response of which only 1 proved fatal.

There is a good indication from these results that there was a better parasite transfer from inoculated cells of either indicus or taurine type to recipient cattle of the same type than to cattle of the differing type. This is further supported by the results of the subsequent stabilate challenges (with the homologous

parasite). There is also evidence from this work that the degree of parasite establishment in the first instance in the recipient animal, may be an important factor in determining the degree of immunity produced subsequently. All the surviving taurines which had initially developed detectable parasitoses and all but 1, a pyrexia, were totally refractory to challenge, whereas 4 indicus animals, none of which showed a temperature response initially, underwent mild disease episodes following challenge. These 4 animals comprised 2 which had originally received taurine cells and 2 which had received indicus cells.

There are at least 2 factors to be considered in these results. There is evidence, as pointed out by the authors, for differing innate resistances in the 2 cattle types to cell culture challenge. This is superimposed on the self-type preference for achieving parasite establishment in the recipient in the LCL inoculation system. Dolan et al., (1982) considered that their results were consistent with the results of previous work (already discussed here) that histocompatibility differences could exert an influence on LCL immunisation procedures.

Of significance in this regard was the finding by Spooner & Brown (1980) that in vitro Theileria-transformed LCLs, in all cases studied except one, continued to express BoLA specificities detected on non-infected donor PBL. Moreover this was found to be the case with one line which had been passaged more than 200 times and where the sensitivity of the transformed cells to typing reagents was almost identical to that of fresh PBL bearing the same specificities.

In summary, it became apparent very early in the work on Theileria-transformed LCLs, that to achieve immunisation in even 80% of recipients, very large numbers of cells were required in the case

of Theileria parva cell lines. This contrasts with the relatively small numbers required to effect T.annulata immunisation.

Evidence which suggests that the MHC may present a barrier to this form of immunisation against ECF is available. Therefore, a study aimed at firstly demonstrating BoLA involvement, and should this prove possible, defining to what extent it is involved in LCL immunisation, appeared to be of major importance in the development of cell line immunisation procedures for ECF. Such a study is, in large part, the subject of this thesis.

CHAPTER 2 General materials and methods

2.1 BoLA typing

2.1.1 Materials

2.1.1.1 The BoLA typing panel

The panel of alloantisera comprised 2 groups. The first group, with which the bulk of the initial typing in Kenya was carried out, was supplied by Dr R.L.Spooner of the A.R.C., Animal Breeding Research Organisation in Edinburgh. They are identified by an Edinburgh number (ED), the number of the animal of origin followed by the date of donor bleeding, which in turn is followed by the numbers of the donor animals of lymphocytes used in absorptions of the original crude serum. The sera and the working dilutions used are listed in Table 2.1.

A number of the sera have been tested in the 2 international comparison tests carried out to date (Spooner et al., 1979; Anon, 1982) and have been designated defining sera with respect to the workshop (w) specificities. A number of other sera are considered by the Edinburgh laboratory of origin to recognise workshop specificities (Spooner, personal communication). The workshop specificities and the sera defining them are listed in Table 2.2.

It should be noted that the Edinburgh panel includes sera defining all workshop specificities presently agreed. Further, as discussed in 1.1.2, specificities are believed to be coded for by alleles at a single BoLA locus.

The second group of sera used was produced specifically to

TABLE 2.1

EDINBURGH PANEL OF BoLA TYPING SERA

Edinburgh no. (ED)	Serum				Working dilution
1	U102A	21:10:74	abs.	HZ155B+GU41	1:4
2	U102A	21:10:74	abs.	HZ163A	1:4
5	U110B	9: 6:75	abs.	U106B+1792	1:4
7	AY22	23:12:74	abs.	1862+1688+GA33	1:4
8	MZ339B	5: 1:86			1:4
9	1804	14:11:74	abs.	1928	1:8
10	1818	5: 1:76	abs.	1984	1:4
11	1819	24:11:75	abs.	JS31+1823	1:8
12	7608	2: 3:76	abs.	1968	1:4
13	7613	2: 3:76			1:8
14	7616	2: 3:76			1:4
16	154	25: 3:75	abs.	SH22	1:8
17	114	25: 3:75	abs.	U112B	1:4
28	271		abs.	1928+1919+1889+KR31	1:16
30	119	16: 9:75	abs.	1984	1:4
38	310	16: 9:75	abs.	1919+W1342	1:8
39	312	25: 6:75	abs.	1984	1:8
40	424	10: 4:75	abs.	1919	1:16
43	2162	19: 6:75	abs.	1819+DS84	1:8
47	2273	16: 9:75	abs.	1830+R01+BF61+KR51	1:4
62	TZ6B	25: 5:76	abs.	BW14+1928	1:16
63	1818	25: 5:76	abs.	1795+1928+LU22+KR31+7073	1:16
64	HZ163A	25: 5:76	abs.	BW14+1862	1:4
65	1862	25: 5:76	abs.	BW14	1:8
66	1432	25: 5:76	abs.	TZ6B+1440+1471+1368	1:16

cont.

TABLE 2.1 CONT. (2)

Edinburgh no. (ED)	Serum				Working dilution
67	1440	25: 5:76	abs.	HZ155B+1823+JS31	1:4
68	1440	25: 5:76	abs.	1470+1594+JS31	1:8
69	1470	25: 5:76	abs.	U110B+GU41+1983+1795+1862	1:4
71	7639	7: 7:76	abs.	1903+1862	1:8
72	7529	7: 7:76	abs.	1823	1:4
73	7698	7: 7:76	abs.	GA41+1471	1:16
74	611	7: 7:76	abs.	1818	1:16
75	7710	7: 7:76	abs.	1818	1:8
76	7718	7: 7:76	abs.	GA41	1:8
77	7652	7: 7:76			1:8
78	7514	7: 7:76	abs.	HZ155B+U106B	1:4
80	646	7: 7:76	abs.	1435+1892+HLk3+LU22	1:4
81	3250	14:10:75	abs.	1368	1:8
82	147	3: 4:75	abs.	1892	1:8
83	1830	24:11:75	abs.	1984+1892+LO21+SX21	1:4
85	U106B	24:11:75	abs.	1984+GU31+RP22	1:4
86	HZ166A	4:12:78	abs.	1928+BW31	1:4
87	1993	20:12:78	abs.	1819+BW31	1:4
88	410		abs.	1903+1892+1889+GU31+AY54 +RO41+BS32	1:4
89	1469	22: 7:73	abs.	HZ156A+1889+1968	1:4
90	2312	16: 9:75	abs.	R41+BW31	1:8
91	1435	20:12:78	abs.	1795+RP22	1:16
92	1804	14:11:74	abs.	1978	1:4
93	1432	25: 5:76	abs.	1993+1440	1:4
95a	7129	9:7:80	abs.	7PP5	1:4
96	7575	20:12:76	abs.	1687+1903	1:16
97	7574	20:12:76	abs.	1471+1862	1:32

cont.

TABLE 2.1 CONT. (3)

Edinburgh no. (ED)	Serum				Working dilution
98	1856	20:1-:76	abs.	1889+Sh22	1:4
99	7655	20:12:76	abs.	1687	1:8
100	88				1:16
101a	427	20:12:76	abs.	1919+SH22+7087+1594+7081	1:16
102	7657	20:12:76	abs.	1471+1862+1919+GA53	1:8
103	7655	20:12:76	abs.	1687+LU22	1:4
104	GU41	9: 7:80	abs.	1978+1809+3489	1:4
105	1889	13:12:78	abs.	1862+BF52	1:4
107	R143				1:8
108	R135		abs.	1862+1809+1432	1:32
95b	7129	9: 7:80	abs.	GA41+7029	1:4
101b	427	20:12:76	abs.	7081+1594+1688	1:4

TABLE 2.2

BoLA WORKSHOP SPECIFICITIES (w) AND DEFINING SERA

w. specificity	Defining sera on basis of comparison test(s)	Additional sera
1	1, 8	83
2	2, 62, 64, 65	
3	39	1
4	82	
5	7, 90	
6	9, 10, 66	75, 97
6.1	12, 92, 93	
6.2	81, 86	
7	11, 17, 67, 68	
8	13, 63, 80	
9	14	
10	69, 71	96
11	76	73, 95a, 102
12	47	
13	5, 16	88, 98, 101a, 109
16	38, 43	
20	78	

enable better BoLA characterisation of East African zebu cattle. The reagents are the result of alloimmunisations carried out in Kenya at the Veterinary Research Department of the Kenya Agricultural Research Institute (KARI), Muguga, during the course of the studies reported here. The Kenyan sera have the prefix KM and are listed in the same way as the Edinburgh reagents, in Table 2.3.

2.1.1.2 Hanks' balanced salt solution (HBSS)

This was supplied in powder form by Gibco Biocult, with phenol red. Sodium bicarbonate was added in the preparation of the HBSS at the rate of 0.35g/L and the pH of the final solution was adjusted to 6.8 as estimated on a Philips PW9409 pH meter, with N HCl and N NaOH. Deionised distilled water (DDW) was used to reconstitute the HBSS. Following preparation, the HBSS was filter-sterilised through a 0.22 uM pore size GS type filter (Millipore Corporation), and full, tightly-sealed half-litre bottles were stored at 4°C until required.

Double strength HBSS (2xHBSS) was prepared in the same way from powdered medium but with the use of half the quantity of DDW. The pH was not adjusted.

2.1.1.3 Ficoll-Hypaque

This was prepared to specific gravity 1.069. 67.5 g Ficoll 400 (Pharmacia Fine Chemicals) was dissolved in 600 ml boiling DDW, and the volume then made up to 750 ml with DDW.

79.8 g of Hypaque Sodium (Winthrop) was dissolved in 120 ml DDW. After the solutions were cooled to room temperature, the hypaque was added to the ficoll with constant stirring until a specific gravity of 1.069 was achieved as monitored with an

TABLE 2.3

KENYA PANEL OF BoLA TYPING SERA

Kenya number (KM)	Serum				Working dilution
1	N994	26:2:81			1:4
2	M777	26:2:81	abs	M993+S344	1:32
3	M639	16:2:81	abs	S337+N019+M638	1:4
4	N023	16:2:81	abs	M762+N097	1:8
5	7514	8:7:76	abs	R128	1:4
6	7616	2:3:76	abs	N019+M686	1:4
7	169	30:4:81	abs	M655	1:4
8	169/81	30:4:81			1:4
9	S413	4:6:81			1:4
10	M655	27:5:81	abs	M662	1:16
11	M642	10:4:81	abs	M802+M778	1:4
12	M993	10:4:81	abs	M802+M638	1:4
14	N770	22:4:81	abs	M616+M756	1:8
15	7514	7:7:76	abs	M810	1:4
17	N775	10:4:81	abs	M679	1:4
18	188/80	30:4:81	abs	N774	1:16
21	M642	10:4:81	abs	M638+M686	1:4
22	N546	22:7:81	abs	M639+N019+N774	1:16
23	S333	3:9:81	abs	S411	1:8
24	M762	25:5:81	abs	M778+N023+N756+N097	1:16
25	S122	22:7:81	abs	N042+N770+N511	1:8
26	7616	2: 3:76	abs	M793	1:4
27	M781	3:9:81	abs	M666	1:8
28	N729	3:9:81	abs	N023+N913	1:16
29	N774	14:4:81	abs	M638+N097+S48	1:8
30	M688	17:9:81	abs	N019+M778	1:8
31	M666	3:9:81	abs	N774+M642+N913	1:8
32	M681	8:6:81	abs	M639+S390+N291+M685	1:4
33	N723	17:9:81	abs	M666+N774+M639	1:16
34	S58	10:8:81	abs	M628+M642+M685+M666	1:2
35	7514	7:7:76	abs	R128	1:2
36	7616	2:3:76	abs	M793	1:2
37	S411	4:6:81	abs	N774+S413+M666	1:8
38	188	30:4:81	abs	M802+M655+M666	1:16
39	M634	14:7:81	abs	M642+M692+S411	1:4
40	N923	27:8:81	abs	M638+N770+M685+N028+M665	1:8

hydrometer.

The ficoll-hypaque was then autoclaved and stored frozen at -18°C until required.

2.1.1.4 Eosin dye

Eosin powder (Koch Light Laboratories Ltd./pure A.R.) was used to make a 5% w/v stock solution in DDW and stored at -18°C.

For use in the microlymphocytotoxicity test a working solution was prepared by adding 1 volume of eosin stock solution to 1 volume of DDW and adding to this, 2 volumes of 2xHBSS.

2.1.1.5 Fixative

To 95 ml of 40% formaldehyde solution was added 5 ml of phosphate buffer prepared as a 0.15 M disodium hydrogen phosphate solution in DDW.

The fixative was stored at 4°C until required for use.

2.1.1.6 Complement (C')

Pooled sera from a number of rabbits served as a complement source. Serum pools were collected by staff of the Animal Breeding Research Organisation from a commercial slaughterer. Pools were tested for complement potency in the lymphocytotoxicity test with panels of typing sera and cells of known reactivity, and were also titrated in the test in the absence of typing sera to assess inherent cytotoxicity. Cytotoxicity in some complement pools was removable with suitable absorptions with selected bovine spleen in powder form.

Batches of complement of sufficient potency and lacking inherent cytotoxicity were stored at -18 or -70°C until required in the test.

2.1.1.7 Non-reactive bovine serum (NBS)

Sera from young male animals were run in the standard test at a 1/2 dilution. Those showing no cell killing were stored at -18°C until required. NBS at a dilution of 1/16 in HBSS was included in all typing panels to allow an estimate of baseline cell viability.

2.1.1.8 BoLA typing sera

These were stored undiluted in small aliquots at -18 or -70°C.

2.1.1.9 Typing plates

Terasaki-type (60 well) plastic plates (Sterilin) were prepared in batches and stored with added typing and control sera at -18°C until required.

Typing and control sera were appropriately diluted with HBSS to a volume of 160 ul and dispensed under liquid paraffin into the plates using a Hamilton 1 ul multidispenser (6 channel). 1 ul of diluted serum was added to each well.

Sera were dispensed into plates in numerical order except that single defining sera (ED 14, 39, 47, 78, 82) were duplicated, but not in adjacent wells.

2.1.2 Methods

2.1.2.1 Cell preparation

Cell preparation from fresh blood samples was essentially as described by Spooner et al., (1979) with a few minor modifications. For this, samples of blood from the jugular or caudal veins were collected aseptically into sterile vacutainers containing

lithium heparin (Becton Dickinson). It was found during the course of this work that extremes of temperature prejudiced the survival of leucocytes in these samples and so they were kept as far as possible at an ambient temperature of 15~20°C until used. This contrasts with the 4°C storage reported by Spooner et al., (1979) which was found to considerably reduce leucocyte recovery and viability.

Lymphocyte separation was achieved by layering 1~1.5 ml of blood onto 1 ml ficoll~hypaque in glass tubes (7 ml nominal volume) followed by centrifugation at 1500 g for 15 min. The lymphocyte layer at the plasma/ficoll~paque interface was removed with a Pasteur pipette and added to HBSS in a second tube and mixed. The samples were then centrifuged at 400 g/5 min. Supernatants were poured off and the cell pellet resuspended in the small volume of HBSS remaining. To achieve lysis of any erythrocytes in the sample, 2 ml of DDW was then added to the cell samples with constant mixing, followed rapidly by 2 ml of 2xHBSS. Having filled the tubes with more HBSS, and following several inversions, the samples were centrifuged at 110 g/5 min. The supernatants were again poured off, the cell pellet resuspended and the tubes refilled with HBSS and inverted several times. The tubes were then centrifuged at 100g for 5 min to leave platelets in the supernatant which was removed to leave 0.5 ml HBSS over the cell pellet.

Following resuspension of the cells, the concentration of viable cells was assessed by counting in a haemocytometer (Neubauer chamber) with phase contrast microcopy (Nikon). On the basis of these counts the volumes of the cell suspensions were adjusted to give a final concentration of 2.5×10^6 cells/ml.

2.1.2.2 Microlymphocytotoxicity test

Following thawing of test plates and their equilibration to room temperature, 1 ul of cell suspension and 1 ul of rabbit complement (serum diluted 1/2 in HBSS) were added to each well (to which the typing sera had previously been added prior to storage).

Following incubation of the plates at 37°C for 80 min, 1 ul of working eosin solution was added to each well. After a further 2~5 min at room temperature, 2ul of fixative were added to each well.

Hamilton single and multiple dipensers were used throughout.

Such prepared plates could be stored at 4°C until reading, which was carried out with phase contrast microscopy at a magnification of 100x (Nikon inverted phase contrast microscope). The following scoring system was used throughout in the reading of the tests;

score	% killed cells
8	80~100
6	60~79
4	30~59
2	10~29
1	0~9

Tests showing 60% or greater cell killing were considered positive and those showing 30~59% cell killing were considered partial reactions.

2.2 Theileria parasites

Species and strains of Theileria parasites used in this work are detailed in the relevant sections, although most of the work involved the use of T.parva parva (Muguga) which is considered a typical T.parva parva strain.

This isolate is described by Brocklesby et al., (1961).

Cryopreserved tick-derived stabilates were the source of all parasites. The method of cryopreservation of ground-up whole infected ticks was a modification of the method of Cunningham et al., (1973a), as described by Radley (1978). Stabilates of T.parva parva (Muguga) were made available by Dr A.S.Young of the Veterinary Research Department, KARI, Muguga.

Stabilates served as a source of parasites for in vivo challenge experiments and to produce infected batches of ticks after passage through cattle which were then used in the in vitro establishment of infected LCLs.

Infected ticks and stabilates were produced by Mr B.Leitch of the Veterinary Research Department, KARI, Muguga.

2.3 Lymphoblastoid cell lines

LCLs were established from PBL and made available by Mr C.G.D.Brown of the Centre for Tropical Veterinary Medicine, Edinburgh and Mr D.A.Stagg of the Veterinary Research Department of the Kenya Agricultural Research Institute. The method of in vitro production was that of Stagg et al., (1981).

The LCLs were maintained essentially as described by Stagg et al., (1976).

The cell lines were initiated and maintained in either RPMI 1640 or Eagles Minimum Essential Medium with Earles salts (MEM).

RPMI 1640 and MEM were supplied in powder form with phenol red by Gibco Biocult Ltd., and Flow Laboratories. DDW was used to reconstitute the media. Sodium bicarbonate was added to RPMI 1640 at the rate of 2 g/L. Penicillin (Glaxo Laboratories Ltd.,) and streptomycin as sulphate (Glaxo Laboratories Ltd.,) were added to the media to a final concentration of 100 iu/ml and 100ug/ml, respectively. After adjusting the pH to 6.8 with N HCl the media were filter-sterilised through 0.22 um pore size GS type filters (Millipore Corporation) and after removal of an aliquot for sterility testing, were stored in full, tightly-stoppered bottles until required.

Immediately prior to use, foetal calf serum (FCS) of selected batches (Gibco Biocult Ltd.,) was added to the media to a final concentration of 17 or 20%. L-glutamine (Gibco Biocult Ltd.,) was also added, to a final concentration of 2 mM.

FCS was filter-sterilised in the same way as the basic media and stored in appropriate aliquots at -18°C.

Following addition of FCS and L-glutamine to the basal media, a further aliquot was taken for sterility testing prior to use of the complete medium in cultures.

2.4 Serology

2.4.1 The indirect fluorescent antibody test (IFAT)

This test to determine levels of antimacroschizont antibody in cattle sera was carried out as described by Burridge & Kimber (1972), by staff of the Veterinary Research Department, KARI, Muguga.

The antigen used was derived from the Muguga C2 cell line which is infected with T.parva parva (Muguga).

2.4.2 The indirect immunoperoxidase test (IIPT)

This was used to assess antibody levels in cattle sera to cell surface antigens on inoculated LCLs. The testing was carried out by Drs K.Cowan and C.Groocock of the Veterinary Research Department, KARI, Muguga.

The method is described in detail by Cowan et al., (1983, in preparation). The relevant cell line cells attached to poly-L-lysine-treated glass slides were used as antigen. The slides used were clean, 8-12 well Multitest slides (Flow Laboratories) and the poly-L-lysine (type I-B or VII-B) was supplied by the Sigma Chemical Company. Aliquots of the LCL cells in culture medium were taken from culture flasks 1-2 days after a 1/10 passage. The culture material was centrifuged at 300 g/6 min and washed 3 times with Hepes buffered KCl saline (HBKS; 0.15M NaCl, 0.01M Hepes, 5 uM KCl, pH 7.4) containing bovine serum albumin (BSA) (fraction V bovine albumin, Armour) to a final concentration of 0.1% (HBKS-A). After washing, the volumes of cell suspensions were adjusted with HBKS-A to give a cell concentration of 5×10^6/ml.

15 ul of cell suspension was placed in each of the multitest wells. The slides were placed in a moist chamber for 20 min to allow the cells to settle and attach to the glass after which the fluid was gently aspirated off. A drop of HBKS containing 0.1% gelatin (HBKS-G) was then added to each well and the slides reincubated as previously. Fluid was again aspirated off and the wells filled with test sera at appropriate dilutions (sera were diluted with HBKS) and the slides incubated at room temperature for 30 min.

Fluid was aspirated off the wells and the cells were washed 3 times for 3 min with HBKS-G. Rabbit antibovine-IgG (diluted

1/200), prepared and supplied by Dr K.Cowan, was then added to the wells and a further 30 min incubation carried out. The washing process was repeated as above, and then the wells were filled with goat-antirabbit-IgG coupled to horseradish peroxidase (prepared and supplied by Dr K.Cowan). The incubation and washing procedure was again repeated. The substrate used was 3-amino-9-ethyl carbazole (AEC) (Sigma Chemicals Co.,) dissolved in absolute ethanol (1 mg AEC/ml). To this was added 2 volumes of Tris-buffered saline with hydrogen peroxide added to a final concentration of 0.01%. This complete indicator was prepared immediately prior to use. The indicator was added to the wells and a further 20-30 min incubation was carried out followed by washing. 2-3 drops of 50% glycerol/0.1% glutaraldehyde in phosphate-buffered saline (prepared from Dulbecco A tablets, Oxoid) were added to each well and cover slips applied which were then sealed with fingernail varnish.

Reactions were read with a light microscope at 400-1000 x magnification.

Included in each assay as controls were known negative and positive sera as well as conjugate and indicator controls.

2.5 Haematology

Haematological parameters of cattle on experiment were assessed on jugular venous blood collected into bijou bottles containing anticoagulant (dipotassium EDTA, 1 drop of 20% w/v in DDW)

Parameters routinely measured were packed cell volume (PCV), leucocyte (WBC) count and haemoglobin (Hb) concentration.

PCV was estimated using a microcentrifuge (Hawksley) technique. WBC counts were performed with an electronic particle counter (Coulter ZB1/Coulter Electronics Ltd.,) and haemoglobin

estimations were performed on a haemoglobinometer (Coulter Electronics Ltd.,).

Haematological parameters were measured on the day of sample collection, by staff of the Veterinary Research Department, Muguga.

2.6 Parasitology

Giemsa-stained blood, gland and internal organ smears were examined for evidence of Theileria parasites in experimental cattle.

The Giemsa stain was prepared by adding 2 gm Giemsa powder (Giemsa's azur eosin methylene blue, Merck) to 108 ml glycerol (BDH Chemicals Ltd.,) which was then heated to 60°C for 2 hr with constant stirring. After cooling to room temperature, 168 ml methanol (BDH Chemicals Ltd.,) was added, followed by a further 2 hr of stirring. 0.56 gm Azur II (Merck) was then added to the stain solution followed by stirring for a further 2 days. The stain was then filtered through a Whatman no.54 filter paper and stored for 1 week in a stained glass bottle prior to testing and use.

Blood smears were prepared by spreading a small drop of freshly obtained venous blood on a clean glass slide, using the edge of a second slide, followed by rapid air drying.

Gland smears were prepared from needle biopsies of selected glands. This involved manual immobilisation of the gland where necessary, and the insertion of a 19G sterile disposable hypodermic needle. After insertion of the needle, it was rotated along its axis several times within the gland and withdrawn following closure of the syringe attachment part of the needle with thumb or finger. The small gland biopsy within the needle could then be expressed onto a clean glass slide and be spread and dried as for a blood smear.

Slides for preparation of smears were as far as possible previously unused and were cleaned by storage in a 1/1 v/v mixture of ethanol and ether (BDH Chemicals Ltd.,).

Prior to staining, smears were fixed in methanol for 1-5 min. Staining involved immersion in the working stain solution prepared by dilution of the stock Giemsa with phosphate-buffered saline (PBS) of pH 7.2. PBS was prepared from tablets (Dulbecco A, Oxoid) and DDW.

A 5% Giemsa in PBS working stain was used. After 20 min in the stain, the staining was differentiated by a brief immersion in PBS. Drops of PBS were removed with blotting paper and the slides allowed to dry prior to microscopic examination.

The microscope used for the examination of smears for parasites was a Leitz Dialux. Examinations were made under oil at 500 and 1000 x magnifications. To establish absence of parasites or assess the levels of low parasitoses, slides were examined for approximately 5 min.

(cont.d)

Degrees of parasitosis were recorded on an arbitrary scale;

\+ = macroschizonts or piroplasms detectable in 1 or only a few fields.

2+ = macroschizonts visible in more than 50% of fields examined.

3+ = more than 1 macroschizont present in most fields examined.

As far as possible, piroplasmaemias were quantified on the basis of the percentage of erythrocytes containing piroplasms.

The collection and preparation of samples for parasitological examination was carried out by staff of the Veterinary Research Department, Muguga.

The examination of the samples was shared with Dr T.T.Dolan of the Veterinary Research Department, Muguga.

CHAPTER 3 The use of BoLA antisera in the modulation of MHC sub-populations in Theileria-infected LCLs

3.1 Introduction

This study was undertaken in an attempt to address a question raised by the first reported work concerned with the expression of MHC-coded antigens on bovine LCLs.
Spooner & Brown (1980) reported the results of BoLA typing of 19 LCLs produced by *in vitro* infection and transformation of bovine PBL by *T.parva* and *T.annulata* (the causative organisms of ECF and tropical theileriosis respectively). The reactivity of the transformed cells with a panel of BoLA typing antisera was compared with the reactivity of fresh uninfected donor PBL and with one exception it was found that the MHC type of the transformed lines was identical with that of the respective donor animals' uninfected PBL. This is in accordance with the results of work in other histocompatibility systems where agents other than *Theileria* spp. have been used to transform cells (Klein *et al.*, 1976).

The exceptional result reported by Spooner & Brown (1980) was obtained on BoLA typing of fresh PBL and cells transformed *in vitro* by *T.parva parva* (Muguga) from a steer twinned to a heifer. Erythrocyte typing suggested that the twins could be chimaeras and BoLA typing of the parents revealed a possible origin for the MHC genotype of the *T.parva*-transformed line . The results were therefore consistent with the possibility that the PBL in this particular case comprised male and female cells of different BoLA types and that the less common genotype (undetected in the BoLA typing of the donor) had been preferentially transformed by the parasite. Fresh PBL from the same donor, when transformed by 2

isolates of T.annulata typed in the same way as the donor.

If T.parva had infected only a subpopulation of cells it would be of significance for the understanding of the cell/parasite interaction, especially in view of the recent results of other workers which also suggest that T.parva has particularly specific requirements with regard to target cell type (Duffus et al., 1978; Black et al., 1981; Pinder & Roelants, 1981; Pinder et al., 1981).

Duffus et al., (1978) suggested that the parasitised cell is a T lymphocyte. Black et al., (1981) elaborated on this when they suggested that the infected cell is an immature-type T cell expressing T cell differentiation antigens, and thus having similarities with MLR and concanavalin A-induced blast cells. Pinder and Roelants (1981) and Pinder et al., (1981) also considered that Theileria-infected lymphoblastoid cells are derived from a T cell subset.

It was felt important to ascertain whether or not cells of an hypothesised minor population (on the basis of BoLA type) within the chimaera had been transformed in preference to those of the major population, for two reasons. First, should such a population of cells be shown not to exist within the donor, the implication would be that T.parva is of itself capable of qualitatively altering the expression of MHC-coded antigens, which would have far-reaching implications in the field of ECF immunology. Second, a possible interpretation should a minor population be shown to have indeed been transformed, would be that the BoLA type of cells has an influence on their capacity to host the parasite, again with important implications.

Two approaches were therefore adopted. In the first instance karyotyping of the donor and its twin was carried out to

establish that they were chimaeras as suggested by erythrocyte typing (Spooner & Brown, 1980). A means was also required of BoLA typing the minor population within the chimaera. For reasons stemming from the methodology of BoLA typing (see 2.1.2) only an overwhelming major population would be successfully typed in any mixture of BoLA types. Should there not be an obvious majority type, relatively large numbers of partial reactions would be expected with clear positive reactions only occurring in the case of shared specificities. A minor population would at best produce a partial reaction with a positive antiserum and if the population were small enough, unique specificities would not be recorded at all. Such a subliminal population, from a BoLA typing viewpoint, was postulated to exist within the chimaeric donor and a means of making this population available for BoLA typing was required. This could not easily be done using standard techniques directly on donor PBL but the possibility existed of BoLA typing such a minor population should it coexist with a major population in a lymphoblastoid cell line. The T.annulata-transformed cell line derived from the chimaera was therefore studied with a view to potentiating any minor population found to a level at which it would be detectable in the microlymphocytotoxicity test. A method of selection of cells was developed for this purpose.

3.2 Materials and methods

3.2.1 Cell lines

The establishment and maintainance of LCLs is detailed in 2.2 and 2.3.

The lymphoblastoid cell lines used were 219TpM, 219TaA and 219TaH, derived from the chimaeric Ayrshire steer (number 219) and 220TaA from its female twin (number 220) as described by Spooner & Brown (1980). 219TpM is infected with T.parva parva (Muguga), 219TaH with T.annulata (Hissar), and 219TaA and 220TaA with T.annulata (Ankara). Briefly, the lines were established by in vitro infection of PBL with tick-derived sporozoites by the method of Brown et al., (1973) and these particular lines were maintained in RPMI 1640 with 25 mM Hepes buffer and 17% foetal calf serum (Gibco Biocult), with added penicillin (100 iu/ml) and streptomycin (100 ug/ml). The lines were established and made available by Mr C.G.D.Brown of the Centre for Tropical Veterinary Medicine, University of Edinburgh, U.K..

3.2.2 Karyotyping of PBL

Peripheral blood lymphocytes of the animals were karyotyped by adding 0.3 ml of venous blood, collected into a vacutainer containing sodium citrate (Becton Dickinson), to a 25 sq cm plastic tissue culture flask (Nunc) containing 5 ml of medium to which had been added 300 ul phytohaemagglutinin (PHA, Wellcome) and 250 iu heparin (Pularin, Evans). The culture was maintained at 37°C for 72 hr after which time 300 ul of 0.01% w/v colchicine solution (BDH Chemicals) was added. After a further incubation for 2.5 hr, cells and medium were transferred to a plastic universal container

(Sterilin) and centrifuged at 200g for 20 min. The supernatant was poured off and the cell pellet resuspended in the small quantity of medium remaining. 5 ml of 0.7% w/v tri-sodium citrate (BDH Chemicals) was then slowly added with careful mixing and a further 25 min incubation carried out. 3 ml of Carnoy's solution (1 part glacial acetic acid/3 parts methanol, BDH Chemicals) were then added slowly and with thorough mixing. After a further 20 min incubation the cells were again centrifuged and the fixative discarded and replaced with fresh Carnoy's solution. Slide preparations of the suspensions were made and stained with Giemsa (Merck) prior to microscopic examination.

3.2.3 Karyotyping of cell lines

0.2 ml of colcemid (Gibco Europe) stock solution (10 ug/ml) was added to a 25 sq cm plastic tissue culture flask (Nunc) containing 10 ml of an actively-growing cell line (i.e., 24 hr after a 1:10 passage). The culture was then incubated for 90 min after which a 5 min centrifugation at 100g was carried out in a glass centrifuge tube. The supernatant was poured off and 5 ml of 0.7% w/v tri-sodium citrate at 37°C was slowly added with gentle mixing. After a further 35 min incubation at 37°C the suspension was centrifuged as above and the pellet resuspended in 5 ml of fresh ice-cold Carnoy's fixative solution. After 10 min at 4°C, cells were spun down and the fixing process repeated once more.

After a final centrifugation the pellets were resuspended in approximately 0.5 ml ice-cold Carnoy's solution and slide preparations were made by dripping the cell suspensions onto wet, ice-cold clean slides, followed by rapid drying. Slide preparations were stained with Giemsa (Merck). Other than the slides all

glassware used in the process was siliconised.

3.2.4 Cell preparation

The preparation of fresh PBL for microlymphocytotoxicity testing was by the method of Spooner et al., (1979), as described in 2.1.2.1.

The preparation of LCL cells for BoLA typing was from actively growing cultures, i.e., 24-48 hr after a 1:10 passage. 2 ml aliquots of cell culture material were removed from the tissue culture flasks after the cells had been resuspended by gentle pipetting. Each aliquot was layered onto 1ml Ficoll/Hypaque, S.G. 1.069 (see 2.1.1.3), in a 7 ml nominal capacity, round-bottomed glass tube and centrifuged at 1500g for 15 min. The layer of viable cells at the culture medium/Ficoll interface was carefully removed with a Pasteur pipette and then added to a second 7 ml round-bottomed tube containing culture medium. Following topping-up of the tube with medium, and inversion, the cells were centrifuged at 110g/5 min. The supernatant was removed to leave approximately 0.5 ml of medium over the cell pellet. The cells were resuspended by applying the tubes to a Whirlimixer (Fisons, Loughborough) and aliquots removed and added to a Neubauer chamber. Following an estimation of viable cell concentration by viewing the chamber with an inverted phase contrast microscope, the volume of cell suspension was adjusted with culture medium to give a cell concentration of 10^6/ml.

3.2.5 Microlymphocytotoxicity testing

This was performed as described by Spooner et al., (1978) with alterations as described in 2.1.2.2.

3.2.6 Cell selection

Selected BoLA typing sera were titrated against cell lines in the microlymphocytotoxicity test. On this basis, antisera positive and negative for the cell lines at a dilution of 1:4 or greater were identified.

Selection of minor populations within the cultures on the basis of BoLA type was accomplished by the incubation at 37°C for 30 min in sterile conditions of 1 ml of culture medium containing 2.5×10^6 cells with 1 ml of the appropriate positive antiserum diluted 1:4 with culture medium. 1 ml of rabbit serum diluted 1:2 in culture medium was then added prior to a further 30 min incubation.

During incubation, the cells and medium were agitated 2-3 times. Following incubation the tubes were centrifuged at 100g for 5 min and the supernatant discarded. After removal of the supernatant, the remaining cells were resuspended in 2.5 ml fresh medium and added to a 25 sq cm plastic tissue culture flask (Nunc) containing a confluent monolayer of IMR 31 (bison lung fibroblastic line, supplied by Mr C.G.D.Brown of the Centre for Tropical Veterinary Medicine, University of Edinburgh, U.K.,) from which medium added 72 hr previously was partially removed to leave 7.5 ml. After 24 hr the culture supernatant was centrifuged to remove cell debris and returned to the culture flask. In this way cells surviving the selection process in low numbers could be propagated.

3.3 Results

3.3.1 BoLA typing of twins 219 and 220 and derived LCLs

The results of typing are shown in Table 3.1.

The workshop specificities detected in the fresh and transformed cells are shown in Table 3.2.

These results confirm those of Spooner & Brown (1980) in that LCLs derived from the twins typed in the same way as the respective donor animals (BoLA phenotype, w6/w12) with the exception of the T.parva-transformed line derived from the steer 219 which had the BoLA phenotype w6/w10.

3.3.2 Karyotyping of 219 and 220 and derived LCLs

This was carried out primarily to establish the chimaeric status of the twin cattle and to ascertain whether or not the 219TaH LCL was a coculture of male and female cells. The results are presented in Table 3.3.

The chimaeric status of 219 and 220 was conclusively shown. It is of interest to note that the proportion of male and female cells in PBL are the same in each of the twins. Further, the animals were of predominantly female PBL type. The LCL 219TaH was also shown to be a predominantly female cell line, whereas the 219TpM line was overwhelmingly a male line.

The BoLA typing results together with the karyotypic analysis supported the view that the 219TpM line could have an origin in the minor PBL population (the male population) of the donor 219; this population being inaccessible to BoLA typing. Further, it was clear from the results that the 219TaH line was indeed a coculture of male and female cells, and the attempt was therefore made to

TABLE 3.1

BoLA TYPING AND REACTIVITY OF CHIMAERIC TWINS 219 AND 220 AND THEILERIA-TRANSFORMED LCLs DERIVED FROM THEM

Serum (ED number)	9	10	12	16	28	47	65	66	69	71	74	75	85
Steer 219													
Fresh PBL	+	+	+	+	+	+	+	+			+	+	
TaH Cells p.3*	+	+	+	+	+	+	+	+			+	+	
TpM Cells p. 13**	+	+	+					+	+	+		+	+
Heifer 220													
Fresh PBL	+	+	+	+	+	+	+	+			+	+	
TaH Cells p.3	+	+	+	+	+	+	+	+			+	+	

\+ Indicates a positive reaction with the typing serum at the standard dilution

* TaH p.3 indicates T. annulata (Hissar) transformed LCL at passage 3

** TpM p.13 indicates T. parva (Muguga) transformed LCL at passage 13.

TABLE 3.2

BoLA WORKSHOP SPECIFICITIES OF CHIMAERIC TWINS 219 AND 220 AND THEILERIA-TRANSFORMED LCLs DERIVED FROM THEM

	w6	w10	w12
Steer 219	+		+
219TaH	+		+
219TpM	+	+	
Heifer 220	+		+
220TaH	+		+

TABLE 3.3

SEX KARYOTYPE ANALYSIS OF 219 AND 220 AND DERIVED LCLs

	Karyotype %	
	Female	Male
Steer 219		
Fresh PBL	80	20
Ta_H p.3	80	20
TpM p.13	2	98
Heifer 220		
Fresh PBL	80	20

Karyotype analysis was based on the % of cells bearing the Y chromosome in 200 cells examined.

potentiate the male cells in this line with a view to ascertaining their BoLA type.

3.3.3 Selection of male 219TaH cells by suppression of female cells in the coculture

The primary requirement for this work was a complement-fixing antiserum which would react with the female cells but not the male cells in the culture. Such an antiserum could then be used for *in vitro* complement-dependent, antibody-mediated cytolysis of the female cells, leaving the male component of the cell line in a viable state to be further cultured prior to ascertaining its BoLA type.

The Edinburgh panel of typing sera used in this work was principally made up of absorbed sera, some of which had been absorbed several times to give operational monospecificity. As a result many of these sera were available in small quantities only and it was therefore decided to titrate the original crude sera, from which sera discriminating between the 219TpM and 219TaH lines were derived, against these lines in order to identify a suitable selective reagent which would be available in sufficient quantity for the work. The crude sera used, the BoLA typing panel sera derived from them and the results of titration in the microlymphocytotoxicity test against the 2 lines, are shown in Table 3.4.

On the basis of this result, antiserum 2273 16.9.75 was chosen as the selective cytolytic agent. This antiserum was used on 2 occasions to treat the 219TaH line. The effect of selection on BoLA and karotype is shown in Table 3.5.

It is clear that treatment of the 219TaH line with antiserum 2273 had a profound effect on the karyotype composition and

TABLE 3.4

TITRATIONS OF POTENTIAL SELECTIVE ANTISERA AGAINST 219TaH AND 219TpM IN THE MICROLYMPHOCYTOTOXICITY TEST

Antiserum	Cell line												Panel derivative sera
	219 TaH p.16						219 TpM p.15						
	Reciprocal titre												
	2	4	8	16	32	64	2	4	8	16	32	64	
Serum 7639 7:7:76	4	-	-	-	-	-	8	8	8	8	8	8	ED 71
Serum 611 7:7:76	8	8	4	-	-	-	6	-	-	-	-	-	ED 74
Serum 2273 16:9:75	8	8	8	4	-	-	-	-	-	-	-	-	ED 47
Serum 1862 25:5:76	8	8	4	-	-	-	-	-	-	-	-	-	ED 65
Serum 1470 25:5:76	8	8	8	8	-	-	8	8	8	8	8	-	ED 69
Serum 7639 7:7:76	8	-	-	-	-	-	8	8	8	8	8	-	ED 71
Serum 611 7:7:76	8	8	8	-	-	-	6	-	-	-	-	-	ED 74
Serum U106B 24:11:75	6	-	-	-	-	-	8	8	8	8	8	-	ED 85
NRS*	-	-	-	-	-	-	-	-	-	-	-	-	

* Non-reactive bovine serum

The reactivity scores are as detailed in 2.1.2.2.

TABLE 3.5

THE EFFECT OF TREATMENT OF 219 TaH WITH ANTISERUM 2273 ON BoLA AND KARYOTYPE

Animal of origin	Cells studied	BoLA Type			Karyotype %	
		w6	w10	w12	♀	♂
219	TaH p16*	+		+	80	
219	TpM p15	+	+			98
219	TaH T47**	+	+			99
219	TaH T47 p3	+	+		10	90
219	TaH p19	+		+	83	17
219	TaH T47x2 p2***	+	+		4	96
219	TaH T47 p6				6	94
219	TaH T47x2 p6				2	98
219	TaH p23				96	4

* Passage number.

** Line 219 TaH treated on one occasion with serum 2273 16:9:75.

*** Line 219 TaH treated on 2 occasions with serum 2273 16:9:75.

NB. 219 TaH p16 was the origin of all the TaH LCL's in this study.

apparent BoLA phenotype in that the cultured cells were completely different from the pretreatment and control cultures in these respects. Moreover, the essentially male line resulting from selection of cells within the 219TaH culture had the same BoLA phenotype, w6/w10, as the male 219TpM line.

3.3.4 Selection for minor populations in the 219TpM and 219TaH T47 lines

On 3 separate occasions the 219TpM and 219TaH T47 (219TaH previously subjected to the selective procedure detailed in 3.3.3) lines were treated with serum 7639 7.7.76 in an attempt to select w12 positive/w10 negative cells. On none of these occasions was it possible to grow up any cells.

3.4 Discussion

The results are consistent with T.parva preferentially transforming cells from a minor population of 20% or less within the PBL of the the chimaeric donor, 219. There is no reason to suppose therefore, that the w6/w10 BoLA type was evolved de novo on transformation. Although it cannot be concluded that no female cells of major population type were transformed, the fact that such cells could not be selected for may be significant. In contrast, in the particular T.annulata (Hissar) line studied, the evidence is that the parasite showed no preference for cells of either a particular sex or BoLA type in the infection and transformation process. These findings could reflect at least 3 possibilities. One could be that the target population for T.parva parva (Muguga) is very restricted but that for T.annulata (Hissar) is less so. The results could then be explained by the fact that such a target subset for T.parva parva

(Muguga) was only present at the time of infection and transformation within the minor population of the chimaera, or if present in both populations, more target cells had the rarer genotype than the common genotype and the latter were overgrown during the production of the cell line. However, the rarer genotype when transformed by T.annulata (Hissar) was still present after more than 20 passages. The target subset for T.parva parva (Muguga) could have been the whole of, or only a part of the minor population; the implication being that BoLA or karyotype per se had no influence but merely allowed the detection of a target preference in the particular system studied.

A second possibility is that the infection and transformation process was directly influenced by BoLA or karyotype. In this regard there is no evidence from previous work of sex restriction in the sporozoite infection system either in vitro or in vivo. However, the possibility that certain BoLA types are more susceptible to T.parva parva (Muguga) infection than others, remains. Indeed links have been found in other species between MHC type and disease susceptibility (Dausset & Svejgaard, 1977; Longenecker & Mossman, 1981). This does not preclude the fact that such a BoLA resistance/susceptibility connection exists for T.annulata (Hissar), especially in view of the fact that only two genotypes were studied. Variation in ECF susceptibility linked to BoLA type would be of significance to the development of livestock industries in ECF areas.

A third possibility is that T.parva parva (Muguga), by comparison with T.annulata (Hissar), is less efficient in the cell infection and transformation process in the in vitro system of cell line production used. Thus in the T.parva in vitro infection system, only very few or even one cell may transform and give rise to a

clonal culture.

With regard to the cell selection technique itself, it is evident that selection of certain BoLA types from within established chimaeric lymphoblastoid cell lines is possible by the method of suppressing other types within such lines by complement-mediated cell killing with BoLA antisera. In this work, total suppression of the majority karyotype was never achieved. However, it was not established that the surviving cells of the sex which was selected against were of the suppressed BoLA type. It is possible that they represent one or more minor populations lacking the specificity detected by the selecting antiserum. The limited evidence that it is not possible to achieve a reversion of cell line type by reverse selection suggests either that all cells are selected by the first treatment or there are too few surviving cells of the original major type left to grow up in the system. The results also suggest that a line once subjected to BoLA selection is subsequently stable.

Selection for human lymphocyte antigen (HLA) variants has also proved possible by means of suppression of non-variants, using HLA antisera in a complement-dependent cytotoxicity system (Pious et al., 1973; Gladstone & Pious, 1978), and in this work the "loss" variants proved to be stable over more than 80 cell doublings in non-selective conditions.

The technique may have uses beyond that reported in this limited study, particularly in the area of immunisation against ECF by inoculation of T.parva-infected LCLs. As discussed in 1.2.3, prior to the work reported in this thesis, there was reason to suspect that the bovine MHC posed a barrier to this form of immunisation. The fact that there is in reality such a barrier is the subject of the work reported in chapters 4 and 5. One possible

way in which such a barrier could be overcome would be to utilise LCLs lacking expression of MHC-coded antigens, analogous to the Daudi cell line of human origin (Bodmer, 1981). Such a bovine cell line has not hitherto been reported. However, using the technique described here it may be possible to potentiate BoLA-negative variants in new and existing T.parva-infected cell lines, should such extant variants occur. The evidence from the work of Pious & Soderland (1977), is that in their HLA system, complement-dependent, antibody-mediated cytolytic techniques select for pre-existing mutants rather than induce modulation in the antigens themselves. Moreover, it may prove possible to increase the chances of isolating such variants in the ECF system by exposure of LCLs to chemical mutagens or ionising radiation. In the human system the use of chemical mutagens has resulted in a 2 orders of magnitude increase in HLA variant frequency (Pious et al., 1977).

In summary, the work reported here has resulted in the clarification of an anomalous T.parva-induced in vitro transformation, and in the development of a technique with potential use in future studies, particularly in the area of LCL immunisation against ECF.

Note

Aspects of the work reported in chapter 3 are the subject of publication,
"Selection, by major histocompatibility type, (BoLA), of lymphoid cells derived from a bovine chimaera and transformed by Theileria parasites.",
Teale, A.J., Kemp, S.J., Young, F., & Spooner, R.L.,
Parasite Immunology, (1983, in press).

CHAPTER 4 A study of the effect of degree of BoLA compatibility between LCLs and recipient cattle on the response to cell line inoculation and subsequent challenge - MHC 1

4.1 Introduction

It has been evident since the early attempts to immunise cattle against ECF with infected tissues that the nature of the inoculated material may have an effect on the efficiency of immunisation. The variability in the results of various workers is reviewed by Wilde (1967) and Cunningham (1977).

Since the development of *in vitro* culture techniques for *T.parva* (Hulliger *et al.*, 1974; Malmquist *et al.*, 1970) and the subsequent techniques for *in vitro* infection and transformation of bovine lymphoid cells with tick-derived sporozoites (Brown *et al.*, 1973; Stagg *et al.*, 1981) the possibility has existed of using parasite-transformed LCLs for the vaccination of cattle against ECF. Vaccination trials have shown however, that in order to ~~reliably~~ reliably produce immunity in the majority of animals to the homologous parasite isolate LCL cell numbers of the order of 10^7-10^9 are required (Brown *et al.*, 1971; Brown *et al.*, 1978a). Even at this dose level however, certain animals die as a result of LCL inoculation and others may remain susceptible to challenge. It would seem therefore, that the variability in response reported by those workers studying the results of inoculation of tissues from infected cattle may also pertain to the LCL immunisation system.

That the acceptability to the recipient animal, in an immunological sense, of the inoculated material may be an important reason for this is suggested by a number of studies carried out to date. Wilde (1967) was the first to suggest that the inoculation of

infected cells could result in a response to the cells themselves as distinct from an anti-parasite response. Wagner and Duffus (1974) drew attention to the fact that the MHC may be important in the LCL/recipient interaction. Their important studies on the immune response to inoculated parasitised lymphoid cells are discussed in 1.2.2.1.

Since that time, two reports have clearly shown a "self-preference" in the LCL inoculation system, (Brown et al., 1978b; Morrison et al., 1981). In the former report, blood infected in vitro during tick feeding immunised only the autologous blood donors, or, as in one case, the twin of such a donor, against homologous stabilate challenge. Morrison et al., (1981) working with LCLs were able to show that doses as low as 10^2 LCL cells may induce immunity in autologous recipients and that doses of 10^6 or higher may kill such recipients. As pointed out by these workers the 10^2 dose level, which may not result in patent infection in the autologous recipient but is capable of inducing immunity to subsequent stabilate challenge, is equivalent to the 10^7-10^8 dose level in some allogeneic recipients in these respects. There would therefore, appear to be approximately a million-fold difference in acceptability of LCLs between some of the autologous and allogeneic systems.

Dolan et al., (1982) broadened this concept to one of a similar-type preference, as opposed to a self preference, when they reported the results of a comparison of responses of B.taurus and B.indicus cattle to B.taurus and B.indicus-derived LCLs. In this study, cattle evidenced a greater response to the same type cell line than to the different type.

All these accumulated results, together with the report of

Spooner & Brown (1980) that Theileria-infected LCLs express BoLA specificities, suggested that the bovine MHC may play an important role in the LCL immunisation system. The work reported in this chapter, and in the subsequent chapter, was undertaken in an attempt to determine whether or not this is the case, and if so, to quantify the restriction on the LCL/recipient interaction imposed by the BoLA system.

4.2 Materials and Methods

4.2.1 Cattle

Cattle were selected for the study on the basis of BoLA type. The reactivity of their PBL with the Edinburgh, and where relevant, the KM panel is shown in Appendix 1.1. Only cattle with no previous exposure to Theileria parasites, as assessed by the IFAT for serum antibody to T.parva parva (Muguga) schizont antigen, were used. The cattle were either bred at the Veterinary Research Department, Kenya Agricultural Research Institute, or purchased from farms in Kenya where strict tick control was practised. Most were Friesians, 3 were Herefords and 3 were B.indicus/B.taurus crossbreds. Two were females and the remainder were steers.

Prior to the experiment, the cattle were treated prophylactically for babesiosis with imidocarb diproprionate (3 mg/kg) (Imizol, Wellcome) and for helminthiasis with oxyclozamide (15 mg/kg)/levamisole (7.5 mg/kg) (Nilzan, Cooper). They were held in a partially covered yard during the experiment and given hay and water ad libitum, and a small quantity of concentrates daily. They were dipped 3 times weekly in dioxathion (Delnav DFF, Cooper) to control ticks.

The cattle were generally well matched for age (1~2 years) and weight.

4.2.2 Cell lines

Cell lines were established and maintained by Mr D.A.Stagg as described in 2.2 and 2.3.

In vitro infections of PBL from six of the animals were the source of LCLs. The ticks from which the sporozoites were harvested to establish the lines had been infected by feeding on cattle infected with T.parva parva (Muguga) stabilate 147.

The LCLs were used at low passage (<10), and were BoLA typed prior to inoculation. The BoLA typing is presented in Appendix 1.1. In all cases LCLs expressed the same w specificities as the donors. For the purposes of inoculation of known cell numbers, cells in aliquots of culture material were counted in a haemocytometer (Neubauer). Viable cells only were counted. Volumes of culture material were adjusted as necessary with growth medium to enable inoculation of the required number of cells in a volume of 10 ml.

Inoculations were made subcutaneously in front of the right shoulder in the region of the right prescapular lymph node.

4.2.3 Experiment Protocol

The 24 animals subjected to LCL inoculation were allocated to six groups of four. A cell line was established in vitro from the PBL of one in each group (ultimately the autologous recipient). Of the three remaining animals in each group, one was BoLA matched with the LCL donor as closely as possible, one was half~matched and one was mismatched. With respect to the degree of matching, particular attention was paid to the workshop specificities and half~matched

animals were considered to have one workshop specificity in common with the group LCL donor/autologous recipient, whereas the mismatched animals did not share workshop specificities with the LCL/donor. Matching was on the basis of sharing of two workshop specificities.

All animals in each group received an identical inoculum of LCL cells derived from the PBL of the donor/autologous recipient in the group. Three groups received 10^3 cells and three groups received 10^5 cells each. The groupings are summarised in Table 4.1. The "w" prefix refers to a workshop specificity and is followed by the agreed workshop number. The notation "~/~" or "w/~" indicates that either two or one of the workshop specificities could not be detected. This notation is used on the assumption that the specificities represent alleles at one BoLA locus.

4.2.4 Challenge

Animals surviving the LCL inoculation phase of the experiment and 4 other steers selected on the basis of negative serum reactivity in the IFAT for antibody against T.parva parva (Muguga) schizont antigen, were challenged subsequently with 1.0 ml of T.parva parva (Muguga) stabilate 147. The stabilate was inoculated subcutaneously below and slightly forward of the right ear, in the region of the parotid lymph node. Stabilate challenge was 70 days after LCL inoculation.

TABLE 4.1

THE GROUPING, BREED AND BoLA TYPE OF CATTLE USED FOR AUTOLOGOUS AND ALLOGENEIC LCL CHALLENGE AND SUBSEQUENT STABILATE CHALLENGE

Group	Inoculum	Animal	Breed	BoLA Type
1	10^3 M980 LCL cells	M980	Hereford	w7/w9
		N894	Hereford	w7/w9
		S334	Friesian	w6.2/w7
		S866	Friesian	w6/w13
2	10^3 S55 LCL cells	S55	Friesian	w6.2/w10
		S59	Friesian	w6.2/w10
		N178	Friesian	w10/ -
		S357	Friesian	- / -
3	10^3 N511 LCL cells	N511	x-bred	w7/w8
		M878+	x-bred	w7/w8
		S340	Friesian	w7/ -
		S54	Friesian	w6.1/w10
4	10^5 S68 LCL cells	S68	Friesian	w11/w20
		S58	Friesian	w11/w20
		N170	Friesian	w11/w16
		S125	Friesian	w5/w6.2
5	10^5 S48 LCL cells	S48	Friesian	w6/(w8)*
		S122	Friesian	w6.2/w8
		N546	Friesian	w6/w10
		M646	Friesian	w5/w20
6	10^5 N989 LCL cells	N989	x-bred	w6/w9/w20
		N886+	Hereford	w6/w9/w20
		S121	Friesian	w6.2/w16
		S127	Friesian	w8/w16

+ Female

* Reacting with 2 of 3 w8 - defining sera

4.2.5 Assessment of response to LCL inoculation and stabilate challenge

4.2.5.1 Haematology

Haematology was performed as described in 2.5.

White blood cell (WBC) levels, haemoglobin concentrations and packed cell volumes (PCV) were assessed on two occasions prior to the experiment and then following LCL inoculation, every 2-4 days to day 28 post-inoculation. The same parameters were measured at 1-4 day intervals from day 10 to day 28 following stabilate challenge.

4.2.5.2 Serology

Serum samples were examined by the IFAT, as described in 2.4.1, for estimation of antibody levels to T.parva parva (Muguga) schizont antigen on two occasions prior to the experiment and then at approximately weekly intervals to day 53 post-LCL inoculation. This was also carried out on four occasions at weekly intervals following stabilate challenge.

Serum samples from animals in groups 3, 4, 5 and 6 were also examined for the development of cell membrane antibodies to the inoculated cell line using the indirect immune peroxidase test (IIPT) described in 2.4.2, using the relevant inoculated LCL cells as antigen.

4.2.5.3 Temperature responses

Rectal temperatures were taken daily from day 1 to day 27 post-LCL inoculation and from day 1 to day 26 post stabilate challenge.

Rectal temperatures were recorded in the morning prior to routine handling and sampling.

A temperature greater than 39.4°C was regarded as a febrile response.

4.2.5.4 Parasitology

Biopsy samples of the right prescapular lymph node (RPG) were taken daily from day 5 following LCL inoculation, and examined for Theileria macroschizonts. A biopsy of the left prescapular node (LPG) and a tail-tip blood smear were examined from the day following observation of parasites in the RPG, to day 35 post LCL inoculation.

The same procedure was followed from day 3 following stabilate challenge except that the right parotid lymph node (REG) was used as a first indicator of parasitosis.

Smears were examined following air-drying, fixation in methanol and Giemsa staining.

Procedures were as detailed in 2.6.

4.2.5.5 Post-mortem examination

Full post-mortem examinations were carried out on the carcasses of all animals dying during the experiment. ECF was confirmed on the basis of the presence of large numbers of parasites in smears of internal organs. Impression smears were routinely made from a superficial lymph node, spleen, lung, liver, kidney and mediastinal and mesenteric lymph nodes.

These were fixed and stained as described in 2.6.

4.2.5.6 Recovery

Recovery was recorded on the day after the last detection of macroschizonts following the return of the rectal temperature to <39.5°C.

4.3 Results

The data concerning haematological parameters, IFAT serology, parasitosis and temperature responses are presented in Appendices 1.2, 1.3, 1.4 and 1.5 respectively.

A summary of the responses to LCL inoculation is given in Table 4.2, and the responses to stabilate challenge are summarised in Table 4.3. One autologous LCL recipient (receiving 10^3 cells) died of ECF as a result of the LCL challenge. All autologous recipients responded serologically to LCL inoculation with the development of antimacroschizont antibodies as measured in the IFAT, and all five which were challenged subsequently with stabilate survived without the development of clinical signs of disease. It is notable that neither of the 10^3 autologous recipients surviving LCL inoculation showed any clinical signs of disease at any stage following LCL inoculation or stabilate challenge although in both phases of the experiment they did evidence parasitosis. The 10^5 autologous recipients evidenced a disease response following LCL inoculation but not on stabilate challenge. All three developed parasitosis following LCL inoculation but not following stabilate challenge.

There was an indication of the development of a degree of leucopenia in the autologous recipients during the first 14 days post-LCL inoculation. The mean WBC level at day 14 (lowest level in

TABLE 4.2

RESPONSE TO LCL INOCULATION ON DAY 0

Group	Animal	LCL and Dose level	BoLA Matching	M	T	R	D	IFAT
1	M980 N894 S334 N866	M980 10^3	a m ½ mis	9	10		21 12*	160
2	S55 S59 N178 S357	S55 10^3	a m ½ mis	8		20		640
3	N511 M878 S340 S54	N511 10^3	a m ½ mis	8		20		
4	S68 S58 N170 S125	S68 10^5	a m ½ mis	10	10	15		10240 10240
5	S48 S122 N546 M616	S48 10^5	a m ½ mis	8	9	16		10240 640
6	N989 N886 S121 S127	N989 10^5	a m ½ mis	8	10	21		2560

M - Days following LCL inoculation to first observed macroschizonts
T - Days following LCL inoculation to febrile response
R - Days following LCL inoculation to recovery
D - Days following LCL inoculation to death
a - Autologous recipient
m - Matched recipient
½ - Half-matched recipient
mis - Mismatched recipient
IFAT - Maximum reciprocal IFAT titre (macroschizont antigen)
* - Accidental death

TABLE 4.3

RESPONSE TO STABILATE CHALLENGE ON DAY 0 (DAY 70 FOLLOWING LCL INOCULATION)

Group	Animal	LCL Previously Inoculated	BoLA* Matching	M*	T*	R*	D*	IFAT
1	M980	M980	a					
	N894	M980	m					
	S334	M980	½	7	10		17	-
	N866	M980	mis	8	11		21	160
2	S55	S55	a	8		9		160
	S59	S55	m	7	11		20	
	N178	S55	½	6	9		23	
	S357	S55	mis	6	9		17	
3	N511	N511	a	7		8		160
	M878	N511	m					160
	S340	N511	½	7	10		17	
	S54	N511	mis	6	10		18	
4	S68	S68	a					640
	S48	S68	m					640
	N170	S68	½	6	10		19	
	S125	S68	mis	6	9		20	
5	S48	S48	a					640
	S122	S48	m	10		11		
	N546	S48	½	8	10	20		160
	M616	S48	mis	7	8		17	
6	N989	N989	a					640
	N886	N989	m	7	10		16	
	S121	N989	½	7	11		21	
	S127	N989	mis	8	12		17	
Chall-	R608	None		7	11		17	
enge	S96	None		6	10		19	
Con-	S114	None		7	10		16	
trols	S170	None		7	10		17	

* - See Table 4.2

IFAT - Maximum reciprocal IFAT titre achieved following stabilate challenge

the first phase of the experiment) for the autologous recipients was 4100/cu mm blood, having fallen from a mean value of 9533/cu mm on the day of LCL inoculation.

Of the six matched recipients, one died at an early stage due to causes not connected with the LCL inoculation. Results were contrasting in the two remaining 10^3 LCL cell matched recipients in that although neither developed anti-schizont antibody as measured by the IFAT, M878 subsequently proved resistant to challenge and S59 did not. It is interesting to note that following challenge M878 neither showed evidence of parasite development or clinical signs of disease. There was however, evidence of a limited serological response to challenge in this animal.

The 3 matched 10^5 LCL cell recipients showed varying responses. As with the 10^3 matched recipients there was no evidence of parasite development or disease as a result of LCL inoculation. Two of the 3 however, developed antischizont antibody as measured by the IFAT. On challenge two of these matched animals survived whereas the serologically poor responder N886 succumbed. Of the two survivors, S58, following a serological response of the same order as the respective autologous recipient, showed no evidence of disease or parasite development in response to challenge. S122 evidenced parasite development but no disease signs on challenge.

Of the six half-matched recipients, none developed a significant IFAT antibody response following LCL inoculation. All but one of the half-matched animals succumbed to challenge. N546 survived after a severe disease episode, and even at this stage did not produce a significant serological response until day 28 post stabilate inoculation. N546 originally received 10^5 LCL cells of the BoLA half-matched S48.

None of the mismatched animals showed any response to LCL cell inoculation and all succumbed to challenge.

All challenge control animals (i.e., those which had not previously been LCL cell recipients) succumbed to challenge.

At the time of challenge, the IFAT titres of all animals which responded serologically to, and survived LCL inoculation, were below the peak levels previously achieved. In no case did they regain these peak levels in response to challenge. The two animals which survived challenge without having detectable IFAT titres in response to LCL inoculation (one matched, and one half-matched) did respond serologically to challenge, but this response was limited.

Table 4.4 shows the serological responses of the recipients to the cell surface antigens of the respective lines as measured by the indirect immunoperoxidase test. None of the autologous recipients developed a detectable response whereas 1 of 4 matched animals, 4 of 4 half-matched and 4 of 4 mismatched recipients responded.

Table 4.5 summarises the responses of the animals, grouped on a BoLA compatibility basis with respect to the inoculated LCLs, to stabilate challenge.

TABLE 4.4

ANTIBODY RESPONSES OF ANIMALS IN GROUPS 3, 4, 5 AND 6 TO CELL MEMBRANE ANTIGENS ON THE INOCULATED CELL LINE CELLS AS MEASURED IN THE IIPT

Group	Inoculated cell and dose	Recipient Animal	Reciprocal maximum IIPT titre*
3	N511 10^3	N511	<5
		M878	<5
		S340	40 (20)+
		S54	40 (14)
4	S68 10^5	S68	<5
		S58	<5
		N170	40 (14)
		S125	10 (20)
5	S48 10^5	S48	<5
		S122	10 (14)
		N546	20 (14)
		M616	5 (20)
6	N989 10^5	N989	<5
		N886	<5
		S121	40 (20)
		S127	20 (35)

+ Figures in parenthesis denote days after cell line inoculation on which maximum titres were first detected.

* A reciprocal of 5 or greater is regarded as a positive response.

TABLE 4.5

NUMBERS OF ANIMALS IN THE AUTOLOGOUS, MATCHED, HALF - AND MISMATCHED CATEGORIES AND CHALLENGE CONTROLS SURVIVING STABILATE CHALLENGE.

BOLA Group	Number Stabilate Challenged	Number Surviving
Autologous LCL recipients	5	5
Matched LCL recipients	5	3
Half-matched LCL recipients	6	1
Mismatched LCL recipients	6	0
Challenge controls*	4	0

* Not previously inoculated with LCL cells

4.4 Discussion

It is evident from this study that the inoculation of defined doses of BoLA-characterised LCL cells into similarly characterised recipient cattle may result in different immune responses in different cattle. This was even evident in the group of six autologous recipients. One of these animals, which received only 10^3 LCL cells, suffered a fatal disease episode as a result. This contrasts with the findings of Morrison et al., (1981) who reported fatal infections occurring following inoculation of 10^6 or more autologous infected cells. However, Morrison et al., (1981) also studied only one or two animals at each dose level down to 10^2.

All of the autologous recipients surviving LCL inoculation were effectively immunised against homologous stabilate challenge effected 70 days later. There is evidence of two types of LCL dose response in this group. First, the 10^5 recipients suffered a mild clinical disease episode after LCL inoculation as evidenced by temperature response, which was not seen in those animals receiving 10^3 autologous LCL cells. This is entirely consistent with the "quantum of infection" hypothesis (Wilde, 1967). Second, there was an apparent difference between the two levels of LCL inoculation in the degree of resistance conferred to subsequent stabilate challenge. Thus, although none of the autologous recipients showed a clinical disease response to challenge, both 10^3 recipients allowed a detectable degree of parasite development on challenge whereas the 10^5 autologous recipients did not. These results suggest that just as the disease itself is a quantum-dependent phenomenon with respect to challenge dose, immunogenesis may also be in the sense that different degrees of priming of the immune system can be achieved with different doses of LCL immunogen. Burridge et al., (1972),

utilising a stabilate challenge in ECF-recovered cattle were not able to relate the severity of primary disease to the response to subsequent challenge. The findings of Barnett (1957) are in agreement with this although Wilson (1950) concluded that the durability of immunity was increased following severe disease reactions by comparison with the immunity following mild reactions. The results reported by Dolan et al., (1982) also suggest a relationship between severity of primary disease response and resistance to subsequent challenge. In this study the primary challenge was with T.parva parva-infected LCLs. The differences in these various findings may be due to the existence of a threshold reaction, at levels above which immunity is optimally induced and below which a relationship exists between disease reaction and immunity. The two autologous 10^3 recipients surviving to challenge in this work suffered mild reactions which resulted in a suboptimal, albeit effective, immune response.

As far as the BoLA-matched LCL recipients are concerned, at the 10^3 dose level one immunisation proved to be effective in that the animal was subsequently totally refractory to challenge. The lack of serological response to schizont material in this animal prior to challenge is a point for consideration. There is a possibility that the animal was immune to the parasite prior to the experiment, especially as it is known that immunity may outlast detectable serum antibodies to schizont material in the IFAT (Burridge et al., 1973b). Furthermore, as reported by Goddeeris et al., (1982, in preparation) immune cattle may not show an antimacroschizont antibody response when challenged. However, the animal concerned was born and reared on a research station where acaricidal control is practised. A more likely explanation of the

response of this animal is that of innate resistance to infection conferred by cross-breeding with B.indicus (Cunningham et al., 1973b; Radley, 1978). It has further been observed that measurable responses to LCL inoculation may be lower in B.indicus animals than in taurines (Dolan et al., 1982). The second 10^3 BoLA-matched recipient showed no evidence of immunity on challenge.

Of the 10^5 LCL cell matched recipients, one succumbed to challenge. The BoLA type of this animal N886, and the autologous recipient to which it was matched, N989, should be considered. The matching was based on the presence on the line and the two animals of w6, w9 and w20. This situation is not consistent with the view that these specificities represent alleles at one BoLA locus. It was however thought at the inception that the specificities w6 and w9 were present and that the single serum detecting w20 (ED78) was cross-reacting with another undefined specificity. This was possible in view of the fact that the w20 serum was suspected to be operationally multispecific. Subsequent absorptions have confirmed this. However, it is now thought that both ED78 and ED14 (the w9-defining serum) cross-react with a single third entity which is defined by KM2. It is not known with certainty that the KM2 specificity is coded for by the first BoLA locus. If it is not, there is a possibility that N989 and N886 were half-matched at the first locus as a result of sharing w6. In this case, the poor immunogenicity of the N989 line in N886 is not inconsistent with the results in the half-matched cattle as a whole. The results of typing large numbers of cattle with the KM panel however, suggest that in all likelihood the KM2 specificity is first locus coded, in which instance this result is viewed in the matched context.

Of relevance here are the serological responses to LCL cell

surface antigens in the recipients. N886 did not respond with detectable antibody levels to the N989 line, which is consistent with good matching at the first locus.

Generally, the responses to cell antigen in the four groups studied are consistent with the degrees of matching assumed at the outset on the basis of the BoLA types as defined in these particular cattle. As expected, there was no evidence of a response in autologous recipients, thus strengthening the view that parasite antigen *per se* on the cell surface was not being measured in this test. This is consistent with the results of previous attempts to demonstrate such antigens (Duffus *et al.*, 1978, Creemers, 1982). It seems likely that the test was indeed measuring a response to MHC-coded antigens, and as expected, a response was detected in all of the half-matched and mismatched recipients studied. One of 4 matched recipients however, also produced a detectable response in the IIPT; animal S122, a 10^5 recipient. It is notable that in this case, as suggested by the response to BoLA, matching was not as good as in the other groups. S48 and S122 were matched for the w6 broad specificity (the presence of the w6.2 subgroup in S122 but not S48 would not be expected to be significant in this context). However, S48 and the derived cell line were positive for two of three sera presently accepted as defining w8 (ED13 and 63) whereas S122 was positive for these sera and the third defining serum, ED80. Additionally, S48 and the S48 LCL are KM22 and KM25 positive whereas S122 is not. It is known that KM22 detects an antigen related to w8 (i.e., reacts with a specificity also detected by ED13 and 63 but not invariably ED80), but the nature of this relationship is not known. It is clear nevertheless, that this slight difference in matching in this group did not prevent the development of a good immune response

following LCL inoculation into S122. Whether the BoLA difference involves the first locus coded entity or the products of a second putative locus is not known.

The different responses in the different groups raise a number of questions concerning immunisation attempts with LCLs. There are a number of possible reasons for the variability observed. First, it could be the result of cell line factors. It is not inconceivable that lines differ in innate aggressiveness following inoculation. This could be the result of host cell subtype and viability on inoculation, amongst other things. Such factors can only be the subject of speculation at present. However, it is also possible that the particular BoLA types involved could be implicated in producing variability of response in recipients, in a number of ways. Certain BoLA w specificities *per se* may be more immunogenic than others and therefore result in a more effective graft response in the recipient leading to earlier elimination of the inoculated allogeneic cell and the parasite which it hosts. It has generally been accepted hitherto that some degree of establishment of the parasite in the cells of the host animal is required to induce immunity (Emery *et al.*, 1981a). This factor would not be expected to pertain with LCL inoculation into BoLA matched recipients, but could be significant with 1/2-matched recipients.

The question of antigenicity of BoLA specificities can be viewed in a second context. Thus it is possible that the parasite is "seen" by the immune system better on some BoLA class I backgrounds than others. This has previously been reported for other pathogens (Doherty *et al.*, 1976), for haptens (Schmitt-Verhulst & Shearer, 1976) and tissue antigens (Zaleski & Klein, 1977). This type of BoLA limitation in LCL

immunisation would operate at a different level from the graft response limitation in that it would only pertain in the autologous, matched and half-matched situations as studied here. Furthermore, it would be expected to operate whether or not parasite transfer from the inoculated LCL to recipient cells occurs. The implication is that BoLA *per se* produces differences in degree of immunocompetence. This also must remain speculation for the present.

The degree of matching itself might be expected to produce the variability in response of the matched animals within the 2 dose level groups in this study, but for reasons already outlined, this would seem to be unlikely. However, effects due to other (putative) specificities and the products of other genetic regions cannot be excluded entirely.

In view of these considerations it can be reasonably concluded from this study that the bovine MHC is a barrier to LCL immunisation of cattle against ECF, although other factors may be superimposed.

CHAPTER 5 A study of the effect of degree of BoLA compatibility between LCLs and recipient cattle on the response to cell line inoculation and subsequent challenge-MHC 2

5.1 Introduction

The study described in chapter 4 suggested that MHC disparity between LCLs and recipients presents a barrier to this form of immunisation against ECF. In that particular study, 6 LCLs were used of different BoLA types and these were inoculated in each case into the autologous recipient and a single BoLA-matched, 1/2-matched and mismatched recipient.

A second large scale experiment was carried out utilising a different approach with 2 principal objectives. First, to confirm the "MHC barrier" hypothesis and second to investigate the possibility that a slightly higher dose level than those used in the first study may be capable of inducing immunity in BoLA 1/2-matched recipients. This investigation was felt to be important because if immunisation could be achieved in BoLA 1/2-match situations the possibility would exist of immunisation of groups of cattle with a relatively limited number of LCLs, each of which would be required at a feasible dose level for large scale production.

For this study therefore, a dose level of 10^6 LCL cells was chosen. A single, BoLA-characterised LCL was used and the recipient groups (grouped on the basis of their BoLA relationship with the LCL) were made as large as the availability of suitable cattle and the logistics of the experimentation would allow.

5.2 Materials and methods

5.2.1 Cattle

Thirty one Friesian steers purchased from Delamere Estates, Manera Farm, Naivasha, Kenya were used in this study. They had been subjected to a strict dipping routine prior to purchase and were considered to have had no exposure to T.parva parva parasites on the basis of the results of the IFAT for serum antibody to T.parva parva (Muguga) schizont antigen.

They were additionally selected on the basis of BoLA type and the reactivity of their PBL with the Edinburgh panel of typing reagents is shown in Appendix 2.1. Further, consideration was given to the parentage of the animals as discussed in 5.2.4.

The cattle were purchased and moved directly to the Veterinary Research Department, Kenya Agricultural Research Institute, in December 1981 after which time acaricidal control was continued with thrice-weekly dipping in dioxathion (Delnav DFF, Cooper). Prior to the experiment they were treated on 2 occasions for helminthiasis with oxyclozamide (15 mg/kg)/levamisole (7.5 mg/kg) (Nilzan,Cooper). During the course of the experiment, those considered strong enough were allowed to go to pasture during the day. All cattle were kept in a partially-covered yard at night where hay and a small quantity of concentrates were made available. Water was given ad libitum.

At the commencement of the experiment, the cattle ranged in age from 18-30 months.

5.2.2 Anaplasmosis control

Anaplasmosis proved to be a recurrent problem during the course of the experiment. Treatment and prophylaxis was undertaken with imidocarb diproprionate (Imizole, Wellcome) at a dose rate of 3 mg/kg. This chemotherapy also serves to treat and prevent Babesia infections.

On day -12 (day 0 being the day of LCL inoculation) animals S806, S817 and S835 were found to be clinically affected by Anaplasma marginale infection, and were treated. On day -5, the remaining cattle were treated prophylactically and S817 was treated for a second time. On day 0, all animals were considered to be free of clinical signs of anaplasmosis and only 1 animal, S817, evidenced an A.marginale parasitosis, but at a low level (less than 1% infected erythrocytes).

On the day of stabilate challenge (day 29) animals S807, S808, S820, S824, S830 and S836 were found to have A.marginale parasitaemias; these being severe in the cases of S807, S808 and S820 (10%, 50% and 80% erythrocytes parasitised, respectively). These 3 animals were treated on day 29, but S808 died with acute anaplasmosis on the same day. All remaining experiment animals were treated prophylactically on day 30 and S834 was retreated on day 34 when it was found to have 20% erythrocytes infected with A.marginale.

5.2.3 Cell line

A single LCL was established for this study by the in vitro infection and transformation of PBL from animal S816. The ticks from which the sporozoites were harvested to establish the line, had been infected by feeding on cattle infected with T.parva parva (Muguga), stabilate 147. The cell line was established by Mr D.A.Stagg as

described in 2.2 and 2.3.

The reactivity of the LCL (S816TpM) with the Edinburgh BoLA typing panel is presented in Appendix 2.1.

5.2.4 Experiment protocol

Thirty of the cattle were inoculated with the LCL, S816TpM. The animal S816 served only as a souce of PBL for the establishment of the cell line, i.e., all recipients were allogeneic with respect to the LCL.

The 30 cattle formed 4 groups on the basis of their BoLA relationship with the LCL. One group shared 2 workshop BoLA specificities (w8/w11) with the line (the matched group). One group shared the w8 specificity and a third group shared the w11 specificity (2, 1/2-matched groups). The fourth group did not share any workshop specificities with the line (mismatched group).

In an attempt to reduce the possibility of sharing by line and recipients of inherited characteristics coded for outside the MHC, the line was made on the S816 background. The sire of this animal did not sire any of the LCL recipients. Further, because of the fact that the animals were reasonably matched for age, it is unlikely that any of the recipients had a 1/2 sib relationship with S816 through the dam. The groups were also constructed as far as possible to ensure genetic heterogeneity. Thus 4 bulls sired the 30 recipients and all 4 were represented through their progeny in 3 of the 4 groups. Sire W was not represented in the w8, 1/2-matched group.

The cattle, their grouping, BoLA workshop specificities and sires are listed in Table 5.1.

Aliquots of the S816TpM line were inoculated into the 30

TABLE 5.1

THE LCL, RECIPIENTS, THEIR SIRES, BoLA w SPECIFICITIES AND GROUPING ON THE BASIS OF SHARING w SPECIFICITIES WITH THE INOCULATED LCL

Group	Animal no.	Sire	Workshop specificities
Matched	S811	W	8,11
	S817	AD	8,11
	S821	J	8,11
	S828	N	8,11
	S830	J	8,11
	S832	AD	8,11
w8, ½ matched	S812	AD	8,13
	S815	J	8,13
	S818	N	8,6
	S820	N	8,6
	S824	N	8,20
	S825	J	8,13
	S831	N	8,20
	S836	AD	8,13
w11, ½ matched	S804	J	11,16
	S807	AD	11,13
	S808	N	11,13
	S813	J	11,6
	S819	AD	11,16
	S826	J	11,6
	S834	W	11,6
	S835	J	11,6
	S996	AD	11,10
	S997	AD	11,16
Mismatched	S806	AD	6,13
	S809	N	6.13
	S814	N	13,20
	S822	J	6,13
	S823	W	10,13
	S829	AD	13,16
S816 TpM LCL*	S816	E	8,11

* The animal number and sire refer to the animal from which the line was derived. The workshop specificities 8 and 11 were present on theLCL cells and the PBL of the donor animal.

cattle on experiment, on day 0. Stabilate challenge of the 30 cattle and 2 additional steers (ECF-naive on the basis of the IFAT) was effected on day 29.

On day 44, a number of the cattle were treated for ECF with halofuginone as lactate (Hoechst). The cattle not treated, and therefore running the full course of the experiment, were all 6 matched animals, the first 2 cattle (on a numerical basis) in each of the 3 other groups and the 2 challenge control cattle which had not previously been inoculated with the LCL.

5.2.5 LCL inoculation

The line was utilised at passage 9.

The concentration of viable cells in the culture material was assessed by counting dye-excluding cells in an aliquot in a Neubauer chamber, using trypan blue (0.5% in culture medium) as a diluent.

10^6 viable cells were inoculated subcutaneously in front of the right shoulder of each recipient animal in 5 ml culture medium.

A 50 ul aliquot of the culture material was deposited on a slide immediately prior to the inculations using a cytocentrifuge (Shandon Cytospin/600r.p.m for 10 min). Following Giemsa staining of the preparation, the following characteristics of the inoculated material were determined,

mean number of schizont particles/cell	10.4
mitotic cells	3%
multinucleate cells	5%
schizont-infected cells	97%

5.2.6 Stabilate challenge

All 30 LCL recipients and 2 additional steers were inoculated with 1 ml of T.parva parva (Muguga) stabilate 147 on day 29. S808 died on the same day as a result of anaplasmosis.

The stabilate was inoculated subcutaneously below and in front of the right ear, in the region of the parotid lymph node.

5.2.7 Assessment of response to LCL inoculation and stabilate challenge

5.2.7.1 Haematology

White blood cell (WBC) levels, packed cell volumes (PCV's) and haemoglobin (Hb) concentrations were assessed on 2 occasions prior to day 0 and then as far as possible, every 3-4 days until day 59.

The method is detailed in 2.5.

5.2.7.2 Serology

Serum samples were examined in the IFAT (detailed in 2.4.1) for the development of antimacroschizont antibody on 2 occasions prior to day 0 and then weekly to day 66.

5.2.7.3 Temperature response

Rectal temperatures were taken daily from day 0 to day 56, in the mornings as the first excercise in routine handling and sampling.

A temperature greater than 39.4'C was regarded as a febrile response.

5.2.7.4 Parasitology

Biopsy samples of the right prescapular lymph node (RPG) were taken daily from day 4 to day 19 and examined for evidence of Theileria macroschizonts.

Samples of the right parotid node (REG) were similarly examined from day 34 to day 58. Biopsies of the left prescapular node (LPG) and tail tip blood smears were prepared and examined from the day first following observation of parasites in the REG on an individual animal basis. The LPG was then examined daily to day 58. Blood smears were examined for evidence of parasites to day 62.

Smears were examined following air-drying, fixation in methanol and Giemsa staining.

Parasitological examinations were carried out as detailed in 2.6.

5.2.7.5 Post-mortem examinations

Full PM examinations were caried out on all cattle dying during the course of the experiment. The procedure followed was as outlined in 4.2.5.5.

5.2.7.6 Recovery

Recovery was recorded on the day after the last detection of macroschizonts following the return of the rectal temperature to less than 39.5°C.

5.2.7.7 Treatment

The cattle treated are given in 5.2.4 and listed in Table 5.3.

The first treatment with Halofuginone lactate was administered on day 44.

5.2.8 Mixed lymphocyte reactivities between LCL donor and recipient cattle

In order to assess whether or not the recipient cattle had putative Ia type differences with respect to the inoculated cell line cells of S816, MLR assays were performed utilising PBL of the recipients as responder cells to irradiated LCL donor PBL. The reverse MLRs using S816 PBL as responders to each of the recipients irradiated PBL, were also carried out.

PBL were prepared from jugular venous blood collected through 14G hypodermic needles into plastic disposable syringes preloaded with Alsever's solution (described in 6.2.1.2). Sufficient blood was drawn into each syringe to give a 1:1 blood/Alsever's solution mixture. Blood collected in this way served as a source of both responder and stimulator cells.

The separation of PBL on ficoll-hypaque and subsequent washing procedures was as described in detail in 6.2.2.1.2. After washing and counting of viable cells in an aliquot in a Neubauer chamber, responder cells were suspended in complete medium at a concentration of 10^7/ml and 5 ml volumes were added to 25 sq cm area tissue culture flasks (Nunc) and incubated at 37°C for 1-2 hr after which non-adherent cells were removed with the medium. After counting of the cells in the non-adherent fraction, the volume was adjusted to give a cell concentration of 5×10^6 cells/ml.

Stimulator cells were prepared as responder cells but without depletion of adherent cells. After adjusting the concentration of stimulator cells to $5x10^6$ cells/ml, they were irradiated for 7.75 min in a Caesium 137 source to give a radiation dose of 5000R.

100 ul aliquots of stimulator cell suspension were added to 100 ul aliquots of responder cell suspension in 96 well, flat-bottomed plastic tissue culture plates (Linbro) and incubated for 4 days in a 5% carbon dioxide/95% air atmosphere. On the fourth day the wells were each pulsed with a 0.5 uCI dose of Iodine 125-labelled deoxyuridine (Amersham International Ltd.). 25 ul of pulse material was added to each well after appropriate dilution of the labelled nucleotide in culture medium.

Harvesting of the well contents onto glass fibre paper was done on day 5 using an automated multiple sample harvester (Automash, Dynatech). Emissions were quantified by 1 min counts of the paper-adsorbed samples in a Gamma 5500 counter (Beckman).

Individual MLRs were established in duplicate. Background uptake of radiolabel by responding cells was assessed in wells containing 100 ul responder cell suspension and 100 ul complete medium. These control wells were also established in duplicate.

Stimulation indices were calculated as,

$$\frac{\text{counts/min in test wells}}{\text{counts/min in medium control wells}}$$

The medium used for the work was based on RPMI 1640 (Flow Laboratories Ltd.,) with 10% FCS, 25 mM sodium bicarbonate, 10 mM Hepes, $5x10^{-5}$ M 2 mercaptoethanol, 50 ug/ml gentamycin and 2mM L-glutamine.

The stimulation indices and background counts are presented in Table 5.2 and are the means of duplicate assays.

TABLE 5.2

MIXED LYMPHOCYTE REACTIVITIES BETWEEN LCL DONOR (S816) AND RECIPIENT CATTLE

	S816 as stimulator		S816 as responder	
Recipients	Background count *	Stimulation index	Background count *	Stimulation index
S804	6800	2.5	6600	5.5
S806	1875	37.6	6600	8.8
S807	1950	13.4	6600	2.9
S808	485	4.9	6600	11.5
S809	1350	77.8	6600	3,8
S811	8200	4.4	6600	17.2
S812	1300	17.6	1885	11.1
S813	14200	5.2	6600	7.0
S814	27500	3.5	6600	9.2
S815	855	27.5	1270	1.5
S817	6500	3.0	1885	3.4
S818	1600	25.5	1885	19.1
S819	1350	2.6	6600	4.9
S820	436	19.7	1885	6.0
S821	401	7.2	6600	6.1
S822	890	11.7	7850	4.4
S823	746	10.0	7850	2.4
S824	3080	13.7	7850	10.4
S825	650	5.1	6600	7.9
S826	830	3.0	7850	9.2
S828	3100	6.9	7850	7.0
S829	500	17.4	7850	2.6
S830	7280	3.7	7850	2.1
S831	780	10.0	7850	7.5
S832	800	4.9	7850	6.3
S834	675	12.7	7850	4.0
S835	2335	2.1	7850	3.0
S836	560	6.2	7850	5.7
S996	16000	3.5	6600	2.6
S997	5026	4.6	1885	3.9

* "Responder" cells with medium only.

On this basis, S816 is considered to show MLR reactivity with respect to all recipients. Putative Ia-like antigen differences are therefore considered likely between the donor and recipients.

5.3 Results

The data concerning haematological parameters, serological changes with respect to response against macroschizont antigen, and daily rectal temperature recordings are presented in Appendices 2.2, 2.3 and 2.4 respectively.

No significant changes were observed in response to LCL inoculation in any of the animals, with the exception of a slight degree of lymph node hypertrophy which was reflected in a blastogenic response observed in gland smears. The observed response was maximal on the first 2 days that gland smears were examined and had disappeared by day 9. This response occurred in all of the LCL recipients and was not apparently different in degree between the groups of recipients.

The responses of the cattle to stabilate challenge are summarised in Table 5.3. The results of daily observations on parasitosis are presented in Appendix 2.5.

All cattle suffered a severe disease response. On day 44 (day 15 following stabilate challenge), 17 of the cattle were treated for ECF as indicated in Table 5.3. It was considered that all of the treated cattle were undergoing a fatal disease response. Of the untreated cattle, 3 survived the challenge and developed a detectable immune response as measured in the IFAT; all 3 being in the group of 6 matched LCL recipients. None of the untreated 1/2-matched or mismatched LCL recipient cattle survived the challenge, and both challenge control cattle succumbed to infection.

TABLE 5.3

RESPONSE TO STABILATE CHALLENGE

Group	Animal	Treated (day 15)	M	T	P	R	D	IFAT
Matched	S811		8	12	16	29		640
	S817		9	12	15	21		640
	S821		10	14	15	22		10240
	S828		8	9	15		20	-
	S830		10	14	15		20	-
	S832		10/11**	12	15		21	-
w8, ½ matched	S812		7	10	15		17	-
	S815		7	9	15		20	-
	S818	+	7	15	15			
	S820	+	9	12	15			
	S824	+	8/9**	13	15			
	S825	+	7	12	15			
	S831	+	7	12	15			
	S836	+	7	12	15			
w11, ½ matched	S804		7	13	12		21	-
	S807		5	10	15		19	-
	S808*							
	S813	+	7	10	15			
	S819	+	8	10	15			
	S826	+	7	12	15			
	S834	+	8	12	15			
	S835	+	9	12	15			
	S996	+	8	12	15			
	S997	+	10	12	15			
Mismatched	S806		7	11			16	-
	S809		7	11	15		19	-
	S814	+	7	10	15			
	S822	+	8	12	15			
	S823	+	7/8**	15	15			
	S829	+	9/10**	13	15			
Challenge Control	S590		7		13		19	-
	S605		7		15		22	-

M - Number of days following stabilate inoculation to first observed macroschizonts

T - Number of days following stabilate inoculation to first observed pyrexia.

Cont.

TABLE 5.3 CONT. (2)

P - Number of days following stabilate inoculation to first observed piroplasms.

R - Number of days following stabilate inoculation to recovery.

D - Number of days following stabilate inoculation to death.

IFAT - Maximum reciprocal IFAT titre.

* - S808 died as a result of Anaplasmosis on the day of challenge.

** - Macroschizonts observed on the later day; biopsies not examined on the previous day.

There is evidence that the development of parasitosis was slower in the matched group of cattle than in the other groups, as shown in Table 5.4.

When the matched group is compared in this respect with all the other groups on a combined basis, the time to macroschizont detection in the matched group of 9.2 days contrasts with the mean for all the other cattle of 7.5 days. For the purposes of these comparisons, when parasites may have been detectable on the day prior to first observation, should suitable material for examination have been available, the earlier day is used in the calculations.

The delay in development of macroschizont parasitosis in the matched group was not reflected in the time to development of pyrexia or to the time to first observation of piroplasms.

Typically, of the parameters recorded, changes in the levels of leucocytes were the most obvious. On day 15 post-challenge, the mean WBC counts in the matched, w8 1/2-matched, w11 1/2-matched and mismatched groups had fallen to 37%, 31%, 16% and 29% of the levels on the day of challenge, respectively.

A serological response to schizont antigen as measured in the IFAT was observed only in the 3 surviving matched animals (serological responses were observed in the treated cattle, but only subsequent to treatment and these data are not recorded here).

The 3 surviving animals were still alive 5 months after challenge, at no time having received treatment for ECF.

TABLE 5.4

DAYS TO FIRST OBSERVATION OF MACROSCHIZONTS IN THE DRAINAGE LYMPH NODE OF STABILATE - CHALLENGED CATTLE

Group	Mean number of days to macroschizont detection ± S.D.
Matched (6)*	9.2±1
w8, ½ matched (8)	7.4±0.7
w11, ½ matched (9)	7.7±1.4
Mismatched (6)	7.5±0.8
Challenge control (2)	7

* Figures in parenthesis indicate number of cattle in group.

5.4 Discussion

The single LCL used in this study at the 10^6 dose level did not engender protective immunity to the challenge in any of the cattle. However, 3 of 6 animals sharing 2 workshop BoLA specificities with the LCL survived the challenge, albeit after a severe disease response. It is felt that the treated animals in the 1/2 and mismatched groups were undergoing a fatal disease response at the time of treatment. If this is accepted, a survival rate of 3 of 6 in the matched group compares with 0 of 23 in the other groups. If treated cattle are excluded, the 3 of 6 survival rate compares with 0 of 6 for the 1/2 and mismatched groups.

That a degree of immunity was engendered in the matched recipients and not in any of the recipients in the other groups, is supported (apart from the difference in survival rates) by the different character of the responses in these 2 types of recipient. Most significantly, the time taken to develop a detectable degree of parasitosis in the local drainage lymph nodes was longer by 1.5–2 days in the matched group than in any of the other groups. Second, the disease response in the matched animals was not as great as in animals in other groups as indicated by the degree of leucopaenia developed. This parameter is generally considered the most sensitive in assessment of ECF disease response.

The character of the immunity engendered in the matched group should be considered in view of the fact that it was apparently incomplete against the challenge given. The factors of delayed development of parasitosis and less severe leucopaenia in the matched animals may well be connected. The outcome of an ECF disease episode is largely dependent on the speed with which an immune response can

be mounted relative to the speed at which the parasite is able to propagate itself within the tissues ultimately involved in that immune response (Pearson et al., 1979; Morrison et al., 1981). Thus it is not inconceivable that a delayed early development of the parasite in the matched animals was the major factor in enabling 50% of them to develope a response before the lymphoid tissues were irreversibly compromised.

It seems likely that the delay in early parasite development in the matched group was in some way the result of the previous inoculation of the S816 LCL. How this occurred in view of the fact that there was no evidence of parasite transfer from inoculated cells to recipient cells, and further, no evidence of a detectable degree of parasite development as a result of the inoculation in the recipients, is unclear.

The development of cytotoxic effector T lymphocytes in PBL has been correlated with the degree of resistance to stabilate challenge (Emery et al., 1981a) and it has been suggested that the cytotoxic T lymphocyte is one immune effector agent in ECF (Pearson et al., 1979; Eugie & Emery, 1981; Emery et al., 1981a & b). Further, it appears that the parasite, in the process of immunisation, must be "seen" by the immune system on a self background (Emery et al., 1981a); the implication being that the immune response in ECF is, at least in some respects, MHC-restricted.

It is possible therefore that 1 of 2 events (or both) occurred following LCL inoculation into the matched recipients which did not occur in the other recipients. First, there may have been a limited degree of parasite transfer to matched recipient cells sufficient to induce a small effector cell population specific for parasite-altered cell targets. The assumption is that such a low

level transfer would not have been detected with the relatively insensitive techniques used to detect parasitosis. The limited transfer hypothesised could have been the result of a better survival of inoculated cells in the matched recipients than in the other animals, and simply a reflection therefore of a BoLA graft response difference. This accomodates the view of absolute self-restriction in the CTL effector induction phase.

It is also possible however, that no parasite transfer occurred (and hence a significant degree of parasite development was not detected) and that the difference in degree of immunity engendered in the groups was the result of differences in BoLA background between inoculated cells and self cells, but in this instance not in the graft response context but in the immune induction context. This view supposes that an immune response (effector cell population) can be generated against a subsequent infection on a self background (the result of tick or stabilate challenge) directly by inoculated LCL cells providing that a degree of BoLA compatibility exists between inoculated cell and recipient, i.e., the inoculated parasitised cell can induce immunity in the same way as a cell infected in vivo (autologous) without the necessity for parasite transfer. It should be emphasised that this has never been shown to occur in the ECF LCL immunisation system, but the necessary studies have not been carried out to the present time.

Such an hypothesised mechanism of immune induction might also be expected to operate in 1/2-matched LCL/recipient combinations. There was no evidence from this study of development of immunity in the 1/2-matched recipients. However, whatever mechanism was operative in the matched group was clearly at a low level. If it is accepted that a clonal response to parasite/MHC

antigens occurs (Zinkernagel & Doherty, 1974b; Doherty et al., 1976) it could be argued that in view of the limited response in the matched group, it is not surprising that a response was not detected in the 1/2-matched groups.

Whatever the mechanism of induction of immunity in the matched group, it occurred at a low level. Why this should have been the case in this study where 10^6 cells were inoculated into each recipient when in the previous study (MHC 1, Chapter 4) 2 of 3 matched recipients receiving 10^5 cells and 1 of 3 receiving 10^3 cells proved refractory to challenge, should be considered. Moreover the level of immunity in the surviving matched animals in the previous study was sufficient to prevent significant disease responses on stabilate challenge and was apparently reflected, in 2 of the 3 animals concerned, in the development of antimacroschizont antibody responses prior to the stabilate challenge phase of the experiment.

A number of factors are known to be different from those operative in the first experiment (MHC 1). Principal among these is the time to challenge in relation to LCL inculation. In MHC 1, challenge was on day 70 whereas in this experiment challenge was on day 29. If it is accepted that the development of a specific CTL response is indicative of immunity, there is no reason to suppose that the earlier challenge was itself responsible for the poorer resistance conferred, as Eugie & Emery (1981) reported a peak in CTL activity at 14 days after a primary challenge, which had disappeared by day 21. However, Eugie & Emery (1981) were using a different immunisation procedure. Nevertheless it is reasonable to suppose that 29 days is sufficient for the development of an immune response, whatever the immunisation protocol or effector mechanism involved. Other immunisation procedures for ECF have proved effective when

stabilate challenge was effected after 1 month (Radley et al., 1975a).

Second, it is known that the cattle used in MHC 2 did not share either parent with the LCL donor. It is posssible that the matched and autologous recipients in MHC 1 were related, but because of their diverse origin (4.2.1), this would seem unlikely.

MLR responsiveness between line donors and allogeneic recipients in the MHC 1 experiment are not known whereas it is known that the recipients in MHC 2 and the LCL donor were mutually-responsive in all cases. Assuming a reasonable degree of polymorphism at loci controlling MLR, it would not be expected that the differences in response to LCL inoculation in the 2 experiments would be attributable to this factor.

As far as the degree of matching is concerned, and possible differences in this respect to MHC 1, it would appear that, if anything, the matching was better in MHC 2 than in MHC 1 on the basis of serological definition with the BoLA typing panel. It was however possible to obtain a retrospective measure of compatibility between donors and recipients in the first experiment (serological response to cell surface antigens measured with the IIPT) which was not possible in the second experiment. However, by using the 3 surviving matched MHC 2 recipients and a cell line derived from a matched animal which succumbed to challenge in a study of alloreactive cytotoxic cell specificity (reported in detail in Chapter 6) further definition of the w8 and w11 specificities on the cells was possible. This work confirms that at least with respect to cytotoxic cell targetting, there are no significant differences in the w8 and w11 entities concerned, between donor and recipients in MHC 2. As discussed in 6.4, there is some evidence of differences between

animals of the MHC 2 w8/w11 group, possibly involving the products of a second, putative, locus. Such differences however do not correlate with responses to LCL inoculation in MHC 2.

Of all the possible variables which could contribute to the differences in responses to different cell lines in defined BoLA situations, the most likely to be responsible are cell line factors per se (also discussed in 4.4). It is conceivable that different lines vary in performance in any given BoLA situation once inoculated into the recipient. Innate aggressiveness (rate of multiplication, invasiveness etc.,) may be particularly important. Another possible factor in variability is the type of host response invoked by the inoculated cell which could be dependent on the cell phenotype (predominant lymphocyte subpopulation infected) in different cell lines. Studies of such factors are in progress, and hopefully will lead to a better understanding of the cell line/recipient interaction.

In conclusion, the results of MHC 2 support the contention that degree of BoLA compatibility between line and recipient is an important factor in determining the recipient response. Undoubtedly other factors also operate, which at the present time are unclear. With the ability to apply BoLA typing in future experimentation, attention can now be better focussed on these other factors, hopefully resulting in resolution of the problems involved in the development of LCL-derived immunogens for ECF.

The results of this work suggest that immunisation of cattle sharing 1 BoLA w specificity with inoculated LCLs may not be possible with "low" cell dose levels. However, for reasons discussed here the cell line used in this study may be poorly immunogenic in all BoLA defined situations. It is possible that at the 10^6 dose

level other lines may successfully immunise BoLA 1/2-matched recipients.

CHAPTER 6 A study of target specificity of bovine alloreactive cytotoxic cells

6.1 Introduction

As discussed in detail in 1.1.2, attempts to date to define the bovine MHC have concentrated on the use of alloantisera raised against BoLA specificities although there are some published results of attempts to define putative Ia-equivalent (class II) specificities using lymphocyte activation studies (MLR). There are no published results of investigations using alloreactive cytotoxic cells.

An investigation of *in vitro*-generated alloreactive cytotoxic cell specificity was felt to be of importance for a number of reasons. First, to establish that agreed SD specificities show homology with those of other species in being important targets for alloreactive CTL (Klein *et al.*, 1981). Second, to establish the fundamental principle of MHC restriction in a bovine system (this has not been achieved hitherto) and by implication demonstrate that already defined BoLA specificities are restrictive elements in cytotoxic cell effector function. This would have relevance for any studies of the bovine immune system which must recognise the role played by the MHC in the immune response. Third, to establish a system of possible use in the further definition of BoLA specificities.

Considerations of possible MHC restriction are of particular relevance to further studies of the immune response in ECF. It has not been possible to demonstrate that currently recognised humoral responses play a protective role in the ECF syndrome (Wagner *et al.*, 1974; Emery *et al.*, 1981a; and reviewed by Wilde, 1967). Further it has not been possible to demonstrate the

presence of parasite-induced antigens on the surface of macroschizont-infected cells with serological techniques (Duffus et al., 1978; Creemers, 1981) and it is the intracellular macroschizont stage of the life cycle which is believed to be responsible for the principal pathogenesis of the disease (Wilde, 1967).

However, evidence for the fact that cell-mediated immune mechanisms are associated with immunity to ECF is furnished by the results of Emery (1981). In this work immunity was passively transferred between cotwins with thoracic duct lymphocytes. Consistent with this, the only immune mechanism which has been demonstrated to have some association with immunity to disease is the CTL effector mechanism. Thus Pearson et al., (1979, 1982) were able to generate, in vitro, CTL for T.parva-infected LCLs, but only from PBL of immune and not naive animals. Eugie & Emery (1981) considered that the generation in vivo of CTL was associated with immunity and the work of Emery et al., (1981a) suggests a correlation between in vivo CTL generation and resistance to the disease.

MHC restriction of ECF-associated CTL function has not been demonstrated and indeed in the absence of a BoLA typing capability it was impossible for workers in this field to do so. It is suggested however, by the self-restriction shown by CTL whereby in vivo generation results in a population of effector cells restricted to the autologous LCL when several cell lines are used as targets in vitro (Eugie & Emery, 1981). The results of Pearson et al., (1979, 1982) suggest that in vitro-generated CTL also have an autologous preference although it was not as marked as that reported by Eugie & Emery (1981).

For these reasons, elucidation of the role played by

MHC-coded antigens on the surface of T.parva-infected lymphoblastoid cells in the immune response is of immediate concern. To achieve this the antigens themselves must be definable, and in particular, those of relevance to CTL effector function.

This study was undertaken as a first step in this direction.

6.2 Materials and methods

6.2.1 Media

6.2.1.1 Culture medium

The medium used in this work was based on RPMI 1640 (Flow Laboratories Ltd.). The RPMI 1640 with phenol red was prepared from powder with DDW. To this was added sodium bicarbonate (2.5 g/L) and Hepes (Flow Laboratories Ltd.,) to give a final concentration of 10 mM. Following adjustment to pH 6.8 with N HCl, the RPMI 1640 basal medium was filter-sterilised through 0.22 um pore size GS type filters (Millipore Corporation) and stored in sealed glass bottles at 4°C until required. Immediately prior to use, foetal calf serum of selected batches (Flow Laboratories Ltd.,) was added to a final concentration of 10%. Also added were 2 mercaptoethanol to a final concentration of 5×10^{-5} M, gentamycin (to 50 ug/ml) and L-glutamine (to 2mM) (all supplied by Flow Laboratories Ltd.).

6.2.1.2 Alsevers solution

This was prepared from DDW by the addition of,

citric acid	550 mg/L
glucose	20500 mg/L
sodium chloride	4200 mg/L
sodium citrate	8000 mg/L

The solution was autoclaved and stored at 4°C until required.

6.2.2 Cell-mediated lympholysis

6.2.2.1 Preparation of responder cells for mixed lymphocyte cultures

6.2.2.1.1 from defibrinated blood

Venous blood (50 ml samples) from the jugular vein were collected aseptically through sterile 14G hypodermic needles into 60 ml sterile plastic disposable syringes. These were immediately emptied into sterile 250 ml conical flasks containing sufficient glass beads to cover the bottoms of the flasks. 50 ml of blood was added to each flask. The flasks were rotated manually to keep the beads in motion until a recognisable discrete clot was formed (10-15 min agitation). The defibrinated blood was removed into sterile plastic tissue culture flasks (Nunc).

Lymphocyte separation was achieved by layering 5 ml of the defibrinated blood onto 5 ml ficoll 400/diatrizoate sodium with EDTA (Ficoll-Paque, Pharmacia Fine Chemicals Ltd.,) in plastic conical centrifuge tubes (Sterilin) of 10 ml nominal volume. These were centrifuged at 1200g/30 min.

The leucocyte layer at, and just over, the plasma/ficoll

interface, was harvested into plastic conical centrifuge tubes (nominal volume 10 ml), so that each tube contained not more than 5 ml of the harvested material. Tubes were topped up with RPMI 1640, closed, inverted several times and centrifuged at 300g/10 min.

The supernatant over the pelleted cells was poured off and the cells resuspended by gentle pipetting in 1.5 ml RPMI 1640. The tubes were again topped up with RPMI 1640 and the contents mixed by repeated inversion. Following centrifugation at 150g/10 min the supernatents were again poured off leaving the cell pellets. These were resuspended in an appropriate volume of complete medium and aliquots from individual donors were pooled. Viable cells in an aliquot were counted in a Neubauer chamber following dilution in trypan blue medium (0.5% trypan blue in RPMI 1640) and complete medium was added to the cell suspensions to give a concentration of $5x10^6$/ml.

6.2.2.1.2 from non-defibrinated blood

Venous blood from the jugular vein was collected aseptically through 14G hypodermic needles into plastic syringes preloaded with Alsever's solution to give a 1/1, blood/Alsever's solution mixture.

The separation of lymphocytes using density gradient centrifugation was as for the defibrinated samples (6.2.2.1.1).

Following harvesting of the cells from the density gradient, the tubes were topped up with Alsever's solution, inverted several times and centrifuged at 300g/10 min. After discarding the supernatant, the cells were resuspended in 1.5 ml Alsever's solution. The tubes were filled with further Alsever's solution, mixed and centrifuged at 150g/10 min. The cells were resuspended and the

process repeated twice more to remove platelets.

Following the third wash, the pellets were resuspended in complete medium, appropriately pooled and counted and adjusted to a cell concentration of $5x10^6$/ml, as previously described.

All cell preparation procedures were carried out aseptically using sterile reagents and containers. Manipulations were done at room temperature (20-25°C). Centrifugations were carried out at 20°C.

6.2.2.2 Preparation of stimulator cells for MLC

Stimulator cells for MLC were obtained from blood collected into Alsever's solution as described in 6.2.2.1.2.

6.2.2.3 Mixed lymphocyte culture (MLC)

Stimulator cells were irradiated with a Caesium source (Cs137) for 7.75 min to give a radiation dose of 5000R. The culture suspensions were placed into the radiation chamber in sterile polythene tubes or plastic tissue culture flasks (Nunc).

1 ml aliquots of stimulator cell suspensions ($5x10^6$/ml) were added to 1 ml aliquots of responder cell suspensions ($5x10^6$/ml) in 24 well (3.5 ml volume/16 mm diameter) flat-bottomed tissue culture plates (Linbro) and incubated at 37°C in a 5% carbon dioxide/95% air atmosphere for 6 days.

6.2.2.4 Cell-mediated lymphocytotoxicity assay (CML)

A chromium isotope (Cr51) release assay with *in vitro* MLC-generated effector cells and Cr51-labelled target cells was used.

Cells in 24 well plates (2 ml cocultures, prepared as described in 6.2.2.3) were resuspended in the wells by gentle

pipetting, harvested, and relevant wells pooled in 50 ml polythene tubes. These were centrifuged at 300g/10 min, the supernatant over the cell pellet poured off, and the cells resuspended in an appropriate volume of complete medium. Viable cells in aliquots of cell suspensions were counted in Neubauer chambers after dilution in trypan blue medium and complete medium was added to the suspensions to give a cell concentration of 4, 2 or $1x10^7$/ml.

T.parva parva (Muguga)-infected LCLs were used as sources of target cells. The methods of establishment and maintenance are described in 2.2 and 2.3. Cultures were used in log phase growth (24 hr after a 1:10 passage) when cell concentrations were approximately 10^6/ml. The contents of the culture flasks were taken into conical centrifuge tubes and centrifuged at 300g for 10 min. The supernatants were poured off the cell pellets and the cells resuspended in an appropriate volume of RPMI 1640 with added foetal calf serum (20%). Viable cells were counted as previously described and the cell concentration of targets was adjusted to $2x10^7$/ml. An equal volume of isotope in medium (Cr51 as sodium chromate, Amersham International Ltd., added to RPMI 1640 to give a 1 mCi/ml solution) was added to the cell suspensions. Labelling was carried out in plastic conical centrifuge tubes with a 60 min incubation at 37°C, during which time the tubes were agitated 2-3 times.

Following incubation, the tubes were topped up with complete medium and centrifuged at 300g for 10 min. The supernatants were poured off the cell pellets which were then resuspended in complete medium and again centrifuged at 300g/10 min. The washing process was repeated twice after which the volumes of suspensions were adjusted to give a cell concentration of 10^6/ml in complete medium, based on prelabelling counts.

The BoLA types of LCLs were assessed at least once (as described in 2.1) after passage 4 and prior to use as targets. BoLA workshop specificities were as for fresh PBL from the respective donors.

The basic assay was performed by adding 50 ul target cell suspension to 200 ul effector cell suspension ($4x10^7$/ml for a 160/1 effector/target ratio, $2x10^7$/ml for an 80/1 ratio, 10^7/ml for 40/1, $5x10^6$/ml for 20/1, $2.5x10^6$/ml for 10/1, and $1.25x10^6$/ml for 5/1) in individual wells of a 96 well, flat-bottomed tissue culture plate (Linbro).

All assays were done in duplicate, i.e., each effector/target combination at each effector/target (E/T) ratio.

50 ul of each target cell suspension was also added to 200 ul of tap water in Beckman tubes and also to 200 ul complete medium in the plates, in triplicate in each case. In early experiments water lysis controls were established in the plates in triplicate.

The plates were incubated at 37°C in a 5% carbon dioxide/95% air atmosphere for 4 hr, after which the cells and debris were resuspended by gentle pipetting. The plates were then centrifuged (300g/10 min) and 125 ul supernatant from each well was taken into an individual polycarbonate counting tube.

During the incubation of the plates, the Beckman tubes containing the targets in tap water were snap frozen in liquid nitrogen and thawed to room temperature, twice. When incubation of the plates was completed, the Beckman tubes were centrifuged in a Beckman microcentrifuge to pellet debris and 125 ul of supernatant removed for gamma counting with the supernatants from the plates.

Cr51 release during the assay was assessed by counting the emissions from the supernatant aliquots over a period of 1 min in a Beckman Gamma 5500 gamma counter.

Percentage chromium release (and hence, degree of cytotoxicity in the assay) was calculated as,

$$\frac{(\text{cpm, test supernatant} - \text{cpm, medium control supernatant})}{\text{cpm, freeze thaw supernatant} - \text{cpm medium control supernatant.}} \times 100$$

cpm = counts per minute.

6.2.2.5 Cold target blocking in CML

The method was as for the basic CML assay (described in 6.2.2.4) with the exception that effector cells were added to the wells in 100 ul unit volumes. Cold targets (unlabelled with isotope) at the appropriate concentration in complete medium were also added in 100 ul unit volumes. The preparation of cold targets from tissue culture material was as described for the "hot" (isotope-labelled) targets with the exception of the labelling procedure which was omitted.

Cold/hot target ratios used were 20/1, 10/1, 5/1, 2.5/1, 1.25/1. Following washing after removal from tissue culture flasks, the concentrations of cold target cells in complete medium were adjusted appropriately, i.e., 10^7/ml for a 20/1 cold/hot ratio, etc..

When combinations of 2 cold targets were used in individual assays, a 20/1 cold/hot ratio implies an effective ratio of 10/1 for each cold target and correspondingly for other cold/hot ratios.

All individual blocking assays were performed in duplicate.

6.2.3 Study protocol

A series of 11 experiments were performed which fall into 3 groups. All involved investigations of the specificity of *in vitro*-generated cytotoxic cells.

6.2.3.1 Experiments A1-6

These form the basis of the study and were designed to demonstrate that currently-defined workshop class I BoLA specificities are restrictive targets for alloreactive cytotoxic cells.

4 donor animals of responder cells (ultimately, effector cells) were used, of different BoLA types. A variety of stimulator PBL and targets were used although most stimulator PBL were obtained from cattle previously used in experiment MHC 2 (described in detail in chapter 5).

Cell lines for use as targets, with the exception of S816TpM, were established and maintained specifically for this work by Mr D.A.Stagg of the Veterinary Research Department, Muguga, by methods described in 2.2 and 2.3. All were transformed by *T.parva parva* (Muguga). S816TpM was an extant cell line previously studied for its *Theileria*-associated immunogenicity in MHC 2. Some of the cattle used as donors for stimulator PBL and target LCLs in this work had therefore previously been inoculated with S816TpM LCL cells (10^6 cell inoculum).

None of the responder cell donors had previously experienced a *Theileria* infection as judged by negative serology in the IFAT for antimacroschizont antibodies (Burridge & Kimber, 1972), or been inoculated with material, infected or otherwise, from stimulator cell donors. This applies to all 11 experiments reported

here.

6.2.3.2 Experiments B1-3

These experiments were performed to study cytotoxic cell capability of effector cells generated in MLCs of BoLA-matched responder/stimulator combinations. The BoLA matching implies sharing of 2 workshop-defined specificities by responder and stimulator combinations. For reasons discussed in 1.1.2 such combinations can be considered to have identity for both BoLA-A locus alleles, as defined by the Edinburgh panel of typing reagents which includes sera accepted as defining at workshop level.

The specificities studied were w8 and w11, as these alleles were both detectable in individuals of a substantial group of cattle, most of which were survivors of previous studies (MHC 2).

6.2.3.3 Experiments C1 and 2

In these 2 experiments, specificity of cytotoxic cell targeting was assessed by cold target inhibition with a panel of cold targets matched, 1/2-matched and mismatched with the respective "hot" targets. In experiment C2, combinations of 2, 1/2-matched targets were used to give both relevant workshop specificities on different cell backgrounds in individual assays i.e., this constitutes a contrived, matched blocking situation.

The experiments were again based on the w8 and w11 specificities.

The responder, stimulator and target combinations for all experiments are summarised in Table 6.1, where the BoLA workshop specificities are also detailed. The reactivities of the donor cattle and LCLs with the full panel of typing reagents, are given in

TABLE 6.1

RESPONDER/STIMULATOR COMBINATIONS IN MLC GENERATION OF CYTOTOXIC CELLS, AND TARGETS FOR CML ASSAYS. BoLA WORKSHOP SPECIFICITIES (w) OF THE PBL AND LCL TARGETS ARE ALSO SHOWN

Experiment	Responder			Stimulator			Target		
A1	S191	w7	w10	S814	w13	w20	S814	w13	w20
				S816	w8	w11	S816	w8	w11
				S821	w8	w11	S821	w8	w11
A2	B487	w5	w20	S814	w13	w20	S814	w13	w20
				S821	w8	w11	S821	w8	w11
A3	B807	w1	w6	S821	w8	w11	S821	w8	w11
				B470	w6	w7	B470	w6	w7
A4	S191	w7	w10	S811	w8	w11	S811	w8	w11
				S821	w8	w11	S821	w8	w11
							S816	w8	w11
							S817	w8	w11
							S828	w8	w11
							S814	w13	w20
							S823	w10	w13
							S829	w13	w16
							B470	w6	w7
							B166	w4	w10
A5	S191	w7	w10	S811	w8	w11	S811	w8	w11
				S821	w8	w11	S821	w8	w11
							S803	w8	w13
							S815	w8	w13
							S818	w8	w6
							S836	w8	w13
							S804	w11	w16
							S807	w11	w13
							S819	w11	w16
							S834	w11	w6
							S823	w10	w13
							S829	w13	w16
							B166	w4	w10
A6	B470	w6	w7	S811	w8	w11	S811	w8	w11
				S821	w8	w11	S821	w8	w11
							S816	w8	w11
							S817	w8	w11
							S828	w8	w11
							S803	w8	w13
							S815	w8	w13
							S818	w8	w6
							S836	w8	w13

cont.

TABLE 6.1 CONT. (2)

Experiment	Responder			Stimulator			Target		
							S804	w11	w16
							S807	w11	w13
							S819	w11	w16
							S834	w11	w6
							S814	w13	w20
							S823	w10	w13
							S829	w13	w16
							S191	w7	w10
							B166	w4	w10
B1	S995	w8	w11	S811	w8	w11	S811	w8	w11
				S817	w8	w11	S817	w8	w11
							S821	w8	w11
							S816	w8	w11
							S828	w8	w11
							S829	w13	w16
	S995	w8	w11	S811	w8	w11	*S191	w7	w10
	S995	w8	w11	S811	w8	w11	*B487	w5	w20
B2	S816	w8	w11	S811	w8	w11	S811	w8	w11
				S821	w8	w11	S821	w8	w11
							S817	w8	w11
							S828	w8	w11
							S814	w13	w20
							B470	w6	w7
							B487	w5	w20
							B807	w1	w6
	S816	w8	w11	S191	w7	w10	*S191	w7	w10
	S816	w8	w11	S191	w7	w10	*B470	w6	w7
B3	S995	w8	w11	S811	w8	w11	S811	w8	w11
				S821	w8	w11	S821	w8	w11
							S816	w8	w11
							S817	w8	w11
							S828	w8	w11
							S823	w10	w13
							S829	w13	w16
							S191	w7	w10
	S995	w8	w11	S191	w7	w10	*S191	w7	w10
	S995	w8	w11	S191	w7	w10	*S828	w8	w11
	S995	w8	w11	S191	w7	w10	*S829	w13	w16

cont.

TABLE 6.1 CONT. (3)

Experiment	Responder	Stimulator	Target	"Cold" target
C1	S191 w7 w10	S816 w8 w11	S816 w8 w11	-
				S816 w8 w11
				S821 w8 w11
				S836 w8 w13
				S834 w11 w6
				S829 w13 w13
			S814 w13 w20	-
C2	S191 w7 w10	S821 w8 w11	S821 w6 w11	-
				S821 w8 w11
				S811 w8 w11
				S816 w8 w11
				S817 w8 w11
				S828 w8 w11
				S818 w8 w6
				S836 w8 w13
				S807 w11 w13
				S834 w11 w6
				S814 w13 w20
				S829 w13 w16
				S807 & S818
				S836 & S834
	S191 w7 w10	S821	S829 w13 w16	

* Target used in assay of cytotoxicity generated in the MLC combination specifically stated. Otherwise, within experiments all targets were used for all responder/stimulator combinations.

Appendix 3.1.

The responder cells from the S191 donor were prepared from defibrinated blood (6.2.2.1.1). All other responder and stimulator cells were prepared from blood collected into Alsever's solution (6.2.2.1.2).

6.3 Results

The results of all experiments are summarised diagrammatically in figures 6.1-6.9. The data from which these figures are derived (counts of emmissions in assay supernatents) are presented in Appendix 3.2.

6.3.1 Experiment A1

Results are summarised in fig. 6.1.

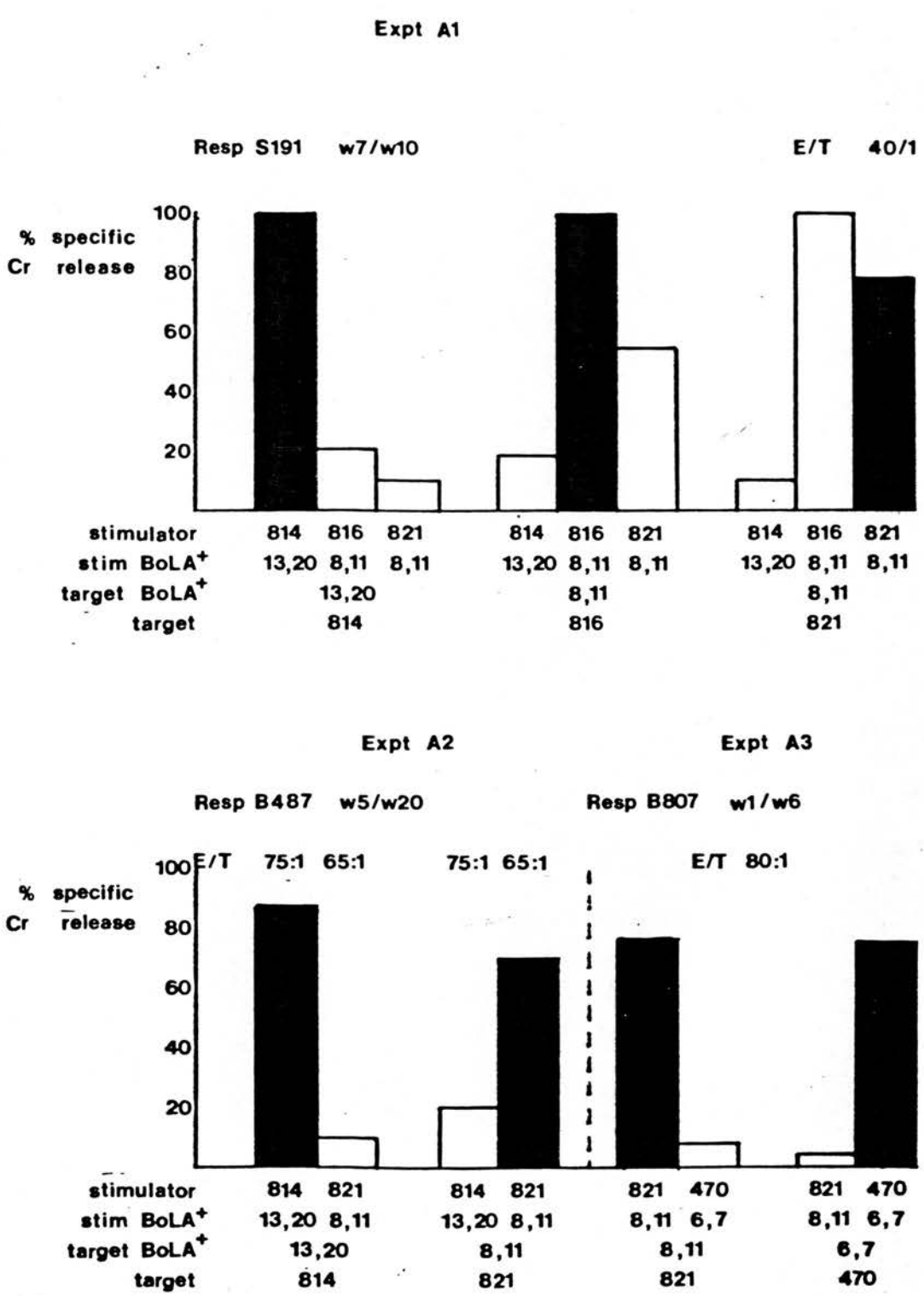
The responder S191 was used in 3 MLC situations. Two stimulators were matched to each other at the BoLA-A locus (w8/w11) and one was of a different BoLA type (w13/w20).

In this experiment a control in the form of culture of S191 responder cells with no allogeneic stimulator cells was included (MLC established with S191 cells plus medium alone). These cells were assayed for effector function subsequently, together with the allostimulated S191 responder cells. LCLs from all 3 stimulator donors were used as targets for all responder populations.

It is clear that the cytotoxicity generated distinguished the w8/w11 cells from the w13/w20 cell, and there was absolute cross-reactivity between the w8/w11 cells. Significant cytotoxicity was not generated in the absence of stimulator cells.

Although maximum cytotoxicities are recorded as 100%, they were in fact greater than this. For this reason, the water lysis

FIG. 6.1



+ w specificities

control was replaced by a freeze/thaw control in subsequent work.

6.3.2 Experiment A2

Results are summarised in fig. 6.1.

A second responder, B487 (w5/w20) was used with a single w8/w11 and the w13/w20 stimulator used in A1. The responder was 1/2-matched through the w20 specificity with one stimulator.

Cytotoxicity again clearly distinguished the 2 stimulator cell types.

6.3.3 Experiment A3

Results are summarised in fig. 6.1.

A third responder of a third BoLA type was used, again with a w8/w11 stimulator and with a third BoLA type stimulator (w6/w7). In this latter case, there was sharing of the w6 allele by the responder and stimulator.

The stimulator types were distinguished, and the cytotoxicity in the 1/2-match MLC situation was as good as that in the mismatch situation.

6.3.4 Experiments A4 & A5

Results are summarised in figs. 6.2 and 6.3.

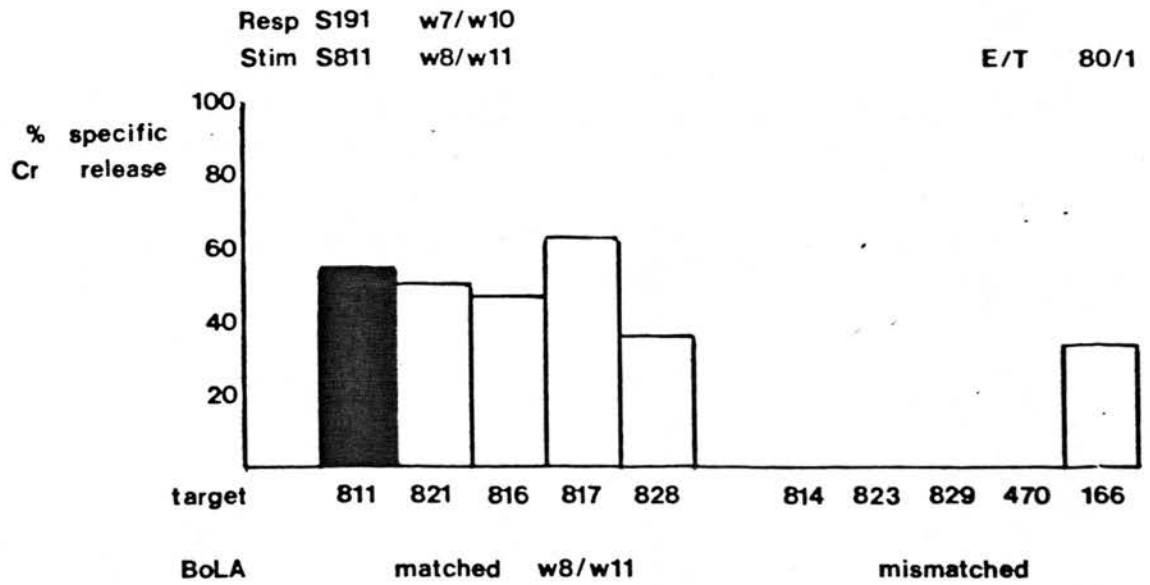
The responder S191 was cocultured with 2 stimulator cells, both of w8/w11 phenotype. The MLC-generated cytotoxicity in the 2 populations was assayed on a panel of w8/w11, w8 1/2-matched, w11 1/2-matched and mismatched targets. The 1/2-matched targets were used in A5 and the full panel of w8/w11 targets in A4.

The results show that,

1. By comparison with mismatched targets, cytotoxicity was

FIG. 6.2

Expt A4



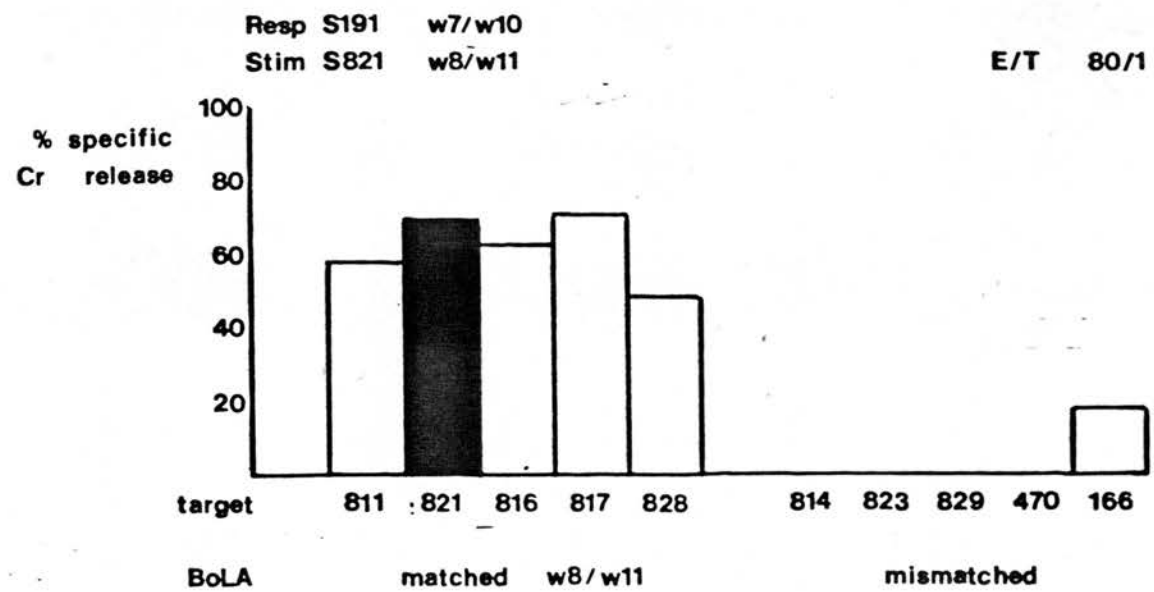
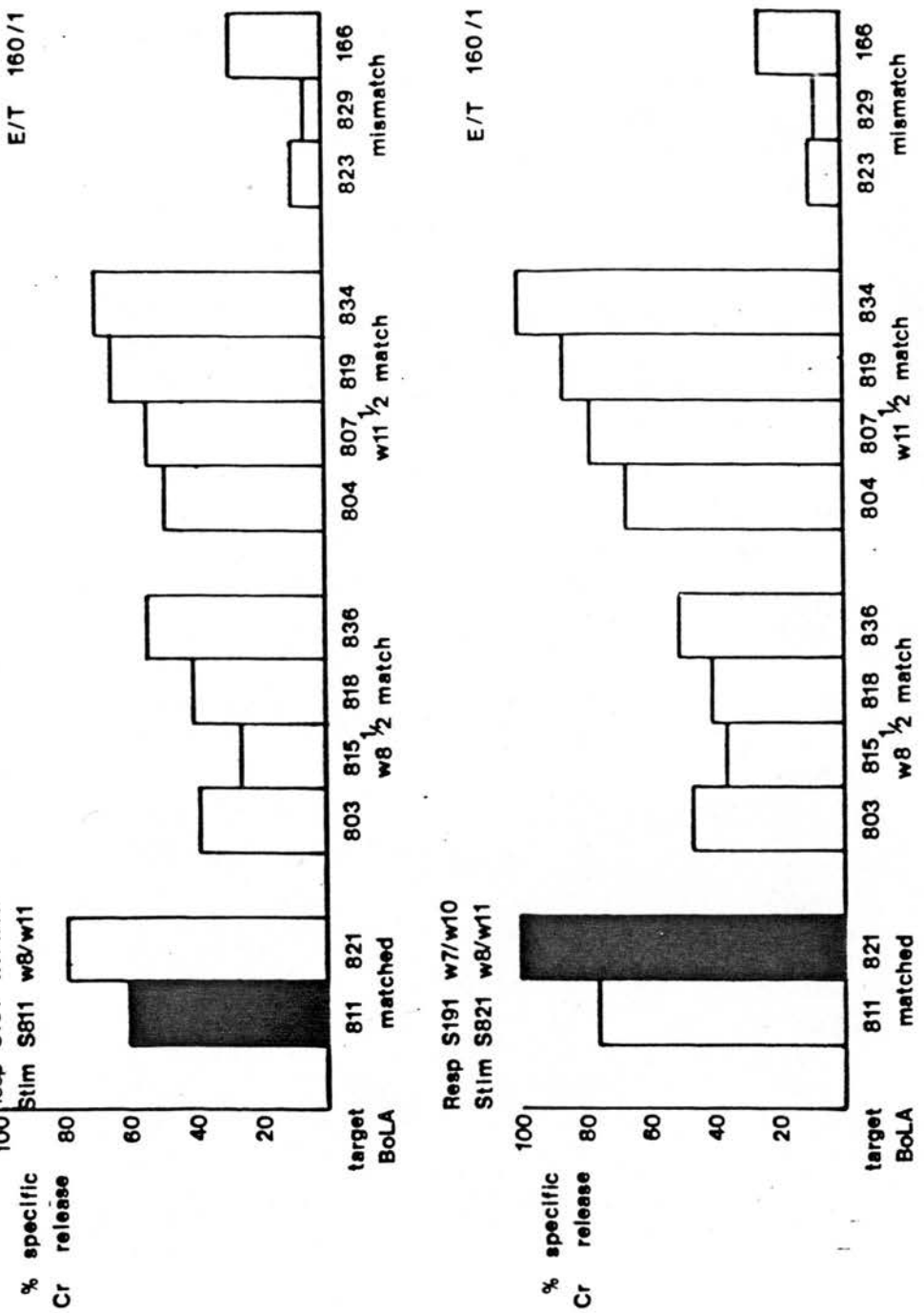


FIG. 6.3
Expt A5



apparent with all w8/w11 and 1/2-matched targets.

2. Cytotoxicity generated in the S191/S821 MLC was greater on all matched and 1/2-matched targets (mean 66%) than cytotoxicity generated in the S191/S811 MLC (mean, 52%), with one exception.

3. Both responder populations gave greater cytotoxicity with w11 1/2-matched targets than w8 1/2-matched targets. Thus the mean cytotoxicities were 40% and 44% with the S191/S811 and S191/S821 combinations respectively on w8 1/2-matched targets, and 59% and 83% with w11 targets. The difference was clearly greater with the S191/S821 population than with the S191/S811 population.

4. The cytotoxicities achieved on w11 1/2-matched targets approached closely those achieved on w8/w11 targets and in some individual cases, exceeded the cytotoxicity achieved on the S811 target with both effector cells. Mean cytotoxicities in A5 on w8/w11 targets were 70% and 88% with the S191/S811 and S191/S821 effector populations respectively, and on the w11 1/2-matched targets, these were 59% and 83% respectively.

These results again reflect the better recognition of w11 1/2-matched targets by S191/S821 effectors than S191/S811 effectors.

5. Of 8 mismatched targets used in the 2 experiments, there was no significant cytotoxicity in 7 cases. The exception was the B166 target in both experiments. Mean cytotoxicities on the B166 target in A4 and A5 were 31% with the S191/S811 effector and 22% with the S191/S821 effector. It is interesting to note that exceptionally, the S191/S811 effector produced greater cytotoxicity in both experiments on this target than the S191/S821 effector.

B166 is unique among the mismatched targets in having reactivity for panel sera ED13 and ED63 (2 of the 3 w8~defining sera).

The degree of cytotoxicity was somewhat less than with the 1/2~matched targets.

6.3.5 Experiment A6

Results are summarised in figs 6.4/1 and 6.4/2.

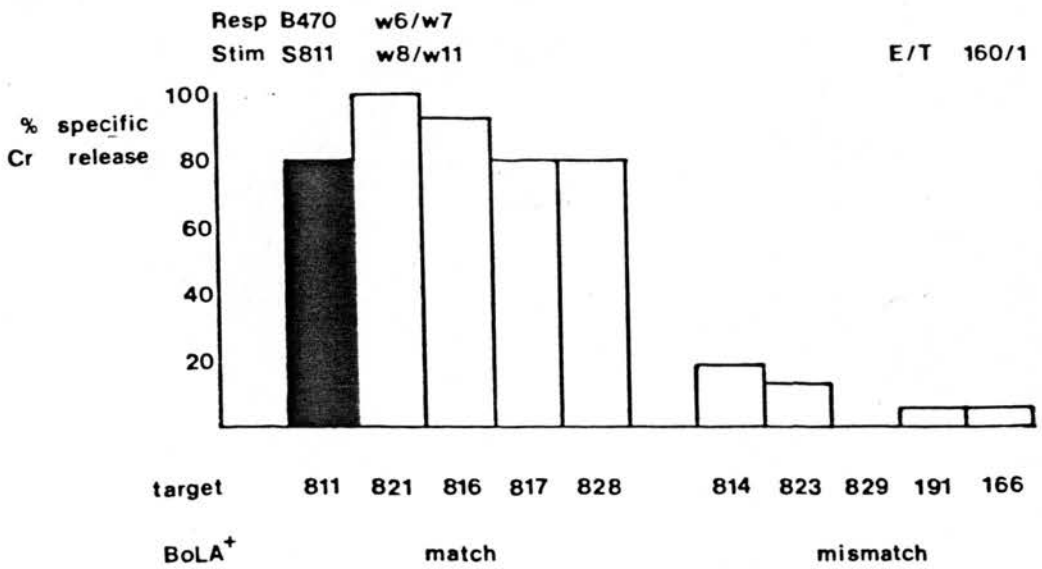
This experiment used essentially the same panel of targets (differing in only 1 mismatch target) and the same stimulators as A4 and A5; the responder in this case being B470, replacing S191. One target, S818 was 1/2~matched at the w6 allele with the responder.

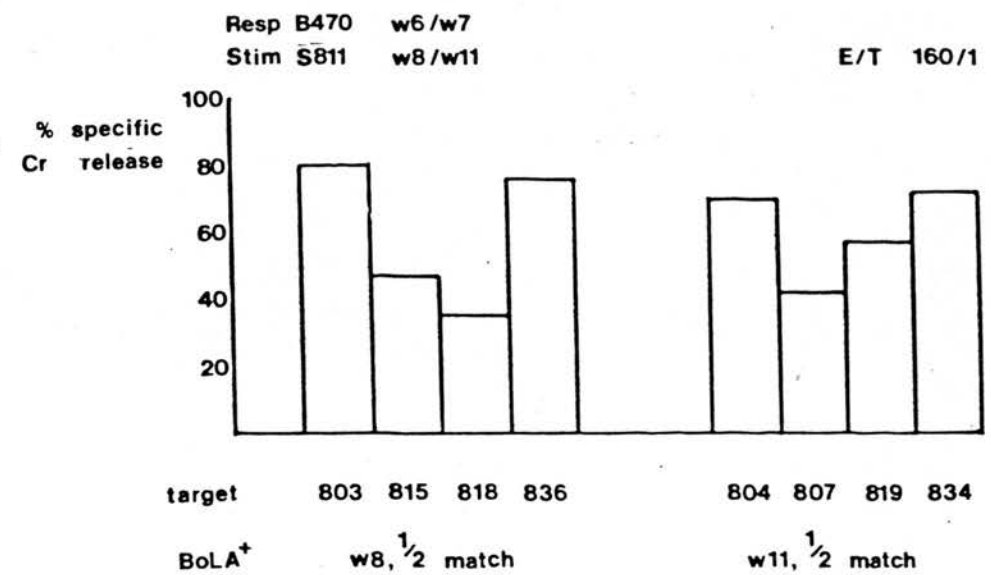
The results show,

1. Matched and 1/2~matched targets were clearly distinguished from mismatched targets.
2. In all cases, cytotoxicity was greater with matched than 1/2~matched targets. The B470/S811 effector gave a mean cytotoxicity of 87% on matched and 59% on 1/2~matched targets. The B470/S821 effector gave 85% and 51% respectively.
3. There was no significant difference between the 2 effectors in degree of cytotoxicity developed with matched targets although it was slightly greater with the B470/S811 effector (70%, as opposed to 64% with the B470/S821 effector).
4. Cytotoxicity on the B166 target was not significant. This contrasts with the situation in A4 and A5 with the S191 responder.
5. The only mismatched target to give a significant cytotoxicity was S814TpM. This was significantly less than that obtained with all matched and 1/2~matched targets.

FIG. 6.4/1

Expt A6

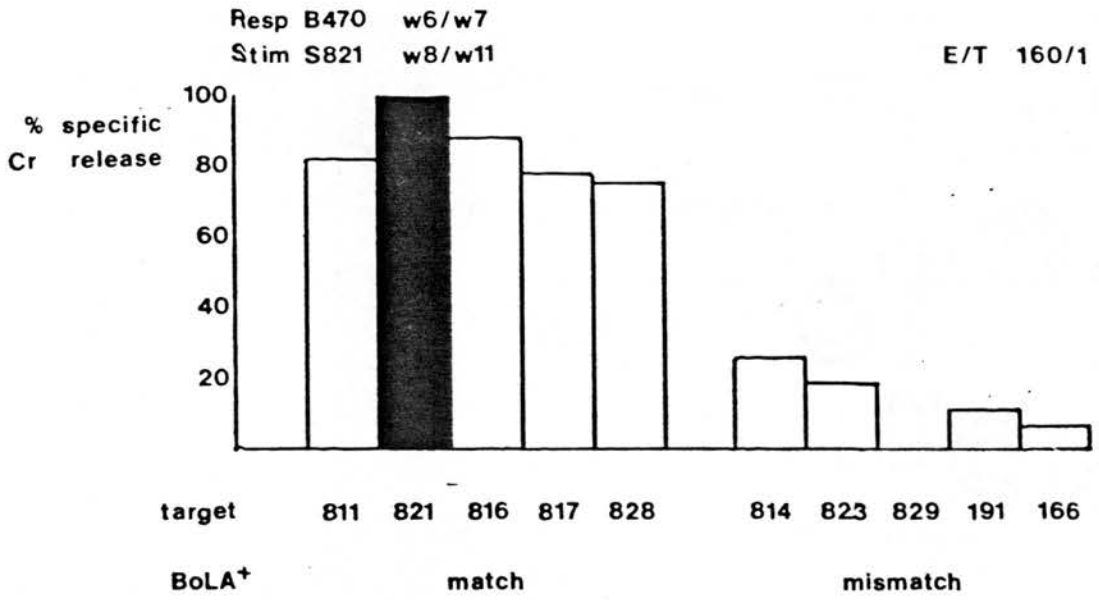


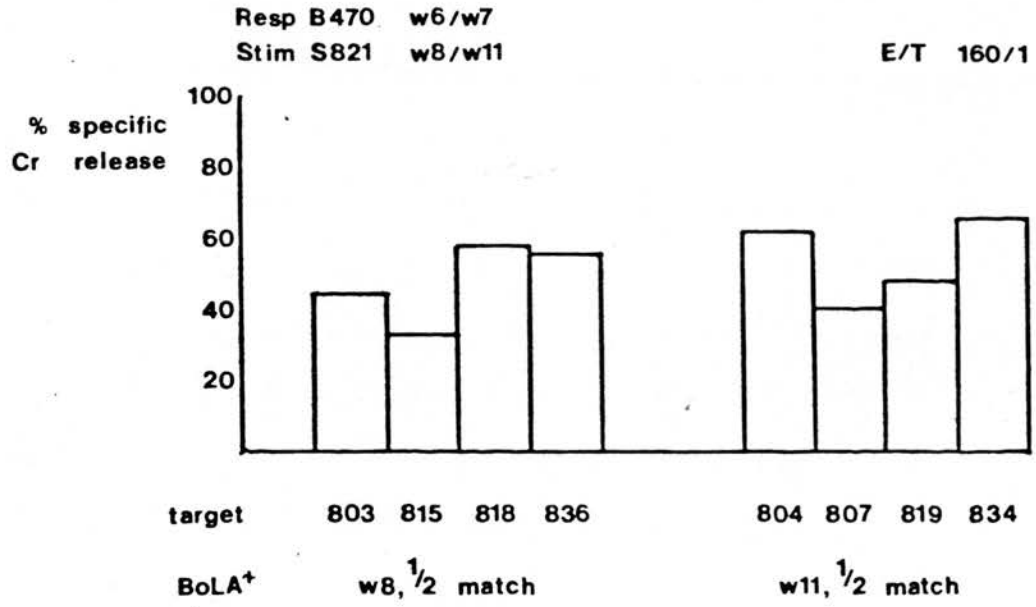


+ target/stimulator relationship

FIG. 6.4/2

Expt A6





\+ target/stimulator relationship

6. There was no significant difference between the w11 and w8 1/2-matched targets although the tendency was for a slightly greater cytotoxicity with w11 than w8, shown by both effectors (mean 58% for w11 and 53% for w8).

6.3.6 Experiment B1

Results are summarised in fig. 6.5.

The aim of the B series of experiments was to study cytotoxicity should it be developed, resulting from coculture of cells matched for both the w8 and w11 specificities.

It is clear that cytotoxicity can be developed in this situation.

In the first experiment of the series, using 2 effector populations resulting from coculture of S995 with S811 and S817, the pattern of cytotoxicity produced by the 2 effectors was the same. Significant cytotoxicity (mean, 52%) was produced with w8/w11 targets S811, S817 and S828 with both effectors. There was no cytotoxicity with the w8/w11 targets S816 or S821 or with mismatched targets S191 and S829.

A limited degree of cytotoxicity (15%) was recorded with mismatched target B487 (w5/w20).

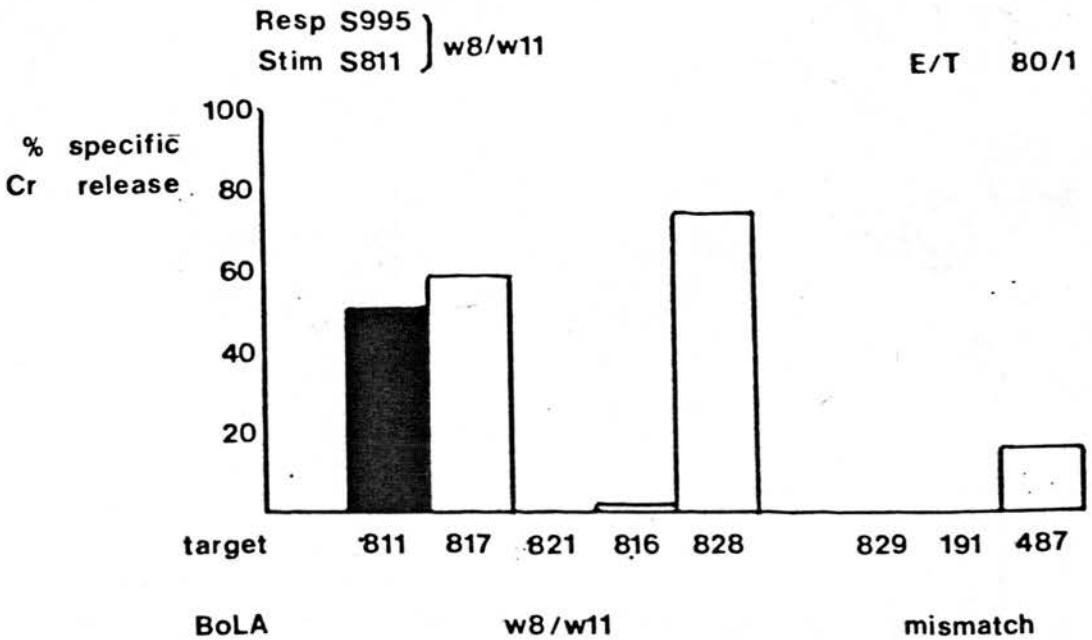
6.3.7 Experiment B2

Results are summarised in fig. 6.6.

Using responder S816 (w8/w11) and stimulators S811 and S821, it is clear that the S816/S811 effector gave the same pattern of cytotoxicity on matched targets as seen with both effectors in B1. Thus, mean cytotoxicity with targets 811, 817 and 828 was 96%. Further, cytotoxicity with mismatched target B487 is clearly shown at

FIG. 6.5

Expt B1



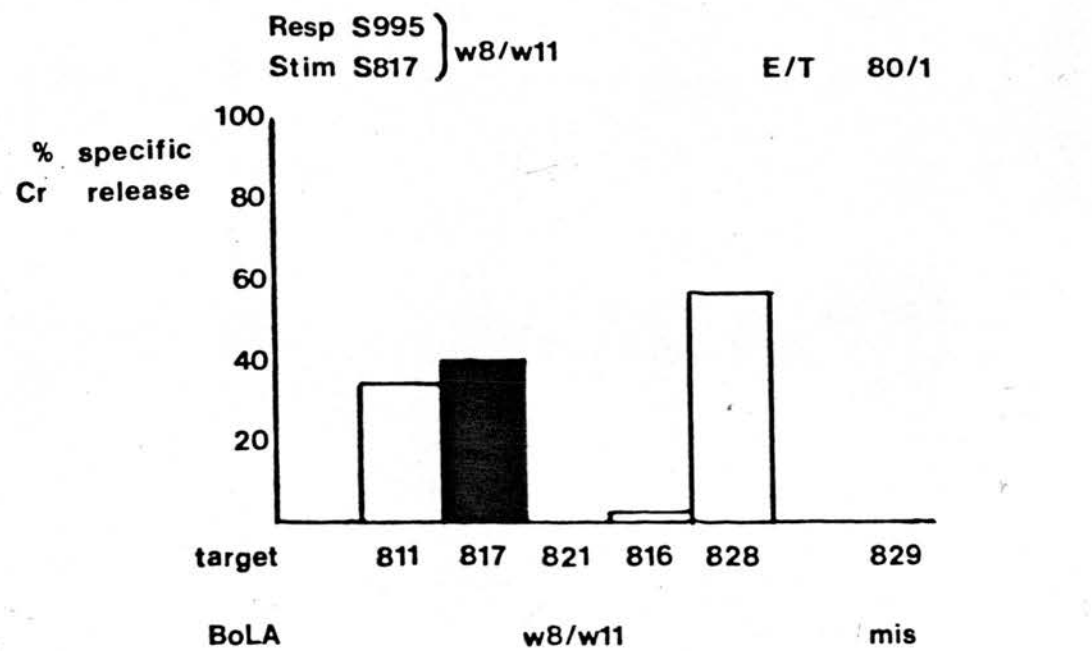
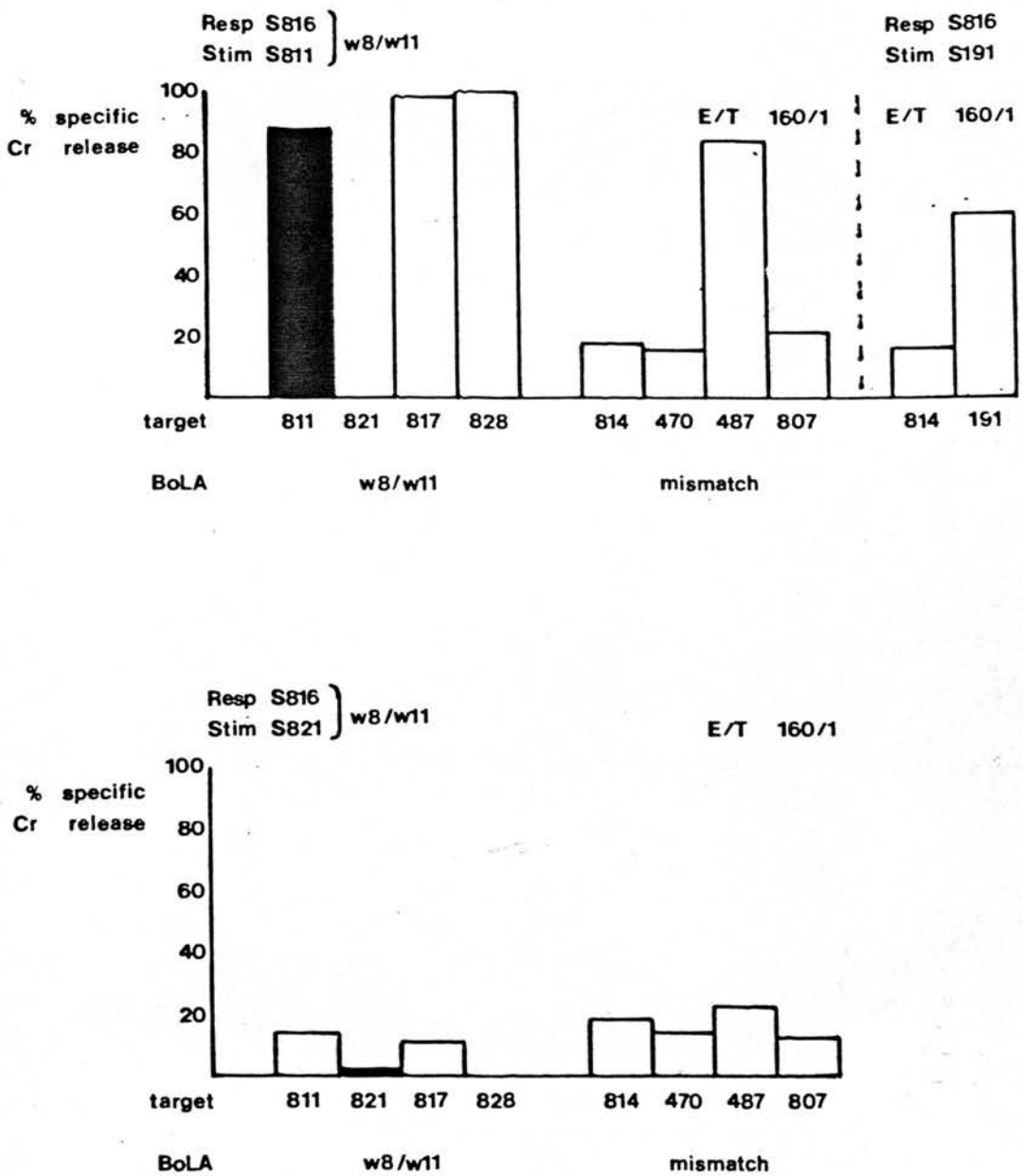


FIG. 6.6


Expt B2
Resp S816
Stim S811
w8/w11
Resp S816
Stim S191
% specific
Cr release
100
80
60
40
20
E/T 160/1
E/T 160/1
target
811 821 817 828
814 470 487 807
814 191
BoLA
w8/w11
mismatch
Resp S816
Stim S821
w8/w11
E/T 160/1
% specific
Cr release
100
80
60
40
20
target
811 821 817 828
814 470 487 807
BoLA
w8/w11
mismatch

85%. There was no cytotoxicity with the S816/S811 effector on the S821 target (the S816 target not being used in this experiment).

The S816/S821 effector produced no significant cytotoxicity.

With the exception of B487, all mismatched targets gave low or insignificant levels of cytotoxicity.

6.3.8 Experiment B3

Results are summarised in fig. 6.7.

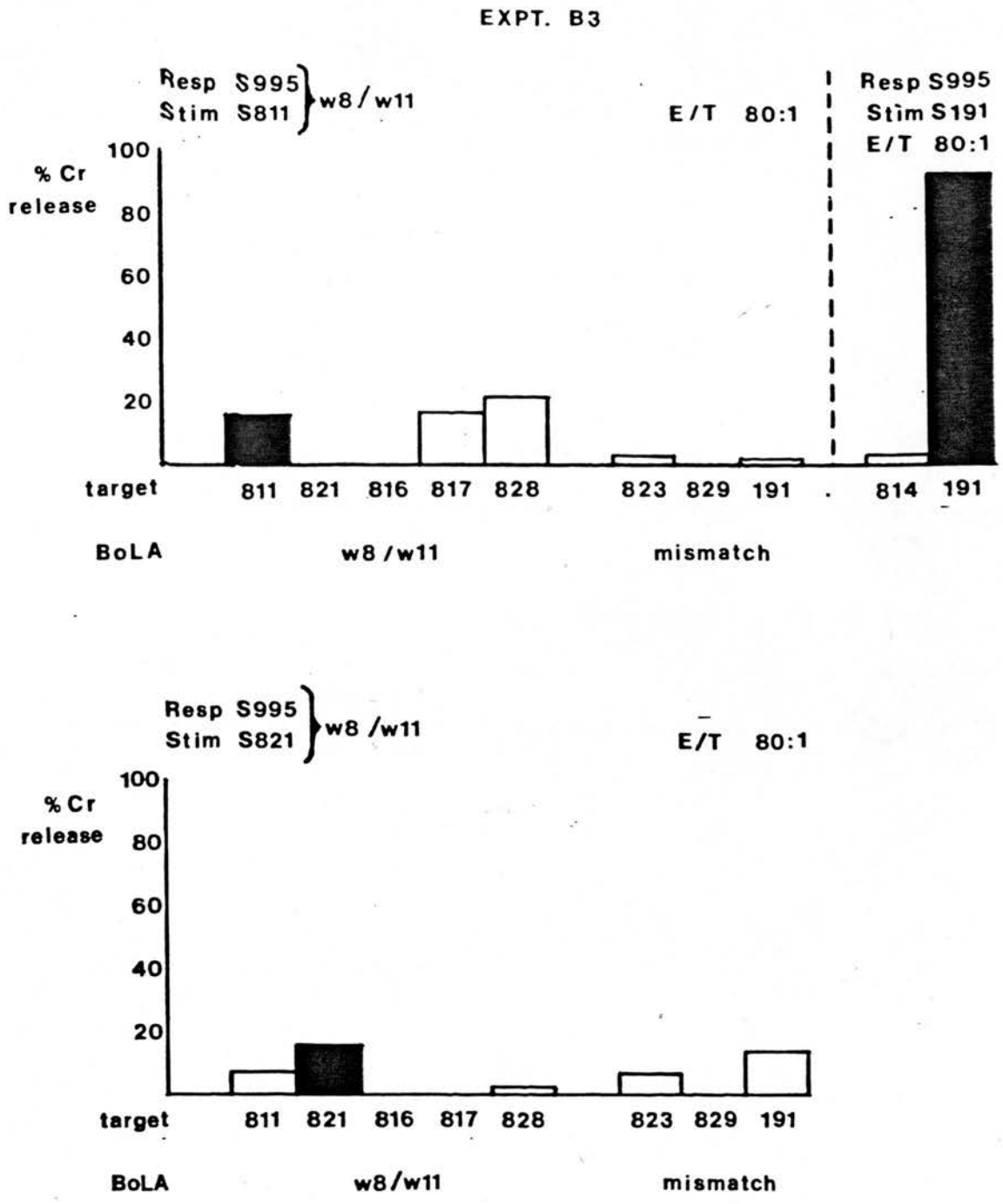
Levels of cytotoxicity generated in this experiment were low. However, it is felt that some of these were significant in view of the clear differences in cytotoxicities achieved by the different effectors on identical targets.

S811 and S821 were used as stimulators for the matched responder, S995. The patterns of cytotoxicity produced by the 2 effectors were distinct. Thus the S995/S811 effector is cytotoxic for w8/w11 targets S811, S817 and S828 and not for targets S816 and S821. This is the same pattern as seen in B1 with the S995/S811 and S995/S817 effectors and in B2 with the S816/S811 effector.

The S995/S821 effector gave significant cytotoxicity with the autologous S821 target and possibly with the S191 target (w7/W10) only.

No other significant levels of cytotoxicity were generated.

FIG. 6.7


EXPT. B3
Resp S995
Stim S811
w8/w11
E/T 80:1
Resp S995
Stim S191
E/T 80:1
% Cr release
100
80
60
40
20
target
811 821 816 817 828 823 829 191 814 191
BoLA
w8/w11
mismatch
Resp S995
Stim S821
w8/w11
E/T 80:1
% Cr release
100
80
60
40
20
target
811 821 816 817 828 823 829 191
BoLA
w8/w11
mismatch

6.3.9 Experiment C1

In this first cold target blocking study, results are recorded and considered for the 40:1 effector target ratio (the highest used) and for cold/hot ratios of 10:1, 5:1, 2.5:1 and 1.25:1. The effector was from the S191 responder/S816 stimulator combination and results in inhibition assays are based on the autologous S816 target. In the unblocked situation, a 38% cytotoxicity was achieved which compares with a 5% cytotoxicity on the mismatched S814 target.

The levels of cytotoxicity in the unblocked and blocking situations (cold/hot ratio of 10:1) are summarised in fig. 6.8. The percentage levels of blocking at all cold/hot ratios are summarised in Table 6.2.

It is evident that,

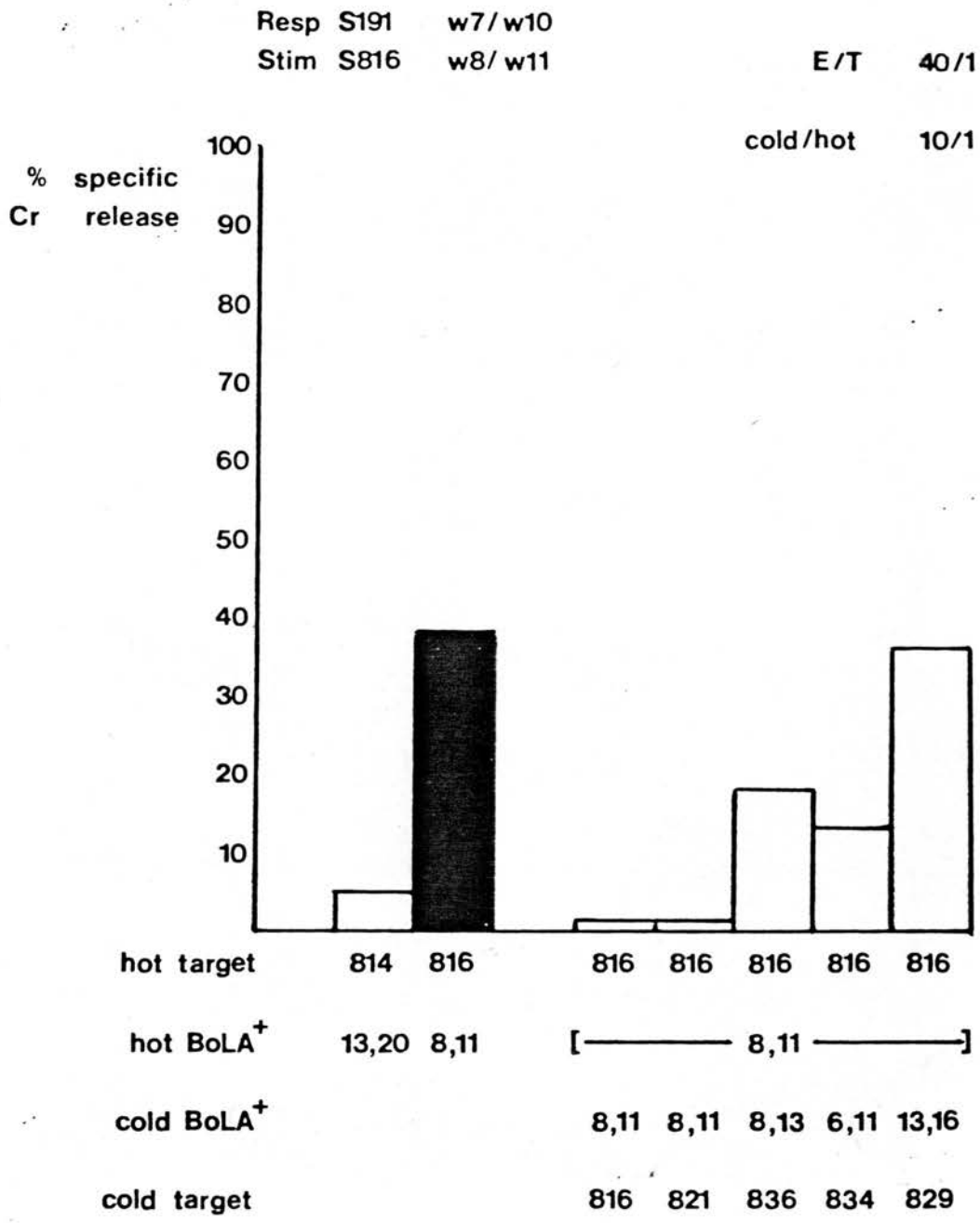
1. No significant blocking was achieved with the mismatched S829 cold target.
2. The w8/w11 cold targets produced more blocking at all levels than 1/2-matched cold targets.
3. There was no significant difference between the w8/w11 cold targets in the degree of inhibition produced. At 3 levels however, the matched cold target S821, gave more blocking than the autologous cold target.

6.3.10 Experiment C2

In this cold target inhibition assay, the same responder was used as in C1 with a different stimulator, S821, but of the w8/w11 type as previously. A larger range of cold targets was used, i.e., 5 matched, 2 of each 1/2-match, and 2 mismatch. Additionally, the 1/2-match targets were used in 2 combinations of w8 and

FIG. 6.8

Expt C1



+ w specificities

TABLE 6.2

EXPERIMENT C1: INHIBITION OF CYTOTOXICITY OF Cr^{51} LABELLED TARGETS (HOT) BY UNLABELLED TARGETS (COLD) AT AN EFFECTOR/TARGET RATIO OF 40:1, EXPRESSED IN PERCENTAGES

	Cold Target				
Cold/Hot ratio	S816	S821	S836	S834	S829
10:1	92	92	53	66	5
5:1	76	76	32	66	0
2.5:1	50	66	26	45	0
1.25:1	32	47	8	26	0

$$\% \text{ inhibition} = \frac{\% \text{ Cr release in control assay} - \% \text{ Cr release with cold target}}{\% \text{ Cr release in control assay}} \times 100$$

Hot Target - S816

w11-bearing cells.

Cold/hot ratios of 20:1, 10:1, 5:1 and 2.5:1 were used with an effector/target ratio of 80:1. In combinations of 1/2-matched blocking cells, each cell was added to the assay at 1/2 the cold/hot ratio stipulated.

Table 6.3 summarises inhibition levels and fig. 6.9 represents diagrammatically the levels of cytotoxicities achieved in the unblocked and blocking situations at the 20:1 cold/hot ratio.

The results reflect and extend those obtained in C1.

Thus,

1. At all levels, inhibition was greater with matched targets (mean value) than with 1/2-match targets (mean value).
2. Significant levels of inhibition were achieved with all matched targets.
3. The w11 1/2-matched targets at all levels, resulted in greater inhibition than the w8 1/2-matched targets, giving on average 22% greater blocking and therefore approximately twice the inhibitory effect. These figures correlate closely with those in C1.
4. The 1/2-match combinations of cold targets resulted in degrees of inhibition very close to those achieved with matched cold targets. At the 20:1 cold/hot ratio the combinations gave greater inhibition than the mean level for the matched targets.
5. A reflection of the result reported in 3 above, is that in certain circumstances, the w11 1/2-match targets gave greater inhibition than individual matched cold targets.
6. There were clear differences between cold, matched targets in the degree of inhibition produced, with the highest level of inhibition occurring in the autologous situation.

TABLE 6.3

EXPERIMENT C2: INHIBITION OF CYTOTOXICITY OF Cr^{51} LABELLED TARGETS BY UNLABELLED TARGETS AT AN EFFECTOR/TARGET RATIO OF 80:1 EXPRESSED AS PERCENTAGES

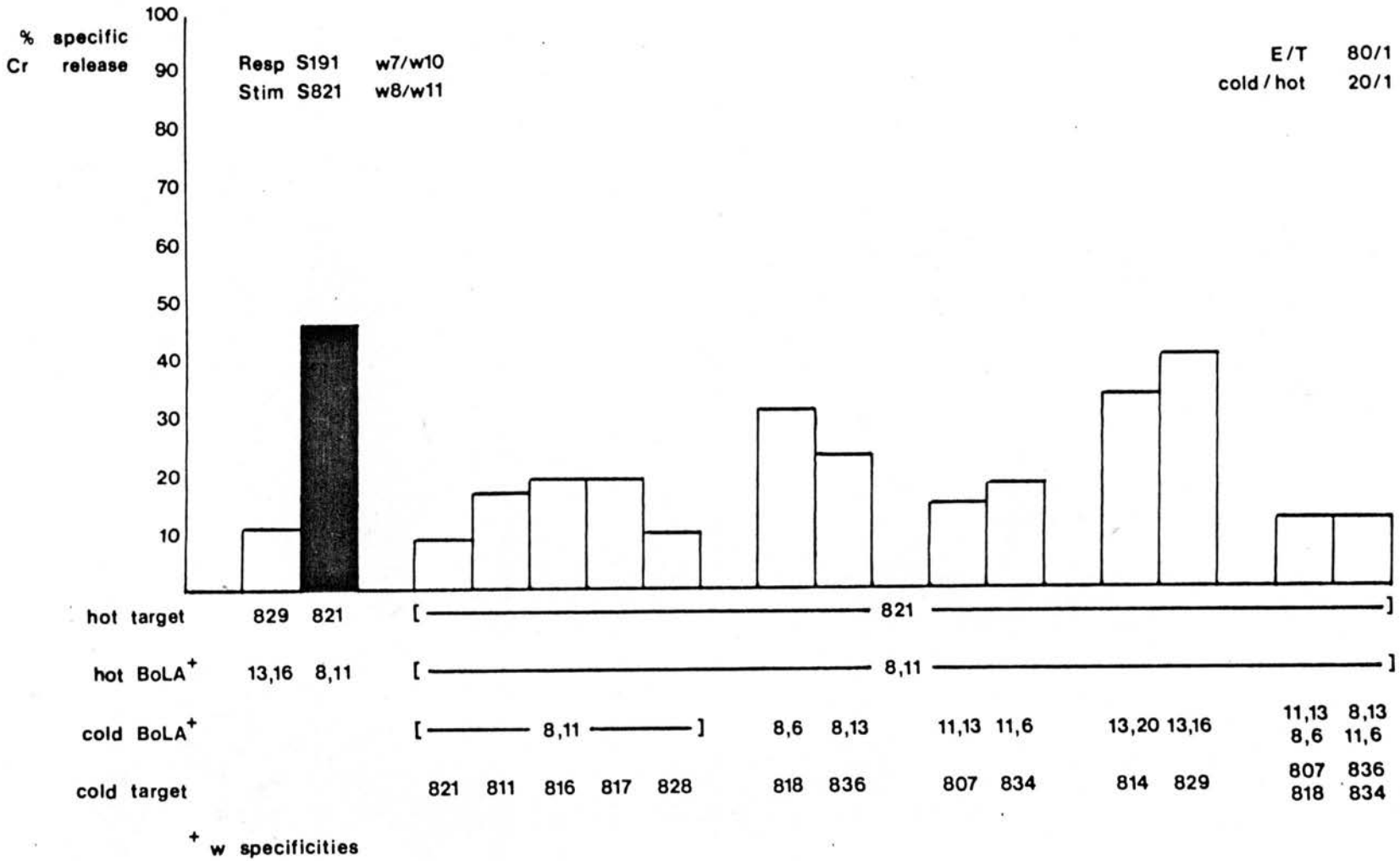
	Cold Target				
Cold/Hot ratio	821	811	816	817	828
20:1	80	63	59	59	78
10:1	63	54	41	61	70
5:1	72	48	39	52	61
2.5:1	35	48	28	37	43

	Cold Target					
Cold/Hot ratio	807	834	818	836	807 +818	834 +836
20:1	67	61	33	50	84	74
10:1	54	46	27	27	59	52
5:1	46	28	26	9	35	43
2.5:1	46	24	15	27	26	28

For calculation of % inhibition, see Table 6.2

Hot Target - 821

FIG. 6.9
Expt C2



6.4 Discussion

In the first series of experiments, 4 responding cells were used of 4 different BoLA phenotypes which between them expressed 6 different BoLA workshop-specificities of the currently-recognised 15. Five stimulator cells were used of 4 BoLA phenotypes representing 6 BoLA specificities. In 2 responder/stimulator combinations, there was sharing of 1 BoLA specificity by the cocultured cells in the cytotoxic cell generation.

All the BoLA phenotypes of stimulator cells were clearly discernible on the basis of susceptibility to cytotoxic cell activity generated in all responder cells. Further, it would seem that only partial differences between responder and stimulator cells, in the 2 cases studied (with the w7 and w13 specificities not shared), does not prejudice the generation of a cytotoxic cell population, and in such cases the level of cytotoxicity developed is of the same order as that produced in mismatched combinations.

The results of experiments A4, 5 and 6, in which targets sharing a single w specificity with stimulator cells were used, suggest that populations of cytotoxic cells are generated in this system which require recognition of only single w specificities on target cells for effect. This is consistent with the clonal response reported in other systems where cytotoxic cells are generated against autologous and allogeneic transformed lines bearing virus antigens; the responding clones being specific for single MHC-coded specificities and virus antigen (Zinkernagel & Doherty, 1974b, 1975) and thus showing dual recognition and classical MHC restriction.

The development of techniques for establishing cloned cytotoxic T lymphocyte lines in human and mouse systems has now placed beyond doubt the fact that a clonal response with respect to

MHC specificity occurs. This work has been done using virus-infected (Townsend et al., 1983) and hapten-modified autologous cells (Siliciano et al., 1983) and allogeneic, "unmodified" cells (Teh et al., 1978a; von Boehmer & Turton, 1983) as stimulator and target cells where the in vitro generation has been both primary and secondary.

It could be argued that the cytotoxic cells generated in the experiments reported here may have been specific for the stimulator specificities on the basis of requiring recognition of stimulator specificities either singly (but both being recognisable) or together. The evidence agaist this is based on the cytotoxicity result with the 1/2-matched targets. In this instance, levels of cytotoxicity are almost invariably less than with matched targets. The most likely explanation for this is that fewer cells are acting in an effector capacity in the 1/2 match than in the full match situation. This is strongly supported by the results of the cold target inhibition experiments in which 1/2-matched cold targets, in most instances, result in a lower level of blocking in any given defined situation than fully-matched cold targets.

In all these experiments, the target specificities were w8 and w11, and the evidence is that at least 2 populations of effector cells are generated against w8/w11 cells by mismatched responders. The possibilty that more are generated against such cells cannot be excluded.

The question of the existence of 1 or more other loci controlling cytotoxic cell specificity may be considered in the context of the results reported here. All of the matched and 1/2-matched animals used in these studies originated from the same source. A degree of haploidentity in this group cannot be excluded.

However the group was the progeny of 5 sires, and certainly a larger number of dams. Three of the 5 sires are of the w11/w13 phenotype, 1 is w8/w13 and the fifth (siring only S816) is w8/w11, w8/wx or w11/wx. There is no correlation in the A and C series of experiments between sire and susceptibility to cytotoxicity or cold target inhibitory activity. This fact, allied with the unlikelihood of any significant haploidentity in the group for reasons of parentage, suggests that the w8 and w11 specificities are recognised as discrete entities in these studies. It does not exclude the possibility that specificities coded for at other putative loci are recognised in the same way. It would seem however, that the responders S191 and B470 used in this work generated cytotoxicity principally against the w8 and w11 specificities on all 3 w8/w11 stimulator cells used (having 3 different sires) and if against other specificities, only in a minor way.

There would appear to be quantitative differences between the responders S191 and B470 in their responses to different w8/w11 cells and to the 2 specificities. Thus S191 evidenced a clearer "preference" for S821 cells over S811 cells as stimulators on each occasion studied. This was not so apparent with the B470 responder. Both responders produced greater degrees of cytotoxicity against the w11 specificity than the w8 specificity, although again this was more obvious in the case of S191. The apparently greater antigenicity of w11 over w8 with S191 was clearly reflected in the cold target inhibition assays from which it would appear that there was a two-fold greater effect with w11 than w8. The possibility of effects of other loci again cannot be excluded, but for reasons already outlined, would seem to be unlikely. In addition to this, the results of combination blocking with different w8 and w11 cells are

not consistent with effects at other loci. In this instance the stimulator for S191 derived neither haplotype from the same source as any of the 1/2-matched blockers.

A qualitative difference in the response of S191 and B470 is evidenced by the different cytotoxic effects on target B166 following stimulation with w8/w11 cells. A degree of recognition of B166 was achieved by S191 but not B470. B166 expresses some serologically detectable cross-reactivity with w8. Why this should be recognised by one responder and not another is not clear, although such results do suggest that this system may offer a particularly sensitive way of further defining BoLA class I specificities. It is apparent that in other systems cytotoxic T lymphocyte clones are indeed capable of exquisite specificity (Krangel et al., 1983). It would be expected that cloning would greatly facilitate studies in this area in the bovine system.

The results of the B series of experiments lend support to the view that in vitro-generated cytotoxic cells may have a use in antigen definition in the bovine system. In these experiments w8/w11 matched stimulator/responder combinations were used to generate cytotoxic cells and in these circumstances there is some reason to believe that a second locus may be controlling cytotoxicity, when it is generated. The results suggest that 3 of the w8/w11 animals form a group distinguishable from the other w8/w11 cattle; the 3 being S811, S817 and S828. Cytotoxicity generated by both S995 and S816 was apparent with these 3 but not with S821 or S816 (S995 was not used as a target). Further, this cytotoxicity was also evident with the B487 target (w5/w20).

The fact that cytotoxicity generated in w8/w11-matched situations is evident with the w5/w20 target as well as with some,

but not all, w8/w11 targets, is perhaps the strongest evidence for non BoLA-A locus control in these circumstances. It is reasonable to suppose that a public component on w8 or w11 would be shared by other w8/w11 cells as well as cells of different A locus type.

To investigate this further, a number of approaches could be adopted which include identifying the haplotypes involved in the w8/w11 animals by using 1/2-matched targets where the shared haplotypes are of common origin with respect to the haplotypes in the S811, S817, S828 group (these are the progeny of 3 different sires). Second, other w5/w20 targets could be used with effectors produced in the appropriate matched-generation system, and third, a larger panel of other mismatched BoLA types could be used.

Cross-immunisations between the different w8/w11 "types" might enable serological definition of the differences observed with the alloreactive cytotoxic cells.

For reasons already discussed, in those mismatch responder/stimulator combinations studied, cytotoxicity generated appeared to be directed against the products of the A locus alleles with no detectable activity directed against the products of other MHC regions. It is possible that had B487 been used as a target in these circumstances, cytotoxicity would have been detected. However it is also possible that in mismatch generation combinations in the system studied, immunodominance of A locus products over the products of other putative loci occurs, and that such second locus products are only stimulatory to a detectable degree where A locus products are shared by stimulator and responder.

Finally, it can be concluded that the objectives of this study as outlined in 6.1, have been achieved and the opportunity now exists to extend this work to further definition of the bovine MHC

and for further studies on the cell-mediated immune response in cattle, with a sound BoLA-orientated approach. This will obviously greatly benifit from the establishment of cloned cytotoxic cell lines in the bovine system, which is the logical next step in furthering this line of research.

CHAPTER 7 Summary and conclusions

The work reported in this thesis in large part involved the application of a BoLA typing capability to two related problems in the field of ECF immunisation with Theileria parva-infected lymphoblastoid cell lines. Discussion of immunisation against, and chemotherapeutic control of, ECF in general terms places the LCL approach in context.

Until relatively recently it has not been possible to reliably and safely treat infected cattle. Chemotherapeutic agents are however now becoming available (reviewed by Dolan, 1981) and will undoubtedly have a role to play in ECF control. As with most chemotherapeutic agents with most infections, they will not control the infection on every occasion and this may even be the case when multiple treatments are effected. Further, in an ECF outbreak, particularly in an extensive livestock rearing situation, which is frequently the norm in East Africa, the disease is likely to take a toll not only in terms of deaths directly attributable to T.parva infection, but also in terms of lost production even when chemotherapy is instituted optimally and at the first sign of disease. Not infrequently treated and recovered cattle are severely checked for a considerable period (personal observation). By the time cattle show clinical signs the infection process is well advanced and as a result, in the absence of access to laboratory diagnostic facilities, chemotherapy on a whole herd basis must be considered which is not only expensive in terms of antibiotics but also in terms of the associated effort involved.

The chemotherapeutics have, and are being used as

components of certain immmunisation regimens (the so-called "infection and treatment" methods, reviewed by Radley, 1981). In the original system, cattle are infected by inoculation with cryopreserved infected tick material and simultaneously inoculated with the chemotherapeutic agent (long-acting formulations of certain tetracyclines). This method has been shown to induce immunity to the homologous parasite isolate in a high proportion of cattle although the incidence of "breakthroughs", i.e., where the chemotherapeutic does not prevent the development of frank disease resulting from the parasite inoculation, is a slight cause for concern.

The newer chemotherapeutic agents, particularly of the quinone and quinazolinone types, may find applications in "infection and treatment" approaches and as a result of their better anti-Theilerial activity, may prove safer than the tetracylines. They may also be administered several days following infection rather than on the same day and one can speculate that as a result, immunogenesis may be improved.

As discussed in detail in 1.2.3, LCL-based immunisation systems have been the subject of a considerable research effort from the time that in vitro culture of infected cells was first reported. Despite this, a field-applicable technique is not available and among the reasons for this, and probably the most important at the present time, is the requirement for very large numbers of cells to achieve immunisation in the majority of recipients and an unacceptably high death rate directly attributable to cell line-induced infections at these dose levels.

Interest in Theileria-infected LCLs has however been maintained despite disappointing results in immmunisation trials and the fact that infection and treatment has greater

field-applicability, for several reasons. First, there is as yet no ideal means of vaccinating against the disease. Second, and related to the first reason, presently available immunisation systems necessarily rely on inducing infection with field isolates. This gives rise to a number of problems, the most important of which is the induction of the carrier state and other dangers inherent in any system which can potentially produce the disease.

The production of a non-infective immunogen would be a major advance in ECF control. There is no single obvious candidate at the present time. Inactivated sporozoite preparations are a possibility but despite efforts to produce an effective vaccine based on non-infective tick-derived material of this type, the vaccine has not so far been forthcoming.

The other principal possible source of non-infective immunogens is the infected lymphoid cell. As discussed in 1.2.2, parasite-associated antigens are induced on these cells although nothing is known of their nature at the present time. Their existence is confirmed by the ability of infected cells to induce lymphocyte activation *in vitro* and a cytotoxic cell response *in vitro* and *in vivo*. Moreover, there is limited evidence that the generation of CTL is correlated with resistance to subsequent challenge. It is for these reasons that the surface of infected lymphoid cells may be expected to eventually yield effective immunogens in preparations devoid of infective parasite material.

The third reason for continued interest in the *Theileria*-infected LCL is that it provides a useful subject for use in studies of the bovine cell-mediated immune response *per se*. The genus *Theileria* offers transforming agents for bovine cells which are readily available and with the appropriate technology, relatively

easy to apply.

In chapters 4 and 5 of this presentation, results are reported of experiments in which a BoLA typing component was introduced into studies of response to cell line inoculation and subsequent challenge. This was a direct attempt to define the role played by histocompatibility antigens in cell line immunisation procedures. An hypothesis had been evolved on the basis of the results of work going back to the early part of the century and culminating in the study of Brown et al., (1978b), that an immune response to histocompatibility antigens on the surface of inoculated infected cells was at least in part responsible for the requirement for large numbers of cells in the inoculum. The results of the work reported here support this hypothesis. As discussed however in 4.4 and 5.4, other factors almost certainly contribute, but nevertheless the results of inoculations in autologous and matched donor/recipient combinations suggest that if the "BoLA barrier" to LCL immunisation could be overcome, the other factors may be relegated to a position of little significance. This is paticularly evident when the difference in cell number requirements of autologous and mismatched recipients would appear to be of the order of 1 million fold.

How the BoLA barrier might be overcome is not within the scope of this discussion and a number of possibilities have already been raised in the relevant chapters.

The work reported in chapter 3 has relevance to all studies of the cell line immunisation system. An accepted view in cell line immunisation research is that Theileria-transformed cell lines express histocompatibility antigens, particularly since the report of Spooner & Brown (1980). The implications, should it occur, that the transformation process can qualitatively alter BoLA antigens (as

detected by current serological defining procedures applicable to the uninfected cell) would be far-reaching, and indeed of considerable interest to ECF immunologists. Spooner & Brown (1980) reported a particular transformation, the product of which appeared to differ in its reactivity with a BoLA typing panel with respect to uninfected donor PBL. With the development of a cell selection technique based on the use of BoLA antisera, the anomalous transformation could be explained without the need to invoke the concept of parasite-induced modulation of histocompatibility antigens. Moreover, the technique developed may have uses in the production of BoLA-negative variants for future immunisation studies (one possible approach to the BoLA barrier problem).

The third area in which the involvement of bovine histocompatibility antigens was studied was in the target specificity of alloreactive cytotoxic cells resulting from primary in vitro generation (chapter 6). This was of interest for a number of reasons, but principally the objective was to further an understanding of the BoLA system per se.

It is felt that this was achieved in that the results are entirely consistent with BoLA-A locus-associated restriction of cytotoxic cell effector function and the demonstration that of the serologically-defined antigens, at least those studied are also lymphocyte definable. This latter fact clearly strengthens the view that the workshop specificities examined, which are in essence the result of the application of statistical analyses to the results of assays of serological activity, are real entities in functional terms. Furthermore, limited evidence suggestive of the existence of a second locus controlling cytotoxic cell effector function was found. Despite considerable efforts, good evidence for a B locus

(class I) has not been forthcoming using other techniques. It cannot be concluded with certainty however, that the products of a second class I locus, rather than class II, were being detected, although it is reasonable to assume that this would be the case in the circumstances described.

The definition of target antigens in the ECF immune response is of immediate concern not least because this may shed some light on improved approaches to vaccination. As discussed in 1.2.2 and 6.1, there is evidence that the only response with any association with protection is MHC-restricted. An obvious candidate for further studies is therefore the cell surface of parasitised lymphoid cells and in this work, the bovine MHC may not be ignored. The application of cloning techniques in the generation of cytotoxic cells specific for membrane antigens of parasite-transformed cells may prove to be a particularly rewarding area for further study. The work reported in chapter 6 indicates that the serologically-defined specificities may be restrictive elements in the targetting of such effector cells, or have a close association with them. It is possible that parasite transformation may induce alterations in the histocompatibility antigens themselves, particularly in the event that some form of interaction antigen is formed. Clear definition of the MHC antigens in the non-transformed state is therefore a prerequisite for meaningful studies on transformation-associated changes.

There are a number of other possibilities as far as parasite-induced changes on the cell surface are concerned, but nevertheless, BoLA definition will be required in their investigation.

Definition of the changes induced by parasitisation and the

involvment of histocompatibility antigens in these changes is also particularly important in the context of the BoLA barrier to LCL immunisation. Separation of parasite and host cell surface antigenic components of transformational changes, may or may not prove possible (which is of interest in its own right for the insight it offers into basic immunological processes). Should it prove possible however, a step towards avoiding the barrier associated with host cell/recipient histoincompatibility, may have been made.

It is clear that the opportunities for further studies of ECF immunology with an immunogenetics component are legion, and the potential rewards in terms of an understanding of fundamental immune processes are great. However, the most pressing problem is that faced by the East African farmer and his stock in an ECF outbreak and their circumstances constitute the raison d'etre of continuing research efforts.

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APPENDIX T.1

BoLA TYPING OF MHC 1 EXPERIMENTAL CATTLE AND LCL's

Animal/LCL	Workshop Specificities	ED & KM (where stated) codes of sera giving positive Reactions (60% kill)	
		Workshop Specificity Defining	Additional Reactions
M980 LCL	7 9	11,17,67,68/14	85,
M980	7 9	11,17,67,68/14	76, 85,
N894	7 9	11,17,67,68/14	76, 85,
S334	7 6	11,17,67,68/9,10,66,75	85,
N866	8 13	13,63,80/5,16	28, 72, 74,
S55 LCL	6.2 10	9,10,66,75,81,86/69,71	17, 85,
S55	6.2 10	9,10,66,75,81,86/69,71	17, 72, 85,
S59	6.2 10	9,10,66,75,81,86/69,71	1, 17, 40,43, 85,
N178	- 10	/69,71	1,2 7, 17, 67,68, 72, 85,88
S357	- -	/69,71	13, 30, 77,
N511 LCL	7 8	11,17,67,68/13,63,80	72, 74, 85,
N511	7 8	11,17,67,68/13,63,80	30, 72, 74, 85,
M878	7 8	11,17,67,68/13,63,80	72, 74, 85,
S340	7 -	11,17,67,68	1, 13, 28,30, 76, 95,88 91
S54	6.1 10	9,10,66,75,12,92,93/69,71	17, 72, 85,88
S68 LCL	11 20	72,76/78	40, 72, 91
S68	11 20	73,76/78	1, 30, 40, 72, 91
S58	11 20	73,76/78	30, 40, 72, 85,88,89,91
N170	11 16	73,76/38,43	30, 40, 72, 74, 85, 91
S125	5 6.2	7,9/9,10,66,75,81,86	
S48 LCL	6 (8)	9,10,66,75/13,63	72, KM22,KM25
S48	6 (8)	9,10,66,75/13,63	5, 72, 74, KM22,KM25
S122	6.2 8	9,10,66,75,81,86/13,63	72, 74,
N546	6 10	9,10,66,75/69,71	30, 72, 85, 91
M616	5 20	7,90/79	5, 85,
N989 LCL	6 9/20	9,10,66,75,12,81/14/78	69, 76, 91
N989	6 9/20	9,10,66,75,12,81/14/78	69, 76, 91
N886	6 9/20	9,10,66,75,12,81/14/78	69, 76, 91
S121	6.2 16	9,10,66,75,81,86/38,43	72,73, 91
S127	8 16	13,63,80/38,43	30, 72,73,74 88, 91

APPENDIX 1.2

MHC1 HAEMATOLOGY : HAEMOGLOBIN LEVELS (mgm%) PRIOR TO AND FOLLOWING LCL INOCULATION ON DAY 0

Day	-17	-1	0	3	7	10	14	17	20	22	24	28
M980	10.5	10.0	10.6	10.6	8.9	9.4	11.1	8.2	9.1			
N894	9.7	9.1	8.9	10.0	9.9	9.2						
S334		12.6	11.2	11.8	12.3	11.4	11.6	10.4	11.8	11.7	11.1	9.7
N866	8.6	7.5	7.7	9.6	9.9	9.4	9.7	8.7	9.6	10.6	9.7	11.0
S55	11.3	11.9	11.7	11.3	11.4	10.8	10.7	9.6	10.8	11.1	9.8	9.2
S59		13.4	12.7		13.9	11.3	12.2	11.4	12.4	13.4		11.0
N178	12.2	10.9	10.6	10.0	8.8	8.8	9.2	8.2	10.1	9.8	9.1	9.1
S357		11.5	11.4	12.1	11.4	12.2	12.1	11.7	13.0	12.8	12.1	11.0
S68	12.3	13.2	11.8	11.7	11.9	9.7	8.2	8.1	8.9	9.3	9.3	10.2
S58	12.8	11.0	13.4		11.7	10.6	11.0	9.5	11.8	10.2	10.8	9.8
N170	11.2	11.4	12.0	11.9	11.0	11.2	10.3	11.0	10.4	10.5	9.6	9.5
S125	11.7	11.5	10.7	11.3	11.9	12.2	10.2	9.5	11.4	11.5	11.0	10.2
S48	12.3	11.8	11.9	11.7	11.4	9.8	9,8	8.2	9.0	9.0	7.4	4.7
S122	10.0	12.1	10.6	10.7	11.3	11.3	11.8	9.7	11.0	10.9	9.5	9.9
N546	13.2	13.1	13.0	13.2	12.3	12.4	12.1	10.4	13.2	13.8	11.2	11.1
M616	13.1	13.0	13.9	12.9	11.9	12.3	12.3	11.1	13.5	13.2	12.6	11.7
N511	13.1	12.6	13.1	12.6	12.3	12.1	11.1	10.0	11.6	11.0	10.8	11.4
M878	14.7		15.8	15.3	16.5	14.4	14.6	13.3	14.4	14.7	14.0	13.2
S340		13.0	14.8	14.3	13.7	14.2	12.9	11.5	14.2	13.0	12.0	12.0
S54	13.2	11.9	12.3	12.3	11.5	12.5	11.9	12.8	12.8	12.7	12.1	12.0
N989		13.1	14.9		16.3	14.7	11.2	10.3	11.0	10.2	10.7	10.5
N886		13.1	13.4	12.7	13.2	11.9	12.6	11.8	13.2	12.7	14.2	11.2
S121	10.2			9.0	9.4	10.5	10.6	9.3	9.7	9.7	9.3	8.1
S127	10.7	11.0		10.0	11.3	11.4	11.2	10.8	11.0	11.2	9.9	9.0

APPENDIX 1.2 CONT.

MHC 1 HAEMATOLOGY : HAEMOGLOBIN LEVELS (mgm%) FOLLOWING STABILATE CHALLENGE ON DAY 70

Day	80	82	84	86	88	90	91	94	96	98
M980										
N894										
S334	10.9	9.7	11.1	13.9						
N866	10.5	11.0	11.0	9.0	9.9	9.0	10.9			
S55	6.4	7.0	8.3	8.3	8.6	9.5	9.7	8.8	8.5	9.8
S59	11.3	10.9	11.1	11.7	11.1					
N178	10.4	10.0	9.5	8.9	8.9	8.1	8.0			
S357	7.2	5.5	4.9	5.6						
S68	12.6	12.3	12.2	12.0	12.9	13.1	13.1	12.1	11.4	12.8
S58	12.2	14.0	12.3	12.2	12.3	13.1	12.7	12.4	10.4	11.1
N170	10.2	9.6	10.1	9.3	9.9					
S125	7.9	6.1	7.0	7.6	7.2					
S48	11.5	11.0	12.1	11.5	11.4	10.7	11.9	10.0	9.2	11.5
S122	12.1	11.4	12.4	12.1	11.5	12.0	12.2	11.4	10.4	11.8
N546	10.8	10.9	10.0	8.2	8.9	10.4	10.5	11.4	10.2	11.6
M616	10.6	9.3	10.9	13.5						
N511	13.0	12.7	11.6	11.7	10.6	12.5	12.5	10.7	10.3	11.6
M878	11.5	12.1		12.1	12.6	13.0	13.6	12.6	12.3	12.0
s340	6.8	5.8	5.6	7.1						
S54	12.3	10.8	11.0	11.4	13.1					
N989	13.0	12.8	13.4	13.7	14.0	14.2	14.3	14.0	12.1	13.8
N886	10.4	10.9	13.3							
S121	10.2		9.6	8.8	10.7	9.5	12.4			
S127	8.1	7.3	8.1	8.5						

Cont.

APPENDIX 1.2 CONT.

Day	80	82	84	86	88	90	91	94	96	98
R608	12.3	14.6	14.9	14.2						
S96	10.6	12.4	10.9	11.4	11.7					
S114	11.7	11.4	14.3							
S170	13.1	13.1	12.7	16.6						

APPENDIX 1.2 CONT.

MHC 1 HAEMATOLOGY : WBC (NUMBERS/mm^3 BLOOD) PRIOR TO AND FOLLOWING LCL INOCULATION ON DAY 0

Day	-17	-1	0	3	7	10	14	17	20	22	24	28
M980	9500	7400	6600	8900	5100	6200	4800	5300	1100			
N894	12800	8300	8300	10400	6700	9900						
S334		13100	11700	13500	12400	12300	10000	14700	8400	9300	8100	6500
N966	9900	8100	8800	5300	8200	8200	7600	14200	6300	10100	9400	7200
S55	15600	11500	10700	13100	11900	13100	7900	14300	9100	9200	8900	7600
S59		10400	10200		16800	11700	8100	13100	8000	10600		9300
N178	7600	7000	5600	8200	5800	5800	4300	8500	5100	5800	4500	4500
S357		7600	7400	10300	8800	9100	6500	11400	6000	7200	6300	5600
S68	6700	10600	11600	14500	11700	7300	3300	8700	7200	9900	11100	7900
S58	12600	8100	9900		9800	10100	6800	12200	6000	7400	7900	5500
N170	8200	7200	6600	8000	6300	8700	5300	9200	5200	6100	4700	4700
S125	16800	11900	11500	10800	7400	14200	6600	16700	11100	12600	11300	8800
S48	13200	9000	10100	10500	8700	5300	1400	9600	11400	9700	9700	6500
S122	15100	11700	9300	9700	9200	10900	3800	18900	7200	9800	7400	5800
N546	9200	6100	6900	5700	8600	8700	5600	10700	5500	6900	5400	6100
M616	7600	4800	5500	7600	6000	8300	5900	9300	5600	6400	6200	4700
N511	7500	5200	5700	6400	5900	4500	4000	6700	6000	5800	4900	5200
M878	11300		7600	11500	4300	6700	7500	11800	5800	8100	6300	5500
S340		7700	7100	8900	9800	10900	8400	10500	5200	6200	6600	7300
S54	15900	10900	12200	14300	11600	12800	7200	15900	13800	10400	8100	7500
N989		10900	12500		10900	8900	3200	5000	4700	5700	7600	8100
M886		7800	8300	7700	6200	5400	4200	5600	5800	4400	9800	6000
S121	10500			7900	4700	10000	11200	11500	4400	6000	6000	5900
S127	12200	11600		8600	6700	10700	8000	16300	9100	11600	7000	7300

Cont.

APPENDIX 1.2 CONT.

MHC 1 HAEMATOLOGY : WBC (NUMBERS/mm^3 BLOOD) FOLLOWING STABILATE INOCULATION ON DAY 70

Date	15/6	17/6	19/6	21/6	23/6	25/6	26/6	29/6	1/7	3/7
Day	80	82	84	86	88	90	91	94	96	98
M980										
N894										
S334	7400	3900	500	900						
N866	12400	5300	2100	4400	1500	3400	730			
S55	14900	12800	15700	22800	12100	14800	12400	12500	12600	11800
S59	7200	1500	940	700	500					
N178	5900	4300	1800	1100	690	600	511			
S357	6500	5700	1400	900						
S68	16400	15300	13700	11700	11900	17800	14700	14600	14500	15100
S58	10900	13400	13400	10900	10900	12600	13100	13500	11600	11000
N170	8300	3600	2300	3700	1600					
S125	8300	4200	1700	600	580					
S48	8600	8200	10400	12700	8000	7900	10200	7800	6300	6700
S122	9900	14100	12500	13600	10800	10300	11300	10700	8700	10900
N546	8400	6100	3700	2300	3800	5800	5800	7900	8300	9100
M616	3200	4100	1100	900						
N511	8800	8000	6300	9000	7400	10800	9900	8600	8400	8300
M878	11500	6200		15600	7000	6700	6300	6200	5500	6300
S340	7900	7000	2200	1200						
S54	10300	4000	1000	2200	620					

Cont.

APPENDIX 1.2 CONT.

Date	15/6	17/6	19/6	21/6	23/6	25/6	26/6	29/6	1/7	3/7
Day	80	82	84	86	88	90	91	94	96	98
N989	17700	20800	21800	1800	16800	22500	19600	17000	16500	18700
N886	4100	1100	300							
S121	7100		1000	800	900	300	690			
S127	12100	5900	1500	600						
R608	15600	11100	2000	900						
S96	7900	5100	600	1900	890					
S114	9800	10300	600							
S170	5900	6600	980	900						

APPENDIX 1.2 CONT.

MHC 1 HAEMATOLOGY : PACKED CELL VOLUME (%) PRIOR TO AND FOLLOWING LCL INOCULATION ON DAY 0

Day	-17	-1	0	3	7	10	14	17	20	22	24	28
M980	30	30	28	31	35	28	33	24	27			
N893	37	27	24	28	28	27						
S334		38	32	33	34	34	30	31	34	34	33	28
N866	25	22	27	30	29	28	26	26	30	31	28	34
S55	30	32	33	32	31	32	31	30	30	33	30	27
S59		39	37		36	34	36	33	37	38		33
N178	33	33	33	29	24	27	27	25	33	29	27	27
S357		36	33	35	31	36	36	35	40	38	36	33
S68	36	44	34	33	32	28	23	24	27	27	27	31
S58	35	33	39		32	31	33	30	37	30	31	29
N170	31	36	36	35	30	33	30	33	32	30	28	28
S125	33	33	31	32	31	36	30	30	34	33	23	30
S48	33	32	36	34	30	28	28	24	28	28	21	15
S122	28	36	31	30	30	34	34	30	33	32	27	29
N546	37	39	38	38	33	37	36	31	40	41	33	32
M616	37	38	42	37	32	37	36	33	42	36	37	35
N511	37	37	39	37	33	36	33	30	36	33	32	34
M878	42		43	44	42	43	39	39	43	42	40	39
S340		39	44	43	35	42	37	34	42	39	37	36
S54	33	36	37	38	31	38	36	40	39	38	36	36
N989		40	40		46	43	33	33	31	31	31	30
N886		38	38	36	35	35	37	33	42	37	42	33
S121	29			26	27	31	31	28	30	27	27	24
S127	29	30		28	33	34	31	33	32	33	28	28

Cont.

MHC 1 HAEMATOLOGY : PACKED CELL VOLUMES (%) FOLLOWING STABILATE CHALLENGE ON DAY 70

Day	80	82	84	86	88	90	91	94	96	98
M980										
N894										
S334	35	28	31	38						
N966	33	30	34	27	26	28	30			
S55	18	21	24	25	28	30	28	28	27	30
S59	31	31	32	32	29					
N178	34	28	27	25	27	24	24			
S357	24	17	15	18						
S68	35	35	35	34	36	40	39	36	33	37
S58	34	38	36	34	36	40	39	36	31	34
N170	32	27	29	28	28					
S125	26	19	21	22	21					
S48	30	31	35	32	32	33	36	30	29	33
S122	36	33	36	34	35	35	36	33	30	35
N546	34	28	29	24	27	32	31	33	31	35
M616	33	26	30	38						
N511	41	33	34	34	30	36	36	32	32	34
M878	36	33		35	34	39	39	38	38	37
S340	24	17	17	21						
S54	35	32	33	35	35					
N989	42	35	38	37	40	40	39	41	36	39
N886	35	29	39							
S121	29		27	27	29	30	36			
S127	27	21	24	23						

Cont.

APPENDIX 1.2 CONT.

Day	80	82	84	86	88	90	91	94	96	98
R608	40	39	40	40						
S96	29	35	29	34	33					
S114	32	34	42							
S170	42	37	36	46						

APPENDIX 1.3

MHC 1 SEROLOGY : RECIPROCAL TITRE AS MEASURED IN THE IFAT AGAINST T. PARVA SCHIZONT ANTIGEN

Day	-17	-4	7	14	20	28	35	53	(7) 77	(14) 84	(21) 91	(28) 98
M980	<40	<40	<40	<40	160							
N894	<40	<40	<40									
S334	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40		
N866	<40	<40	<40	<40	<40	<40	<40	40	40	40	160	
S55	<40	<40	<40	<40	40	640	160	160	40	<40	40	160
S59	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40		
N178	<40	40	<40	<40	<40	<40	<40	<40	<40	<40	40	
S357	40	40	40	40	40	40	40	40	40	40		
S68	<40	<40	<40	40	160	640	10240	2560	640	160	160	160
S58	<40	<40	<40	40	<40	640	10240		160	640	160	160
N170	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40		
S125	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40		
S48	<40	<40	<40	160	2560	10240	10240	10240	640	640	640	160+
S122	<40	<40	<40	<40	40	640	160	640	160	160	160	160
N546	<40	<40	<40	40	<40	<40	<40	<40	<40	40	40	160+
M616	<40	<40	<40	<40	<40	<40	640	<40	<40	<40		
N511	<40	<40	<40	<40	2560	<40	640+	160	160	40	40	160
M878	<40	<40	<40	<40	<40	<40	40	<40	40	160	160	40
S340	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40		
S54	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40		

Cont.

APPENDIX 1.3 CONT.

Day	-17	-4	7	14	20	28	35	53	(7) 77	(14) 84	(21) 91	(28) 98
N989	<40	<40	<40	<40	2560	640	640	640	640	640	160	160+
N886	<40	<40	<40	<40	<40	<40	<40	<40	<40	40		
S121	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	
S127	<40	<40	<40	<40	<40	<40	<40		<40	<40		

LCL inoculation on day 0

Stabilate challenge on day 70

APPENDIX 1.4

MHC 1 : PARASITOSIS IN RESPONSE TO LCL INOCULATION ON DAY 0

		Day																										
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
M980	RPG*										+	+	2+	3+	3+	3+	3+	3+	3+	3+	3+	2+								
	LPG*												+	3+	3+	3+	3+	3+	3+	3+	3+	+								
	Blood														+	1%	5%	+	20%	20%	25%	35%								
S55	RPG									+	+	+	+	+	+	+		+		+	+									
	LPG																		+											
	Blood																	+	+											
N511	RPG									+	+	+	+	+	3+	3+	3+		3+	+										
	LPG																		3+		2+									
	Blood														+	+	+	+	+	+	+		+							
S68	RPG											+	2+	+	+	+														
	LPG											+	+	2+	+															
	Blood												+	+	+	+	+	+	+	+	+	+	+		+					
S48	RPG									+	+	2+	2+	3	+	+		+												
	LPG										+	+	2+	2+	2+															
	Blood											+	+	+	5%	15%	10%	20%	30%	+	25%	20%	15%		17%			+		
N989	RPG									+	+	2+	2+	3+	3+		3+	+	+	+										
	LPG											+	+	2+	+															
	Blood													+	+	7%	10%	+	10%	+					2%			+		

* RPG - right prescapular lymph mode

\+ LPG - left prescapular lymph mode

APPENDIX 1.4 CONT.

MHC 1 : PARASITOSIS IN RESPONSE TO STABILATE CHALLENGE ON DAY 0
(DAY 70 FOLLOWING LCL INOCULATION)

		Day																										
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
S334	LEG*								+	+	+	2+	2+	3+	+	+	3+													
	RPG+										+	+	+	3+	3+		+													
	Blood													+	2%	3%	20%												
N866	LEG									+	3+	3+	2+	3+	3+	+	3+		3+	3+	+	3+	3+						
	RPG											+	2+	3+	3+		3+		+	2+		3+	+						
	Blood												+	+	+	2%	5%		40%	50%	35%	35%	40%						
S55	LEG									+																			
	RPG																												
	Blood																												
S59	LEG								+	+		3+	2+	3+	3+	3+	+		+	2+	3+								
	RPG											+	2+	2+	3+	3+	3+				2+								
	Blood														+	2%	5%		2%	10%	20%								
N178	LEG							+	+			2%		2%	+	3+			+	+		3+	3+	3+	3+				
	RPG															+			+						2+				
	Blood														+	2%	10%		10%	40%	40%	20%	50%	50%	50%				
S357	LEG							+	+	2+	2+		2+	2+	2+	3+	+												
	RPG												2+	2+	3+	3+	3+												
	Blood													+	2%	3%	6%												
N511	LEG																												
	RPG																												
	Blood																												

APPENDIX 1.4 CONT.

		Day																										
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
M878	LEG																													
	RPG																													
	Blood																													
S340	LEG								+	3+	2+	+	3+	3+	3+	3+	3+													
	RPG												3+	3+	3+	3+	3+													
	Blood														+	3%	20%													
S54	LEG							+	+	2+	3+	3+	2+	3+	3+	3+	3+			2+										
	RPG											+	2+	2+	3+	3+			3+	3+										
	Blood														+	5%	10%		20%	35%										

* LEG - Left Parotid lymph mode

\+ PRG - Right prescapular lymph mode

APPENDIX 1.4 CONT.

MHC 1: PARASITOSIS IN RESPONSE TO STABILIATE CHALLENGE ON DAY 0
(DAY 70 FOLLOWING LCL INOCULATION)

		Day																										
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
S68	LEG*																													
	RPG+																													
	Blood																													
S58	LEG																													
	RPG																													
	Blood																													
N170	LEG								+	2+	3+	3+	2+	3+		3+	3+		3+	3+										
	RPG							+					+	2+	3+	3+	3+		3+	3+										
	Blood														+	+	5%		25%	50%										
S125	LEG							+	+	+	3+	3+	2+	3+	3+	3+			3+	3+	3+									
	RPG											+	+	2+	3+	3+	3+		3+	3+	3+									
	Blood														+	5%	25%		60%	90%	96%									
S48	LEG																													
	RPG																													
	Blood																													
S122	LEG											+																		
	RPG																													
	Blood																													
N546	LEG									+	2+			2+	+	+	+			2+	+									
	RPG													2+	3+		+													
	Blood														+	+	2%		+	+	+									
M616	LEG								2+	3+	2+	2+	2+	3+	3+	3+	3+													
	RPG													2+	3+	3+														
	Blood														+	+	+													

Cont

APPENDIX 1.4 CONT.

MHC 1 : PARASITOSIS IN RESPONSE TO STABILATE CHALLENGE ON DAY 0
(DAY 70 FOLLOWING LCL INOCULATION)

		Day																										
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
N989	LEG*																													
	RPG+																													
	Blood																													
N886	LEG								+	2+	3+	3+	2+	3+	+	+														
	RPG											+	2+	2+	+															
	Blood												+	+	3%	4%	5%													
S121	LEG								+	2+	2+	3+	2+	3+		3+	+		3+	2+	+	+								
	RPG											+	+	2+	2+	3+	3+		3+	2+		+	3+							
	Blood																		35%	40%	40%	50%	45%							
S127	LEG									2+	3+	3+	3+	3+	3+		3+													
	RPG										+	+	+	2+	3+	3+	3+													
	Blood													+	3%	5%														
R608	LEG								+	3+	2+	3+			2+	3+		3+												
	RPG										+				2+	2+		3+												
	Blood														+	2%		+												
S96	LEG							+	+	3+	3+	3+	2+	3+	3+		3+	3+	3+	3+										
	RPG													2+	3+		3+	3+	3+	3+										
	Blood														+		15%	20%	30%	30%										
S114	LEG										3+	+		2+	2+	2+	3+													
	RPG								+		+	+	+	+	3+	2+	3+													
	Blood												+	+	+	5%	40%													
S170	LEG								+	2+	+	3+	3+	2+	2+	2+	2+	3+												
	RPG												+	2+	2+	2+	2+	3+												
	Blood												+	+	+	+	10%	20%												

Con

* LEG - Left parotid lymph mode

+ RPG - right prescapular lymph mode

APPENDIX 1.5

MHC 1: TEMPERATURE RESPONSES (^{o}C) TO LCL INOCULATION ON DAY 0

	Day												
	1	2	3	4	5	6	7	8	9	10	11	12	13
M980	37.5	38.0	37.5	38.0	38.7	37.1	38.1	39.0	38.3	40.0	39.1	40.1	39.4
N894	37.6	37.2	36.9	38.7	38.7	37.2	39.4	39.3	39.0	41.0	39.9		
S334	36.9	37.5	37.0	37.7	38.0	37.4	37.9	37.6	37.0	37.9	38.2	38.1	37.2
N866	36.9	37.5	37.0	37.7	38.3	37.5	38.7	38.9	38.7	39.0	38.5	38.6	37.7
S55	37.4	37.0	37.4	37.8	38.1	37.6	38.3	38.5	38.0	38.6	38.4	38.9	38.7
S59	37.4	37.3	37.4	37.8	38.3	38.4	38.3	39.0	39.1	39.3	39.0	38.9	38.9
N178	37.3	37.4	37.4	38.3	38.4	37.3	38.2	38.5	38.2	38.0	39.8	38.7	38.2
S357	37.0	37.6	37.0	38.2	38.6	37.6	37.7	38.7	38.0	38.0	38.5	38.5	38.0
N511	36.9	36.8	36.9	37.1	38.5	37.5	37.8	39.0	38.7	38 6	39.2	38.7	38.0
M878	37.0	38.0	37.0	38.2	38.3	37.5	38.2	38.7	38.9	38.6	39.5	39.0	38.4
S340	37.2	37.5	37.2	37.6	38.0	36.5	37.5	38.8	38.3	38.6	38.7	38.7	37.7
S54	37.0	37.4	37.2	37.6	38.2	37.5	38.0	38.7	37.3	37.5	38.9	38.5	38.4
S68	37.0	37.5	37.0	37.0	37.9	36.8	38.0	38.4	38.0	39.5	40.5	39.5	40.0
S58	37.1	37.2	37.1	37.8	37.8	37.5	38.4	38.9	38.4	38.6	38.5	38.5	38.3
N170	37.4	37.6	37.4	38.0	38.7	38.2	38.3	39.1	38.0	37.9	38.6	38.4	38.3
S125	37.3	37.3	37.3	37.5	38.1	37.9	38.4	39.2	38.4	39.9	38.8	38.3	38.0
S48	37.3	37.4	37.3	37.8	38.5	37.0	38.0	38.4	39.9	38.3	40.6	40.0	39.8
S122	37.3	37.4	37.3	37.8	38.6	37.4	37.4	39.0	40.2	39.5	39.4	38.9	38.3
N546	37.0	37.2	37.0	37.7	38.3	37.2	38.0	38.5	37.9	38.0	37.9	38.0	38.4
M616	37.0	37.6	37.0	37.0	37.5	37.8	37.0	38.3	38.1	37.5	37.0	37.7	38.0
N989	37.3	37.0	37.3	38.4	38.2	38.2	38.6	38.2	38.9	40.5	40.0	40.0	40.1
N886	37.0	37.2	37.0	37.1	38.5	37.2	38.4	38.7	38.1	40.2	39.3	38.5	39.0
S121	37.2	37.0	37.2	37.0	38.8	36.5	38.3	38.8	40.3	40.1	38.7	38.5	38.4
S127	37.4	37.0	37.4	37.9	38.2	37.8	38.1	38.5	37.0	39.4	39.2	38.8	38.6

Cont

APPENDIX 1.5 CONT. (2)

	Day													
	14	15	16	17	18	19	20	21	22	23	24	25	26	27
M980	39.4	37.	38.5	40.2	40.7	39.9	39.0							
N894														
S334	38.1	37.8	37.9	38.4	38.1	37.9	37.7	37.9	37.5	37.8	37.0	37.1	38.0	
N866	37.9	38.0	40.2	38.4	38.9	37.9	38.2	38.3	38.5	38.9	37.8	37.9	37.8	38.3
S55	38.4	38.1	37.9	38.0	38.1	38.7	38.5	38.6	38.3	38.0	38.3	38.1	38.2	38.8
S59	38.4	38.4	39.0	38,9	38.5	39.4	39.2	39.0	37.5	37.2	37.7	37.7	39.4	38.8
N178	38.4	38.1	39.0	38.5	38.6	38.0	38.4	38.0	37.5	37.2	38.0	38.2	37.8	38.5
S357	38.0	37.8	38.9	37.7	38.3	37.8	37.9	37.7	37.8	37.5	37.5	37.7	38.0	38.8
N511	37.9	38.0	38.7	38.4	38.6	37.8	38.6	38.7	38.5	38.7	38.0	38.5	38.0	38.2
M878	38.0	38.2	38.2	37.6	38.6	38.0	37.9	37.5	37.2	37.2	37.8	37.9	38.7	39.1
S340	38.5	38.3	39.2	38.6	38.0	38.4	38.6	38.7	38.5	38.0	38.3	37.5	38.4	38.5
S54	37.7	37.9	39.2	38.2	38.7	37.4	37.9	38.0	38.2	38.5	38.0	38.2	38.7	38.4
S68	38.0	37.8	37.0	37.8	38.5	37.8	38.0	38.2	38.0	37.8	37.8	37.6	37.0	37.0
S58	37.9	37.9	38.5	38.6	38.8	38.2	37.9	37.6	37.8	37.6	38.3	38.9	38.0	37,9
N170	38.5	38.1	38.0	38.6	38,9	38.2	37.9	37.5	38.2	38.4	37.5	37.3	37.2	38.8
S125	38.0	37.4	38.4	37.6	37.5	38.0	37.9	38.0	38.2	37.7	37.2	37.0	37.0	37.2
S48	39,7	40.4	38.0	38.4	39.2	38.6	38.0	38.2	38.1	38.0	38.4	38.5	38.4	38.4
S122	38.0	37.4	39.4	37.6	39.1	38.8	38.9	38.7	37.7	37.6	38.2	38.0	38.2	38.0
N546	38.0	38.4	37.4	38.7	39.6	38.9	38.2	37.8	38.3	37.8	38.2	38.0	38.9	37.6
M616	37.2	37.2	38.2	37.9	38.1	37.8	38.0	37.7	37.8	37.6	38.7	38.5	38.3	37.5
N989	40.4	39.3	39.4	38.6	40.1	40.0	39.7	38.9	38.7	38.3	37.7	37.6	37.6	38.7
N886	39.8	39.8	39.7	39.4	39,0	39.7	39.6	39.5	37.5	37.5	37.6	37.5	38.0	
S121	38.4	37.7	37.5	38.5	37.5	38.0	38.5	38.0	37.8	37.2	37.4	37.5	37.4	38.0
S127	38.2	37.7	37.9	38.2	38.0	38.0	38.0	38.1	37.0	37.4	37.0	37.1	37.2	38.0

APPENDIX 1.5 CONT. (3)

MHC 1 : TEMPERATURE RESPONSES (^{o}C) TO STABILATE CHALLENGE ON DAY 0

	Day												
	1	2	3	4	5	6	7	8	9	10	11	12	13
S334	37.5	37.6	37.5	38.0	37.9	37.5	38.5	38.5	37.9	29.5	40.7	39.7	40.5
N866	38.6	38.4	38.1	38.0	38.6	38.4	38.8	38.7	38.5	39.2	40.0	40.5	41.1
S55	37.2	37.2	37.6	38.5	38.7	39.2	38.6	39.0	38.6	37.6	39.5	38.2	38.3
S59	37.4	37.4	38.0	37.5	38.5	38.9	38.7	38.9	39.0	38.4	41.1	41.1	41.1
N178	37.1	37.1	38.0	37.4	38.3	38.5	38.0	38.5	39.5	40.4	40.0	40.0	40.9
S357	38.5	38.2	37.4	38.5	39.0	38.6	39.0	38.8	39.8	40.2	41.0	41.0	41.3
N511	37.5	37.4	37.8	37.8	37.9	38.9	38.4	37.2	37.5	38.2			38.6
M878	38.4	38.2	38.2	37.0	38.6	39.0	38.8	39.4	38.5	38.9			38.5
S340	37.4	37.4	37.3	37.5	37.9	39.0	38.7	38.4	39.1	40.2			40.0
S54	38.0	37.9	38.5	37.8	38.2	38.7	38.5	39.3	38.9	39.9			41.1
S68	38.0	37.8	37.1	37.6	37.8	38.4	37.4	38.5	37.4	38.7	38.5	38.5	38.0
S58	38.0	38.0	37.1	37.1	38.7	37.8	38.9	38.5	39.0	38.2	38.2	38.2	38.1
N170	37.4	37.2	37.0	37.6	39.0	38.8	38.8	38.6	38.3	40.5	41.1	41.1	40.6
S125	37.6	37.7	38.5	38.0	37.8	37.8	38.8	38.4	39.5	38.6	40.1	40.1	38.5
S48	38.0	37.9	37.8	37.4	38.4	38.8	38.8	40.6	38.8	38.6		37.9	38.0
S122	37.2	37.2	37.4	38.8	38.7	39.1	39.0	39.0	38.6	38.0	38.5	38.5	38.1
N546	38.0	38.0	38.0	37.6	38.2	38.1	38.7	38.2	39.4	40.3		39.6	39.8
M616	37.2	37.2	38.3	38.0	38.7	38.8	38.6	40.5	38.9	39.9		40.8	41.1
N989	38.6	38.2	37.6	37.4	37.7	38.6	38.8	39.2	38.4	38.2		39.2	38.8
N886	37.5	37.5	37.9	38.0	38.8	38.8	37.5	38.6	39.2	40.5		41.0	38.8
S121	38.0	38.0	37.7	37.8	38.6	38.4	38.5	39.1	38.0	38.4	39.6	41.0	40.0
S127	37.4	37.2	38.0	37.6	38.4	38.5	38.4	37.2	38.5	38.2	38.7	40.0	40.5
R608	37.5	37.4	37.6	37.2	38.6	38.3	38.2	38.5	38.2	39.5	39.6	39.0	41.3
S96	S96	38.4	37.9	38.4	38.0	38.2	38.0	38.5	39.1	39.0	39.6	38.0	41.5
S114	S114	37.5	37.5	37.5	38.4	38.9	37.8	38.7	39.2	40.0	40.1	40.7	41.1
S170	37.5	37.5	37.4	38.0	38.6	38.4	38.2	38.8	38.4	40.6	40.6	40.4	41.1

Cont.

APPENDIX 1.5 CONT. (4)

MHC 1 : TEMPERATURE RESPONSES (^{o}C) TO STABILATE CHALLENGE ON DAY 0

	DAY														
	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
S334	39.4	38.8													
N866	40.8	39.7	40.0	41.2	40.2	40.3	40.0	39.7							
S55	38.7	38.6	39.0	38.5	38.9	37.0	37.9	38.2	38.5	38.2	38.3	37.8	37.1		
S59	41.2	40.5	39.1	41.5	41.4	41.0									
N178	40.8	41.1	39.7	41.4	41.0	40.2	41.0	40.5	41.3	40.7					
S357	41.5	40.7	39.3												
N511	38,5	38.1	39.0	38.7	38.5	38.4	38.5	38.5	37.9	37.7	38.2	38.4	38.1		
M878	38.8	38.3	38.0	38.5	38.4	38.0	38.0	38.2	37.5	37.6	37.9	37.8	37.5		
S340	41.0	39.3	40.4												
S54	40.6	40.8	39.8	41.0	40.0										
S68	28.6	37.4	38.0	38.4	37.5	37.o	37.9	37.6	37.1	37.4	37.6	37.2	37.8		
S58	38.9	38.6	38.8	38.5	37.8	37.0	37.8	37.7	37,3	37.5	38.5	39.8	38.0		
N170	41.1	40.6	41.0	41.3	37.0										
S125	40.5	39.8	39.5	40.8	38.5	1.0									
S48	39.0	38.5	38.7	38.8	37.9	38.0	37.4	37.8	38.0	37.8	37.3	37.9	37.8		
S122	38.5	38.9	39.2	38.8	38.0	37.4	38.6	38.0	38.1	38.0	38.5	38.2	38.0		
N546	40.5	40.8	40.7	40.8	39.9	38.2	38.9	37.6	37.2	37.3	37.9	37.2	37.8		
M616	39.7	37.7	37.9												
N989	38.2	38.5	38.2	38.4	38.0	38.3	38.4	38.0	38.1	38.2	39.0	37.7	37.9		
N886	39.2	41.0													
S121	41.4	40.5	39.6	40.3	3819	40.3	40.7	39.0							
S127	40.8	39.5	39.9												
R608	38.9	39.8	39.8												
S96	40.5	39.0	40.1	41.2	38.7										
S114	40.7	49.8													
S170	41.3	40.1	39.9												

APPENDIX 2.1

MHC 2 : BoLA TYPING AND SIRES OF MHC2 EXPERIMENT CATTLE AND LCL

Sire		w.specificities	ED codes of non w defining sera
	816TpM	8/11	30 72 74 91 95a 102 105
E	S816	8/11	30 72 74 85 91 95a 99 102 105
W	S811	8/11	72 74 85 95a 99 102
AD	S817	8/11	30 72 74 85 91 95a 99 102
J	S821	8/11	30 72 74 91 95a 99 102
N	S828	8/11	30 72 74 85 91 95a 99 102
J	S830	8/11	30 72 74 85 91 95a 99 102
AD	S832	8/11	30 72 74 91 95a 102
AD	S812	w8/w13	28 72 74 88 101 104
J	S815	w8/w13	28 72 74 88 102 105
N	S818	w8/w6/81,100	72 74
N	S820	w8/w6 12,97,100	72 74
N	S824	w8/w20	30 72 74
J	S825	w8/w13	28 30 72 74 88 91
N	S831	w8/w20	72 74 101
AD	S836	w8/w13	28 72 88
J	S804	w11/w16	30 72 74 91 95a 102 105
AD	S807	w11/w13	28 30 72 74 85 88 91 95a 99 102
N	S808	w11/w13	28 30 72 74 85 88 91 95a 99 102
J	S813	w11/w6 100	30 72 91 95a 99 101 102 105 109
AD	S819	w11/w16	30 72 91 95a 99 102 105
J	S826	w11/w6 100	30 72 95a 99 102
W	S834	w11/w6 100	72 91 95a 101 102 105
J	S835	w11/w6,93,100	30 72 85 91 95a 99 101 102 105
AD	S996	w11/w10	30 72 85 91 95a 99 105
AD	S997	w11/w16	30 72 85 91 95a 99 102 105
AD	S806	w6/w13	28 72 74 88
N	s809	w6/w13	28 30 72 74 88 91
W	S814	w13/w20	28 72 74
J	S822	w6/w13	28 72 74
W	S823	w10/w13	28 72 74 85 105
AD	S829	w13/w16	28 30 72 74 91 105
	S120	w16/-	28 30 72 85 88 91 102 105 109
	S167	w20/-	85 88 105
	S195	w6/-	30 102 109

APPENDIX 2.2

MHC 2 HAEMATOLOGY : WBC COUNTS/mm^3 PRIOR TO AND FOLLOWING LCL INOCULATION ON DAY 0

Date	3/5	10/5	14/5	17/5	21/5	24/5	28/5	31/5	4/6	7/6
Day	-8	-1	3	6	10	13	17	20	24	27
S804	10800	11300	9600	9900	11800	10600	12500		11700	12300
S806	6700	10200	4800	5700	6200	7300	7300	9100	7800	8800
S807	6100	6300	6100	8700	7900	6800	7800		5200	6000
S808	6300	6600	5600	6900	5500	4800	8100		6600	5500
S809	6000	6600	4700	6200	6800	5300	7500		6000	6100
S811	7800	7900	5700	6500	7900	7500	13100		9600	9800
S812	7900	8700	7000	10300	7100	9200	15100		12000	9400
S813	6900	7000	5000	6000	7200	6800	7200		5900	6900
S814	7500	7200	4700	7300	7100	7400	10800		11400	16700
S815	9200	7300	8200	7800	11900	9600	13200		11800	9300
S817	9900	14200	21400	14700	4800	5400	6300		7900	8100
S818	8100	7500	7600	8000	138000	8200	10900		8000	5900
S819	10400	10600	7900	8100	6400	9200	9800			10600
S820	15900	16500	10700	9700	15800	14700	19800		11500	15600
S821	4700	5100	5500	7500	6300	4200	6400		6100	6500
S822	8600	8900	5500	7700	7000	7500	9900		8300	9200
S823	10900	9100	5900	7700	9800	9200	11700		13200	8500
S824	10700	7700	5300	9800	10100	7600	10900		10100	10100
S825	8200	7400	7100	8900	8200	7400	9900		8900	11500
S826	7700	9300	11000	9300	8900	7800	9000		8400	9100
S828	7800	6500	6700	10000	6400	6600	8000		7900	8500
S829	6100	4900	4400	5200	4900	4100			4100	
S830	8900	10700	7800	11800	10800	8000	11800		10000	9000
S831	6400	8100	5600	6900	7800	6900	8300		8300	8100
S832	4800	5400	3700	4000	4000	3700	5800		6200	6800
S834	7300	89000	5500	8300	9200	7200	9700		10000	12200
S835	7100	8200	7100	7600	8100	7300	11700		11300	11900
S836		7400	5200	6100	5600	7200	8800		8400	9100
S996	13200	9700	7200	7600	11100	10300	9300		7300	9600
S997	13800	13400	8600	10800	14600	11200	13300		9100	11300

Cont.

APPENDIX 2.2 CONT

MHC 2 HAEMATOLOGY : WBC COUNTS/mm^3 FOLLOWING STABILATE INOCULATION ON DAY 29

Date	11/6	14/6	18/6	21/6	23/6	25/6	28/6	2/7	5/7	9/7
Day	31	34	38	41	43	45	48	52	55	59
S804	11000	11200		5800	1600	1500	1800			
S806	10900	9500	11900	4800	750	1900				
S807	7600	6200	6700	3400	1900	1320				
S808										
S809	7600	7200	10100	7100	1100	960	2900			
S811	10300	13800	9400	7700	3500	3400	5500	6600	12800	12500
*S812	10400	9300	7300	4900	1400	860				
*S813	9100	10000	11800	5100	630					
*S814	12500	12100	11700	4100	690					
S815	11200	8300	6500	4700	1100	663	645			
S817	8800	8800	5700	4200	1400	840	11300	3500	5200	9100
S818	5600	7300	9200	7500	3000					
*S819	11400		8100	5200	1300					
*S820			10200	7200	6700					
S821	6800	6400	6100	5700	4500	2300	3300	6900	13600	4900
*S822	14300	10200	7800	12000	4700					
*S823	7700	9500	8100	4900	4500					
*S824	7900	11200	9100	7400	2800					
*S825	14700	8600	7500	6100	3600					
*S826	11100	97PP	7900	6300	2000					
S828	15200	6800	5700	5500	1900	1400	1400			
*S829	5100	12600	6900	7200	5000					
S830	9100	6400	7700	8600	3700	672	652			
*S831	11300	8700	7400	4500	1800					
S832	11900		6900	6900	2000	703	926			
*S834	12700	14500	10200	5600	990					
*S835	11600	11300	10800	4800	1000					
*S836	6800	9300	9000	7500	1600					
*S996	14700	11300	8300	6300	2800					
*S997	7300	8300	10100	5800	1500					
S590	7500	7000	11000	4000	1700	545	695			
S605	19100	17800	18900	8500	1800	1600	1202			

* Treated on day 44 (day 15 post-stabilate inoculation)

Cont.

APPENDIX 2.2 CONT

MHC 2 HAEMATOLOGY : HAEMOGLOBIN LEVELS (mgm %) PRIOR TO AND FOLLOWING LCL INOCULATION ON DAY 0

Date	3/5	10/5	14/5	17/5	21/5	24/5	28/5	31/5	4/6	7/6
Day	-8	-1	3	6	10	13	17	20	24	27
S804	10.2	9.7	11.7	10.2	11.5	10.8	11.3		11.2	11.2
S806	7.6	9.2	9.5	9.7	10.5	10.2	10.5	9.3	8.4	8.4
S807	9.4	9.0	10.0	9.8	10.6	9.5	10.6		9.2	8.4
S808	10.1	11.1	9.8	10.5	10.5	9.7	10.0		8.9	6.8
S809	10.9	10.1	9.8	10.9	11.5	9.9	10.8		10.0	9.6
S811	11.1	10.7	10.6	10.5	11.1	9.8	11.3		10.6	10.8
S812	9.7	9.7	10.0	9.2	9.8	9.8	11.5		10.0	10.5
S813	8.9	9.6	9.3	8.9	9.6	8.9	10.0		7.8	7.9
S814	10.6	9.7	10.1	9.4	10.5	9.7	11.4		9.6	9.3
S815	10.1	10.2	9.9	9.4	10.0	10.0	10.9		9.5	9.1
S817	4.6	6.3	6.9	7.3	8.0	7.9	8.6		7.6	7.2
S818	8.8	9.6	9.9	10.1	10.7	9.8	10.4		1.2	9.7
S819	11.1	10.0	10.2	10.2	10.7	10.6	11.0		-	10.8
S820	9.9	9.8	9.3	9.9	9.7	9.0	9.4		8.0	5.5
S821	9.8	8.8	10.1	9.2	10.3	9.4	10.4		9.4	9.6
S822	10.5	9.9	9.5	10.2	10.5	9.8	10.6		10.7	10.8
S823	9.9	9.1	9.2	8.1	9.5	8.7	9.0		9.0	7.6
S824	10.2	9.9	9.5	9.6	10.8	9.5	10.1		10.1	10.5
S825	11.2	10.0	9.6	10.0	10.7	10.4	10.8		10.9	10.9
S826	11.4	1o.5	12.5	11.2	12.2	11.1	12.0		10.8	11.9
S828	9.4	10.0	9.9	10.1	11.1	9.9	11.0		11.0	11.5
S829	10.2	9.6	9.8	9.5	9.8	9.2	-		9.5	
S830	9.7	8.5	9.4	9.5	10.6	9.3	11.6		10.1	9.3
S831	9.8	10.1	9.8	10.5	11.4	9.9	10.8		11.3	10.7
S832	8.9	9.4	9.3	8.8	9.8	8.8	9.6		9.8	10.1
S834	10.9	10.1	10.3	9.6	10.3	9.4	10.5		9.5	10.1
S835	7.5	8.7	9.1	8.9	9.8	9.7	8.9		9.3	10.1
S836	9.2	10.2	10.1	10.7	12.2	11.2	11.9		10.8	10.3
S996	12.0	10.5	10.7	10.3	11.2	11.1	12.1		11.7	11.9
S997	12.7	10.8	10.5	11.2	11.7	10.7	11.6		11.3	10.0

Cont.

APPENDIX 2.2 CONT

MHC 2 HAEMATOLOGY ; HAEMOGLOBLIN LEVELS (mgm %) FOLLOWING STABILATE INOCULATION ON DAY 29

Date	11/6	14/6	18/6	21/6	23/6	25/6	28/6	2/7	5/7	9/7
Day	31	34	38	41	43	45	48	52	55	59
S804	11.2	11.8	11.1	11.0	10.0	9.6	8.8			
S806	8.9	10.1	10.1	8.9	8.5	9.3				
S807	6.6	6.5	7.3	6.5	5.7	5.8				
S808										
S809	6.4	9.8	9.9	9.1	8.8	8.6	11.4			
S811	11.5	11.6	11.0	10.2	8.7	9.2	8.1	8.1	7.1	7.0
S812	10.2	9.7	9.6	8.1	7.3	7.3				
*S813	8.3	8.4	9.6	9.7	8.4					
*S814	7.3	7.4	8.7	8.7	7.7					
S815	10.3	9.7	9.6	9.4	8.9	9.1	8.4			
S817	6.7	8.2	8.4	8.6	7.3	7.3	6.2	5.9	4.9	4.5
*S818	10.6	4.9	10.4	10.6	10.6					
*S819	10.1	-	10.8	9.3	8.6					
*S820	2.9		6.4	6.6	6.4					
S821	9.6	9.6	10.1	10.2	9.5	8.3	7.9	8.3	7.8	6.9
*S822	11.6	10.5	11.2	10.8	9.7					
*S823	5.4	5.2	6.6	6.0	4.4					
*S824	9.5	10.6	10.2	9.5	8.9					
*S825	11.2	11.2	11.2	10.5	8.7					
*S826	12.3	11.9	12.0	11.2	9.8					
S828	11.5	10.4	9.9	10.8	8.4	8.0	6.7			
*S829	5.1	4.2	5.9	5.9	4.4					
S830	9.2	8.1	8.0	9.4	7.8	8.6	7.3			
*S831	11.3	10.1	10.7	9.9	8.8					
S832	10.8		11.1	9.4	9.0	8.3	8.0			
*S834	7.6	5.2	7.2	7.2	6.6					
*S835	9.6	9.2	9.8	8.7	7.2					
*S836	9.4	9,5	9.8	9.0						
*S996	12.5	12.0	11.6	10.3	8.4					
*S997	6.9	5.8	6.9	7.0	6.7					
S590	11.1	12.5	10.7	10.1	9.1	9.5	9.6			
S605	11.7	11.2	11.1	9.8	8.9	10.5	8.9			

* Treated on day 44 (day 15 post stabilate inoculation)

Cont.

APPENDIX 2.2 Cont

MHC 2 HAEMATOLOGY : PCV LEVELS PRIOR TO AND FOLLOWING LCL INOCULATION ON DAY 0

Date	3/5	10/5	14/5	17/5	21/5	24/5	28/5	31/5	4/6	7/6
Day	-8	-1	3	6	10	13	17	20	24	27
S804	27	30	34	30	33	32	33			
S806	21	28	28	29	30	31	30	27		
S807	25	28	30	27	33	29	30			
S808	29	33	29	30	30	30	32			
S809	28	30	29	32	33	30	33			
S811	29	31	32	30	33	30	33			
S812	29	30	30	27	30	31	33			
S813	25	30	28	26	28	27	30			
S814	28	29	30	29	21	29	33			
S815	29	31	30	27	30	30	30			
S817	15	19	20	21	25	25	27			
S818	26	30	30	30	32	30	31			
S819	30	30	30	30	30	32	33			
S820	27	30	28	28	28	28	28			
S821	28	27	30	27	30	29	30			
S822	29	30	27	29	31	29	30			
S823	24	27	27	25	27	28	29			
S824	28	30	27	27	31	29	30			
S825	31	30	28	30	31	31	32			
S826	32	34	36	33	36	33	36			
S828	26	30	29	28	33	29	33			
S829	28	29	29	27	29	28	-			
S830	25	25	28	29	32	28	35			
S831	27	30	29	30	34	30	30			
S832	25	28	27	25	30	28	30			
S834	29	30	30	29	30	30	31			
S835	24	28	27	27	30	30	25			
S836	26	30	30	31	36	33	33			
S996	33	30	32	30	33	34	36			
S997	29	33	31	32	34	32	34			
									NOT DONE	NOT DONE

Cont.

APPENDIX 2:2 CONT

MHC 2 HAEMATOLOGY : PCV LEVELS FOLLOWING STABILATE INOCULATION ON DAY 29

Date	11/6	14/6	18/6	21/6	23/6	25/6	28/6	2/7	5/7	9/7
Day	31	34	38	41	43	45	48	52	55	59
S804	31	34	30	30	28	25				
S806	26	31	29	26	26	28				
S807	18	21	20	18	17	16				
S808										
S809	28	28	28	26	26	25	33			
S811	33	33	30	28	26	27	24	24	21	20
S812	20	28	29	23	21	22				
*S813	25	26	28	25						
*S814	21	22	25	24	22					
S815	28	30	28	27	26	25				
S817	20	25	25	23	21	23	19	18	15	13
*S818	30	29	28	30	31					
*S819	30		30	27	25					
*S820	10		20	19	20					
S821	28	29	30	30	27	25	23	24	24	20
*S822	32	30	31	30	27					
*S823	15	15	20	18	14					
*S824	28	30	29	28	26					
*S825	33	33	32	26	25					
*S826	35	34	33	30	28					
*S828	33	29	27	28	23	23	21			
*S829	14	13	20	17	14					
S830	28	23	23	27	23	25	23			
*S831	33	29	30	28	25					
S832	31		31	27	26	25	24			
*S834	22	15	22	20	21					
*S835	29	27	28	25	22					
*S836	26	27	27	25	26					
*S996	36	35	33	30	25					
*S997	21	16	20	20	20					
S590	31	34	30	28	25	27	28			
S605	33	31	30	26	25	32	26			

* Treated on day 44 (day 15 post stabilate inoculation)

APPENDIX 2.3

MHC 2: RECIPROCAL TITRES IN THE IFAT TO T.PARVA SCHIZONT ANTIGEN PRIOR TO AND FOLLOWING LCL INOCULATION ON DAY 0 AND STABILATE CHALLENGE ON DAY 29

Day	-4	3	17	24	31	38	45	52	59	66
Date	7/5	14/5	28/5	4/6	11/6	18/6	25/6	2/7	9/7	16/7
S804	<40	<40	<40	<40	<40	<40	<40			
S806	"	"	"	"	"	"	"			
S807	"	"	"	"	"	"	"			
S808	"	"	"	"	-	-				
S809	"	"	"	"	"	"	"			
S811	"	"	"	"	"	"	"	640	640	640
S812	"	"	"	"	"	"	"			
*S813	"	"	"	"	"	"	"			
*S814	"	"	"	"	"	"	"			
S815	"	"	"	"	"	"	"			
S817	"	"	"	"	"	"	"	640	640	640
*S818	"	"	"	"	"	"	"			
*S819	"	"	"	"	"	"	"			
*S820	"	"	"	"	"	"	"			
S821	"	"	"	"	"	"	"	10240	2560	10240
*S822	"	"	"	"	"	"	"			
*S823	"	"	"	"	"	"	"			
*S824	"	"	"	"	"	"	"			
*S825	"	"	"	"	"	"	"			
*S826	"	40	40	40	"	"	"			
S828	"	40	<40	<40	40	"	40			
*S829	"	<40	"	"	<40	"	<40			
S830	"	"	"	"	"	"	"			
*S831	"	"	40	"	"	"	"			
S832	"	"	<40	"	"	"	"			
*S834	"	"	"	"	"	"	"			
*S835	"	"	"	"	"	"	"			
*S836	"	"	"	"	"	"	"			
*S996	"	"	"	"	"	"	"			
*S997	"	"	"	"	"	"	"			
*S590	"	"	"	40	"	"	"			
S605	"	"	"	<40	"	"	"			

* Treated on day 44 (day 15 post stabilate inoculation)

APPENDIX 2.4

MHC 2 : RECTAL TEMPERATURES (°C) FOLLOWING LCL INOCULATION ON DAY 0

Date	11/5	12/5	13/5	14/5	15/5	16/5	17/5	18/5	19/5	19/5	20/5	21/5	22/5	24/6
Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13
S804	38.2	38.0	38.3	38.1	38.2	38.0	38.1	37.5	38.0	37.8	37.5	37.8	37.6	38.2
S806	38.1	38.3	38.5	38.4	38.0	37.8	37.6	40.3	38.5	38.0	38.4	38.2	38.0	38.8
S807	38.8	38.7	38.6	38.5	37.8	37.6	37.5	38.1	38.3	38.0	38.9	38.0	37.8	38.8
S808	38.8	38.8	38.7	38.3	38.1	38.2	38.0	37.8	38.6	38.5	39.0	38.9	38.4	38.0
S809	38.9	38.8	38.5	38.6	38.5	38.0	37.9	37.0	37.9	37.7	37.8	38.0	38.6	38.6
S811	38.3	38.3	38.4	38.1	38.2	37.8	38.0	38.9	37.2	37.5	38.0	37.8	37.8	38.3
S812	39.0	39.1	39.0	38.9	38.8	37.8	37.6	38.0	38.8	38.5	38.5	38.3	38.0	37.5
S813	39.1	38.9	38.7	38.7	38.6	37.9	37.8	38.0	38.7	37.5	38.8	38.9	38.5	38.8
S814	38.4	38.5	38.3	38.1	37.9	38.5	37.8	37.5	38.2	38.3	38.0	38.6	38.4	38.8
S815	38.7	38.8	38.6	38.5	38.6	37.5	37.2	37.2	37.9	37.0	38.0	38.5	38.2	38.2
S817	38.6	38.5	38.1	38.3	37.9	37.9	37.7	37.3	38.2	37.0	38.9	38.3	38.0	38.5
S818	38.4	38.0	38.5	38.4	38.1	37.9	37.9	38.9	38.1	38.0	37.8	38.2	38.3	38.0
S819	38.6	38.7	38.4	38.6	37.8	37.5	37.2	38.5	39.0	37.8	39.2	38.8	38.9	38.2
S820	38.7	38.9	38.8	38.7	38.8	38.0	38.0	39.0	40.0	37.8	38.7	38.9	38.0	38.5
S821	38.0	38.0	38.3	38.5	38.2	38.0	37.0	38.5	38.6	38.3	38.5	38.6	38.6	38.7
S822	38.9	38.6	38.4	38.6	38.7	38.1	38.1	37.7	38.4	38.2	38.9	39.0	39.1	38.5
S823	38.1	38.2	38.0	38.4	38.5	38.2	38.0	37.6	39.5	37.6	37.5	38.2	38.0	39.0
S823	38.0	38.1	38.2	38.3	38.1	38.0	38.1	38.2	38.5	38.2	38.0	38.3	38.4	38.7
S825	38.6	38.5	38.7	38.6	38.7	38.5	38.3	38.5	38.4	37.0	38.2	38.2	38.3	38.7
S826	38.3	38.4	38.3	38.2	38.1	37.5	38.0	38.4	38.4	38.5	38.0	37.9	37.8	38.2
S828	38.3	38.4	38.6	38.3	38.2	38.0	38.0	39.3	38.2	39.5	38.8	38.4	38.3	38.3

Cont.

APPENDIX 2.4 CONT.

Date	11/5	12/5	13/5	14/5	15/5	16/5	17/5	18/5	19/5	20/5	21/5	22/5	23/5	24/5
Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13
S829	38.3	38.5	38.5	38.3	38.2	38.0	38.0	39.3	38.2	39.5	38.8	38.4	38.3	38.3
S830	38.5	38.4	38.6	38.7	38.8	38.0	38.1	37.8	37.8	37.0	37.7	38.5	38.5	38.0
S821	38.5	38.6	38.5	38.6	38.5	38.0	38.1	38.3	38.6	38.0	37.8	38.2	38.6	38.9
S822	38.4	38.3	38.2	38.1	38.2	37.8	37.8	37.0	38.5	37.0	37.8	37.6	37.7	37.4
S834	38.3	38.4	38.3	38.2	38.1	38.2	37.0	38.0	37.2	37.8	38.2	38.9	38.8	38.9
S835	38.9	38.6	38.3	38.1	38.0	38.4	37.9	38.5	38.0	37.8	37.5	37.4	37.0	37.4
S836	38.4	38.4	38.3	38.0	37.8	37.5	37.2	38.4	38.5	38.0	38.9	38.8	37.6	38.5
S996	38.5	38.4	38.5	38.7	38.1	37.9	37.8	38.9	38.0	37.1	38.5	38.4	38.6	37.5
S997	38.8	38.6	38.5	38.3	38.2	37.8	38.0	38.2	38.2	37.0	38.2	38.6	38.0	37.5

APPENDIX 2.4 CONT.

Date	25/5	26/5	27/5	28/5	29/5	30/5	31/5	1/6	2/6	3/6	4/6	5/6	6/6	7/6	8/6
Day	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
S804	38.2	37.0	38.0	38.2				38.0	38.5	38.0	38.2	38.4	38.5	38.0	37.9
S806	38.1	38.5				38.0	38.3	38.1	38.7	38.0	37.8	38.0	38.1	38.0	38.2
S807	38.4	37.7	38.9	39.0				37.4	38.7	38.1	38.0	37.5	37.7	37.8	38.0
S808	38.9	37.2	37.5	38.5					38.2	38.1	37.8	38.2	38.2	39.0	38.8
S809	38.7	37.7	37.7	38.4				38.0	38.3	38.2	38.3	38.0	38.2	38.0	37.9
S811	38.0	38.2	37.0	38.6				37.6	37.8	37.3	37.0	37.8	37.6	37.5	37.6
S812	37.6	37.2	37.5	37.6				37.1	38.3	38.0	38.1	38.5	38.3	37.8	37.9
S813	38.9	38.6	38.1	37.8				37.5	37.3	37.7	37.5	37.6	37.3	38.0	38.3
S814	38.8	38.4	38.2	38.3				37.4	37.6	37.8	37.8	37.5	37.2	37.5	37.3
S815	38.0	38.0	37.0	38.0				37.9	38.8	38.1	37.8	38.4	38.6	38.4	38.6
S817	38.0	37.5	38.0	38.4				38.5	38.3	38.2	38.3	38.0	38.4	38.3	38.2
S818	38.2	38.2		37.9				37.4	37.6	37.1	37.6	37.0	37.3	38.1	38 0
S819	37.8	38.3	38.2	38.4				37.9	38.5	37.9	38.0	38.1	38.0	37.8	37.7
S820	38.0	38.4	37.8	38.5				38.4	38.0	37.9	38.0	38.6	38.3	38.5	38.1
S821	37.9	38.2	38.5	37.8				37.2	38.2	38.0	37.8	37.5	37.7	37.8	38.0
S822	38.4	37.5	38.5	38.0				37.1	38.0	38.2	38.0	38.3	38.1	38.2	38.3
S823	38.1	37.5	37.2	38.2				38.0	37.9	37.7	37.8	38.4	38.2	37.8	37.7
S824	38.5	39.2	39.1	38.7				38.4	38.9	38.4	38.2	38.2	38.0	38.0	38.1
S825	38.5	38.0	37.8	38.0				38.5	38.8	38.5	38.0	38.0	38.3	38.4	38.3
S826	37.8	37.9	38.1	37.8				37.4	37.5	37.7	39.1	37.6	38.5	38.5	38.6
S828	38.6	37.7	37.0	37.5				37.3	37.3	37.6	37.5	37.3	37.5	37.6	37.4
S829	39.0	37.8	37.7	38.0				37.5	38.4	37.8	37.7	38.2	38.1	37.8	37.9
S830	38.0	38.5	38.6	38.0				38.0	38.5	38.1	38.4	38.0	38.2	38.3	38.2
S831	37.5	38.2	37.6	38.4				38.5	38.3	38.2	38.5	37.6	37.4	37.5	37.7

Cont.

APPENDIX 2.4 CONT.

Date	25/5	26/5	27/5	28/5	29/5	3035	31/5	1/6	2/6	3/6	4/6	5/6	6/6	7/6	8/6
Day	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
S832	37.8	37.5	38.0	38.1				38.2	39.2	38.5	38.3	38.0	38,4	38.0	37.9
S834	38.5	37.8	38.5	38.2				38.0		38.2	38.3	37.6	37.8	38.0	38.1
S835	38.6	37.6	38.7	38.2				38.1	37.8	38.0	38.1	38.0	38.4	38.0	38.4
S836	38.9	38.5	37.5	38.2				38.3	38.4	38.2	38.2	37.5	37.7	37.8	37.6
S996	38.3	38.2	37.0	38.5				38.7	38.9	38.3	38.0	37.6	37.5	37.9	38.0
S997	38.2	37.7	38.2	38.0				37.7	38.0	37.2	38.2	38.0	38.2	38.3	37.3

APPENDIX 2.4

MHC 2 : RECTAL TEMPERATURE (^{o}C) IN RESPONSE TO STABILATE INOCULATION ON DAY 29

Date	9/6	10/6	11/6	12/6	13/6	14/6	15/6	16/6	17/6	18/6	19/6	20/6	21/6	22/6
Day	29	30	31	32	33	34	35	36	37	38	39	40	41	42
S804	38.8	37.8	38.5	38.0	38.4	37.6	37.0	38.0	37.7	38.4	38.4	38.3	38.0	39.5
S806	37.0	37.5	38.4	38.5	38.2	38.1	37.1	37.6	38.5	38.8	39.4	39.6	39.5	40.4
S807	40.0	39.0	38.4	38.0	38.3	37.5	38.0	38.2	38.3	39.3	39.5	39.7	39.7	39.3
S808	42.1													
S809	38.7	37.0	38.4	38.7	38.5	38.4	38.5	38.7	37.6	38.2	39.4	39.6	39.2	39.8
S811	39.0	37.7	38.9	38.7	38.4	38.4	37.5	38.2	38.5	38.9	38.9	38.6	40.3	39.9
S812	38.2	38.0	38.3	38.6	38.7	37.2	37.6	37.8	38.5	39.0	40.0	39.8	40.3	40.9
S813	38.6	38.4	38.8	38.7	38.3	37.6	38.0	37.7	38.0	38.5	39.5	39.2	40.0	40.0
S814	38.7	38.2	38.5	38.6	38.5	37.9	38.0	38.4	38.6	38.9	39.5	39.7	40.0	40.9
S815	37.0	37.3	37.6	37.1	37.7	38.0	37.1	37.0	38.6	39.5	39.7	39.6	39.5	40.2
S817	38.0	37.4	38.5	38.6	38.1	37.8	37.7	38.3	38.2	39.0	38.2	38.2	40.5	39.9
S818	37.8	37.1	38.8	38.4	38.5	37.1	37.7	38.5	37.8	38.5	37.9	38.2	39.0	39.3
S819	38.8	38.2	38.5	38.4	38.6	38.2	38.4	38.5	38.6	39.0	40.4	39.8	39.7	41.2
S820	39.6	37.8	37.6	37.7	37.2	37.8	38.3	37.5	38.4	38.0	38.9	38.7	39.7	39.2
S821	38.4	37.9	38.5	38.8	37.9	38.0	38.5	38.7	38.0	38.8	38.2	38.4	38.8	39.4
S822	38.5	37.9	38.9	38.7	38.5	38.2	37.8	38.1	37.6	37.9	38.8	38.6	39.7	40.5
S823	38.5	38.0	38.5	37.7	37.6	38.0	37.2	37.7	38.5	38.3	38.7	38.7	39.3	39.4
S824	39.5	37.7	38.2	38.0	37.7	38.0	37.5	38.0	38.0	38.4	38.7	38.8	39.4	40.5
S825	39.0	37.0	38.6	38.8	38.4	37.4	37.0	38.4	38.0	38.6	38.0	38.2	40.0	40.7

Cont.

APPENDIX 2.4 CONT.

Date	9/6	10/6	11/6	12/6	13/6	14/6	15/6	16/6	17/6	18/6	19/6	20/2	21/6	22/6
Day	29	30	31	32	33	34	35	36	37	38	39	40	41	42
S826	38.4	37.7	37.8	37.5	37.7	38.2	38.2	38.2	38.3	38.5	38.5	38.6	39.6	40.2
S828	38.3	37.5	38.4	38.0	37.8	38.0	37.2	38.2	38.6	40.0	40.0	39.6	40.8	41.0
S829	38.5	38.2	39.0	38.9	38.5	37.5	37.8	38.4	38.1	38.7	38.6	38.8	39.4	39.8
S830	39.0	38.0	39.5	38.8	38.7	39.5	39.4	39.4	39.2	39.2	38.3	38.4	39.2	39.0
S831	38.0	37.7	38.6	38.4	38.6	37.7	37.6	37.4	37.4	38.1	38.5	38.6	39.7	40.0
S832	38.3	37.6	38.8	38.4	38.5	38.2	37.0	38.2	38.5	38.9	38.5	38.7	40.3	40.7
S834	38.4	38.2	38.8	37.4	37.7	38.0	37.2	38.4	38.4	38.7	38.6	38.8	39.6	39.9
S835	38.7	37.2	38.2	38.0	37.6	39.9	37.4	38.2	38.4	38.7	38.7	38.9	40.4	40.0
S836	39.6	38.1	38.5	38.3	37.7	38.1	37.9	38.0	38.2	38.6	39.0	39.1	39.7	40.6
S996	38.5	37.5	38.0	37.9	37.4	38.8	37.8	38.0	38.4	39.7	38.3	38.4	39.9	40.3
S997	38.5	38.8	38.8	38.4	38.6	37.9	37.0	38.7	38.2	38.5	37.4	37.7	39.6	39.7
S590		37.8	38.4	38.1	38.0	38.1	38.0	38.0	38.4	38.2	38.0	38.3	39.0	39.5
S605		37.0	37.5	37.7	37.6	37.7	38.4	39.6	38.5	38.8	38.1	38.4	40.8	40.0

APPENDIX 2.4

MHC 2 : RECTAL TEMPERATURES (^{o}C) IN RESPONSE TO STABILATE INOCULATION ON DAY 29

Date	23/6	24/6	25/6	26/6	27/6	28/6	29/6	30/6	1/7	2/7	3/7	4/7	5/7	6/7
Day	43	44	45	46	47	48	49	50	57	52	53	54	55	56
S804	38.5	39.0	41.2	40.9	40.0	40.4	39.6	37.6						
S806	40.0	39.8	41.8											
S807	39.4	39.9	40.1	40.5	40.2									
S808														
S809	40.0	40.5	40.9	40.3	39.6	38.6								
S811	40.4	40.0	41.6	41.5	40.0	39.3	39.0	38.5	38.1	39.0	38.4	38.2	37.7	37.4
S812	39.9	40.3	41.0	40.7										
*S813	40.7	40.8												
*S814	40.5	40.2												
S815	39.3	39.0	41.2	41.5	39.6	39.4								
S817	40.4	39.4	41.2	40.8	40.0	39.7	39.0	38.0	38.1	38.0	37.6	37.9	37.7	38.0
*S818	39.4	40.0												
*S819	41.5	40.7												
*S820	40.0	39.9												
S821	40.0	40.3	39.0	39.4	39.0	38.6	38.5	37.5	38.7	38.5	38.4	38.1	37.0	37.5
*S822	40.7	40.6												
*S823	39.2	40.2												
*S824	40.2	40.4												
*S825	40.5	40.0												
*S826	40.0	39.4												
S828	40.1	39.5	40.6	40.8	39.0	40.1	38.2							

Cont.

APPENDIX 2.4 CONT.

Day	43	44	45	46	47	48	49	50	57	52	53	54	55	56
Date	23/6	24/6	25/6	26/6	27/6	28/6	29/6	30/6	1/7	2/7	3/7	4/7	5/7	6/7
*S829	39.5	40.5												
S830	40.3	40.3	40.4	40.7	40.5	39.3	38.5							
*S831	40.0	40.1												
S832	41.2	39.4	41.0	40.8	39.5	40.3	39.2	40.4						
*S834	40.4	40.6												
*S835	40.1	39.5												
*S836	40.7	39.5												
*S996	40.4	399												
*S997	40.2	39.2												
S590	40.2	40.0	41.4	41.5	40.0	40.9								
S605	39.5	39.3	39.0	39.3	39.5	40.0	40.0	40.2	36.5					

* Treated on day 44 (day 15 post stabilate challenge)

APPENDIX 2.5

MHC 2 : PARASITOSES IN RESPONSE TO STABILATE CHALLENGE ON DAY 29

Day		34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62		BoLA status with respect to previously inoculated cell line
S804	REG			+	+	2+	NE	NE	NE	NE	NE	3+	+	NE	2+	2+	2+	3+												Died day 50	½ match
	LPG						NE	3+	2+	3+	NE	3+	3+	+	NE	+	NE	NE													
	Blood								+	+	+	10%	30%	30%	60%	25%	35%	40%													
S806	REG			+	+	+	+	3+	3+	2+	3+	+	3+																		Died day 45	mismatch
	LPG					+		3+	3+	3+	NE	3+	3+																			
	Blood									NE	NE		NE																			
S807	REG	+	+	NE	2+	2+	2+	3+	3+	2+	+	3+	NE	+	2+																Died day 48	½ match
	LPG		NE				2+	3+	+	2+	3+	3+	3+	+	3+																	
	Blood									NE		7%	20%	20%	50%																	
S809	REG			+	+	+		3+	3+	2+	+	3+	NE	3+	NE	+																Died day 48	mismatch
	LPG							3+	2+	2+	2+	3+	3+	3+	NE	NE																
	Blood											10%	+	40%	50%	50%																
S811	REG				2+	+	3+	+	3+	3+	3+	+	3+	+		2+	2+	2+	3+	3+	3+	2+		NE	+							match
	LPG						2+	NE	+	2+	NE	+	NE	+		+	+			+	+			NE								
	Blood							NE					+	+	2%	2%	NE	2%	NE	1%	2%	1%	NE	+	1%	4%	5%	5%				
S812	REG			+	+	2+	2+	+	3+	+	3+	3+	3+	3+																	Died day 46	½ match
	LPG						2+	2+	3+	3+	3+	3+	3+	3+																		
	Blood											2%	20%	NE																		
S813	REG			+	+	NE	2+	2+	3+	2+	NE	+																			Treated day 44	½ match
	LPG						2+	+	3+	3+	2+	+																				
	Blood											2%																				
S814	REG			+	+	3+	NE	3+	3+	NE	NE	+																			Treated day 44	mismatch
	LPG						NE	3+	2+	NE	2+	3+																				
	Blood									NE		+																				
S815	REG			+	+	2+	2+	+	3+	3+	3+	3+	+	3+	3+	3+															Died day 49	½ match
	LPG						2+	+	+	3+	3+	3+	3+	3+	3+	+																
	Blood											+	20%	40%	50%	35%																
S817	REG					+	2+	2+	3+	2+	2+	3+	2+	+	3+	+								NE	NE	NE						match
	LPG							2+			3+	2+	3+	+	3+	2+	2+								NE		NE					
	Blood											2%	20%	+	30%	35%	25%	20%	NE	20%	10%	10%	12%	10%	10%	+		10%				
S818	REG			+	+	2+	2+	3+	NE	2+	3+	3+																			Treated day 44	½ match
	LPG						+	3+	+	NE	3+	3+																				
	Blood											2%																				
S819	REG				+	NE	NE	+	+	NE	3+	3+																				Treated day 44	½ match
	LPG							+	+	NE	3+	3+																				
	Blood											2%																				
S820	REG					+	NE	+	2+	3+	3+	3+																				Treated day 44	½ match
	LPG							+	2+	+	+	3+																				
	Blood											+																				

Cont.

Day		34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62		BoLA status with respect to previously inoculated cell line
S821	REG			NE			+	+	3+	2+	2+	NE	+	+	+		+	+														match
	LPG						+			2+		+	+	+	2+	+																
	Blood											+	+		+	+	+	+		+												
S822	REG				+	+	2+	2+	3+	3+	3+	3+																			Treated day 44	mismatch
	LPG						2+	2+	2+	NE	3+	3+																				
	Blood											+																				
S823	REG			NE	+	NE	NE	2+	3+	+	3+	3+																			Treated day 44	mismatch
	LPG							+	3+		+	3+																				
	Blood											+																				
S824	REG				NE	2+	+	2+	3+	2+	2+	3+																			Treated day 44	½ match
	LPG						NE	3+	+	NE	2+	3+																				
	Blood											+																				
S825	REG			+	+	+	2+	+	3+	3+	3+	3+																			Treated day 44	½ match
	LPG						2+	+		2+	3+	3+																				
	Blood											+																				
S826	REG			+	+	2+	+	+	3+	2+	+	NE																			Treated day 44	½ match
	LPG					2+	NE	+	+	3+	3+	3+																				
	Blood											10%																				
S828	REG				+	2+	NE	NE	3+	3+	3+	3+	3+	3+	3+	3+															Died day 49	match
	LPG						+		3+	3+	+	+	3+	3+	3+	3+																
	Blood											7%	17%	30%	35%	60%																
S829	REG					NE	+	3+	2+	2+	2+	3+																			Treated day 44	mismatch
	LPG						+	+	2+	+	2+	3+																				
	Blood											2%																				
S830	REG						2+	3+	3+	3+	2+	3+	3+	3+	3+	3+	3+															Died day 49	match
	LPG						+	3+			3+	2+	3+	2+	3+	3+	3+															
	Blood											+	20%	5%	35%	25%	40%															
S831	REG			+	+	2+	2+	3+	3+	3+	2+	3+																			Treated day 44	½ match
	LPG						2+	3+	2+	3+	3+	3+																				
	Blood											2%																				
S832	REG						NE	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	NE													Died day 50	match
	LPG						NE	3+	+	2+	3+	3+	+	3+	3+	NE	NE	3+														
	Blood											+	2%	7%	10%	20%	20%	25%														
S834	REG				+	2+	2+	3+	2+	3+	+	+																			Treated day 44	½ match
	LPG						2+	3+	+	NE	2+	3+																				
	Blood											+																				

Cont.

Day		34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62		BoLA status with respect to previously inoculated cell line
S835	REG					NE	2+	3+	3+	3+	3+	3+																			Treated day 44	½ match
	LPG					+	2+	3+	2+	3+	3+	+																				
	Blood											+																				
S836	REG			+	+	3+	2+	2+	3+	3+	NE	3+																			Treated day 44	½ match
	LPG						2+	2+	3+	3+	3+	3+																				
	Blood											2%																				
S996	REG				+	NE	+	3+	2+	2+	3	3+																			Treated day 44	½ match
	LPG						+	3+	2+	2+	+	3+																				
	Blood											2%																				
S997	Reg						2+	+	NE	2+	3+	3+																			Treated day 44	½ match
	LPG						+	+	2+	NE	2+	3+																				
	Blood											2%																				
*S590	REG			+	+	NE	NE	3+	3+	3+	NE	+	3+	3+	3+	+															Died day 48	challenge control*
	LPG			+			NE	3+	+	2+	3+	3+	3+	3+	3+	NE															
	Blood									+		10%	+	30%	50%	60%																
*S605	REG			+	+	+	2+	3+	3+	3+	3+	+	3+	3+	NE	NE	2+	3+	3+												Died day 57	challenge control*
	LPG			+				3+	3+	3+	3+	3+	3+	3+	NE	3+	3+	+	3+												
	Blood											+	30%	30%	60%	50%	80%	50%	NE													

* Not previously inoculated with LCL

NE - Notexamined

APPENDIX 3.1

REACTIVITIES OF PBL USED IN ALLOREACTIVE CYTOTOXIC CELL GENERATIONS AND TARGET LCLs, WITH THE EDINBURGH PANEL OF BoLA TYPING SERA

	Workshop Specificities	Edinburgh codes of sera giving positive reactions	
		Workshop Defining	Additional Reactions
A. PBL			
B470	6,7	9,10,66,75,12,81,97/11,17,67,68	43,69,96+78,85,91,105
B487	5,20	7,90/78	85,105
B807	1,6.2	1,8,83/9,10,66,75,12,81,86,97	30,91,105
S191	7,10	11,17,67,68/69,71,96	13,85,93,105
S811	8,11	13,63,80/73,76,95a,102	72,74,85,99
S814	13,20	5,16/78	28,72,74,88,98,101a,109
S816	8,11	13,63,80/73,76,95a,102	30,72,74,85,91,99,105
S817	8,11	13,63,80/73,76,95a,102	30,72,74,85,91,99
S821	8,11	13,63,80/73,76,95a,102	30,72,74,91,99
S995	8,11	13,63,80/73,76,95a,102	30,72,74,91,99
B. Target LCLs			
B166	4,10	69,71,96/82	13,63,72,85,105
B470	6,7	9,10.66,75,12,81,92,97/11,17,67,68	96,85,91,96,101,105,109
B487	5,20	7,90/78	85,105
B807	1,6.2	1,8,83/9,10,66,75,12,81,86,97	30,91,105
S191	7,10	11,17,67,68/69,71,96	13,85,105

Cont.

APPENDIX 3.1 CONT. (2)

	Workshop Specificities	Edinburgh codes of sera giving positive reactions	
		Workshop Defining	Additional Reactions
S803	8,13	13,63,80/5,16	72,87,88,91,95b,96,98,101a,101b,102,104 109,110
S804	11,16	73,76,95a,102/38,43	13,72,88,91,101a,104,105,109,110
S807	11,13	73,76,95a,102/5,16	72,91,95b,101b,105,109,110
S811	8,11	13,63,80/73,76,95a,102	72,74,81,95b,96,110
S814	13,20	5,16/78	72,88,95b,98,101a,109
S815	8,13	13,63,80/5,16	72,74,81,88,95b,98,101a,101b,102,104, 109,110
S816	8.11	13,63,80/73,76,95a,102	30,72,74,91,105
S817	8,11	13,63,80/73,76,95a,102	72,74,91,95b,96,101a,102,103,104,109,110
S818	6,8	9,10,66,75,81,97,100/13,63,80	72,74,95b,96,104,110
S819	11,16	73,76,95a,102/38,43	72,88,91,105,109,110
S821	8,11	13,63,80/73,76,95a,102	72,74,91,92,95b,104,105,110
S823	10,13	69,71,96/5,16	72,85,88,95b,98,101a,101b,105,109,110
S828	8,11	13,63,80/73,76,95a,102	72,74,91,95b,110
S829	13,16	5,16/38,43	13,30,72,88,91,95b,98,101a,101b,105,109, 110
S834	6,11	9,10,66,75,97,100/73,76,95a,102	72,91,95b,104,110
S836	8,13	13,63,80/5,16	72,88,95b,98,101a,109,110

APPENDIX 3.2

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	% Cr^{51} release
A1	S814	Water lysis	2993		
			3314		
			3587		
			$\bar{x}$ 3298		
		Medium release	354		
			362		
			283		
			$\bar{x}$ 333		
		S191/S814	3800	40:1	100
			4327		
			$\bar{x}$ 4064		
		S191/S816	973	40:1	21
			928		
			$\bar{x}$ 951		
		S191/S821	648	40:1	11
			664		
			$\bar{x}$ 656		
		S191/medium	364	40:1	2
			403		
			$\bar{x}$ 384		
A1	S816	Water lysis	3442		
			3067		
			-		
			$\bar{x}$ 3255		
		Medium release	267		
			276		
			319		
			$\bar{x}$ 287		
		S191/S814	904	40:1	19
			787		
			$\bar{x}$ 845		
		S191/S816	3147	40:1	100
			3399		
			$\bar{x}$ 3273		
		S191/S821	2624	40:1	75
			2400		
			$\bar{x}$ 2512		
		S191/S816	356	40:1	2
			330		
			$\bar{x}$ 343		
A1	S821	Water lysis	4219		
			4943		
			5343		
			5312		
			$\bar{x}$ 4825		

Cont.

APPENDIX 3.2 CONT. (2)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	% Cr^{51} release
		Medium release	477		
			387		
			533		
			$\bar{x}$ 466		
		S191/S814	915	40:1	10
			884		
			$\bar{x}$ 900		
		S191/S816	4887	40:1	100
			4965		
			$\bar{x}$ 4926		
		S191/S821	4075	40:1	78
			3994		
			$\bar{x}$ 3870		
		S191/medium	370	40:1	0
			318		
			$\bar{x}$ 344		
A2	S814	Freeze/thaw	4909		
			5381		
			5009		
			$\bar{x}$ 5700		
		Medium release	420		
			394		
			398		
			$\bar{x}$ 404		
		B487/S814	4572	75:1	87
			4382		
			$\bar{x}$ 4477		
		B487/S821	864	65:1	10
			911		
			$\bar{x}$ 888		
A2	S821	Freeze/thaw	2950		
			3141		
			2860		
			$\bar{x}$ 2964		
		Medium release	270		
			305		
			298		
			$\bar{x}$ 291		
		B487/S814	787	75:1	21
			989		
			$\bar{x}$ 843		
		B487/S821	2164	65:1	70
			2143		
			$\bar{x}$ 2154		

Cont.

APPENDIX 3.2 CONT. (3)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	% Cr^{51} release
A3	S821	Freeze/thaw	2950		
			3141		
			2860		
			$\bar{x}$ 2964		
		Medium release	270		
			305		
			298		
			$\bar{x}$ 291		
		B807/S821	2401	80:1	76
			2234		
			$\bar{x}$ 2318		
		B807/B470	477	80:1	8
			545		
			$\bar{x}$ 511		
A3	B470	Freeze/thaw	3300		
			3217		
			-		
			$\bar{x}$ 3259		
		Medium release	517		
			436		
			508		
			$\bar{x}$ 487		
		B807/S821	606	80:1	4
			593		
			$\bar{x}$ 600		
		B807/B470	2607	80:1	75
			2545		
			$\bar{x}$ 2576		
A4	S811	Freeze/thaw	5671		
			5756		
			6206		
			$\bar{x}$ 5878		
		Medium release	1370		
			-		
			$\bar{x}$ 1357		
		S191/S811	3944		
			3737		
			$\bar{x}$ 3841		
		S191/S821	3966		
			3907		
			$\bar{x}$ 3937		
	S821	Freese/thaw	10059		
			10173		
			10189		
			$\bar{x}$ 10140		
		Medium release	1274		

Cont.

APPENDIX 3.2 CONT. (4)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	% CR^{51} release
			1173		
			-		
			$\bar{x}$ 1224		
		S191/S811	5329	80:1	50%
			6009		
			$\bar{x}$ 5669		
		S191/S821	7222	80:1	70%
			7670		
			$\bar{x}$ 7446		
	S816	Freeze/thaw	6073		
			6906		
			6584		
			$\bar{x}$ 6521		
		Medium release	1941		
			1413		
			1136		
			$\bar{x}$ 1497		
		S191/S811	3814	80:1	46%
			3808		
			$\bar{x}$ 3811		
		S191/S821	4624	80:1	63%
			4638		
			$\bar{x}$ 4638		
A4	S817	Freeze/thaw	4568		
			5389		
			4977		
			$\bar{x}$ 4978		
		Medium release	2093		
			2035		
			1871		
			$\bar{x}$ 2000		
		S191/S811	3764	80:1	63%
			3985		
			$\bar{x}$ 3875		
		S191/S821	4050	80:1	71%
			4182		
			$\bar{x}$ 4116		
A4	S828	Freeze/thaw	4619		
			4881		
			4836		
			$\bar{x}$ 4779		
		Medium release	2172		
			2112		
			1954		

Cont.

APPENDIX 3.2 CONT: (5)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	% CR^{51} release
			$\bar{x}$ 2079		
		S191/S811	3115	80:1	36%
			2969		
			$\bar{x}$ 3042		
		S191/S81	3414	80:1	48%
			3357		
			$\bar{x}$ 3386		
A4	S814	Freeze/thaw	7375		
			7828		
			6792		
			$\bar{x}$ 7332		
		Medium release	1616		
			1276		
			1172		
			$\bar{x}$ 1354		
		S191/S811	1216	80:1	0%
			1012		
			$\bar{x}$ 1114		
		S191/S821	1087	80:1	0%
			1160		
			$\bar{x}$ 1124		
A4	S823	Freeze/thaw	8497		
			8766		
			8278		
			$\bar{x}$ 8514		
		Medium release	2515		
			1900		
			1479		
			$\bar{x}$ 1965		
		S191/S811	1347	80:1	0%
			1240		
			$\bar{x}$ 1294		
		S191/S821	1082	80:1	0%
			1030		
			$\bar{x}$ 1056		
A4	S829	Freeze/thaw	5333		
			S384		
			5789		
			$\bar{x}$ 5502		
		Medium release	842		
			660		
			526		
			$\bar{x}$ 676		
		S191/S811	505	80:1	0%

Cont.

APPENDIX 3.2 CONT. (6)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	% CR^{51} release
			557		
			$\bar{x}$ 531		
		S191/S821	584	80:1	0%
			694		
			$\bar{x}$ 639		
A4	B470	Freeze/Thaw	4095		
			4398		
			4110		
			$\bar{x}$ 4201		
		Medium release	800		
			680		
			588		
			$\bar{x}$ 689		
		S191/S811	484	80:1	0%
			487		
			$\bar{x}$ 486		
		S191/S821	405	80:1	0%
			442		
			$\bar{x}$ 424		
A4	B166	Freeze/thaw	4430		
			4412		
			4237		
			$\bar{x}$ 4360		
		Medium release	490		
			484		
			398		
			$\bar{x}$ 424		
		S191/S811	1887	80:1	34%
			1634		
			$\bar{x}$ 1761		
		S191/S821	1165	80:1	18%
			1137		
			$\bar{x}$ 1151		
A5	S811	Freeze/thaw	2903		
			2762		
			2822		
			$\bar{x}$ 2829		
		Medium release	300		
			289		
			299		
			$\bar{x}$ 296		
		S191/S811	1732	160:1	60%
			1894		
			$\bar{x}$ 1813		
		S191/S821	2218	160:1	76%
			2221		
			$\bar{x}$ 2220		

APPENDIX 3.2 CONT. (7)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	% CR^{51} release
A5	S821	Freeze/thaw	4059		
			4368		
			4535		
			$\bar{x}$ 4321		
		Medium release	374		
			401		
			414		
			$\bar{x}$ 306		
		S191/S811	3336	160:1	79%
			3661		
			$\bar{x}$ 3499		
		S191/S821	4266	160:1	100%
			4613		
			$\bar{x}$ 4440		
A5	S803	Freeze/thaw	4027		
			4005		
			4188		
			$\bar{x}$ 4073		
		Medium release	877		
			785		
			-		
			$\bar{x}$ 831		
		S191/S811	1861	160:1	38%
			233		
			$\bar{x}$ 2047		
		S191/S821	2303	160:1	47%
			2435		
			$\bar{x}$ 2369		
A5	S815	Freeze/thae	4401		
			3668		
			4003		
			$\bar{x}$ 4024		
		Medium release	390		
			403		
			416		
			$\bar{x}$ 403		
		S191/S811	1351	160:1	27%
			1412		
			$\bar{x}$ 1382		
		S191/S821	1757	160:1	36%
			1675		
			$\bar{x}$ 1716		
A5	S818	Freeze/thaw	2773		
			2570		
			-		
			$\bar{x}$ 2672		
		Medium release	342		

Cont.

APPENDIX 3.2 CONT. (8)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	$\frac{1}{2}CR^{51}$ release
			391		
			448		
			$\bar{x}$ 394		
		S191/S811	1310	160:1	40%
			1281		
			$\bar{x}$ 41296		
		S191/S821	1257	160:1	41%
			1397		
			$\bar{x}$ 1327		
A5	S836	Freeze/thaw	3116		
			3090		
			-		
			$\bar{x}$ 3103		
		Medium release	314		
			359		
			322		
			$\bar{x}$ 332		
		S191/S811	1776	160:1	54%
			1907		
			$\bar{x}$ 1842		
		S191/S821	1756	160:1	52%
			1773		
			$\bar{x}$ 1765		
A5	S804	Freeze/thaw	3187		
			3089		
			-		
			$\bar{x}$ 3138		
		Medium release	415		
			428		
			413		
			$\bar{x}$ 419		
		S191/S811	1741	1:160	49%
			1780		
			$\bar{x}$ 1761		
		S191/S821	2292	1:160	67%
			2180		
			$\bar{x}$ 2236		
A5	S807	Freeze/thaw	2166		
			2173		
			2201		
			$\bar{x}$ 2180		
		Medium release	255		
			331		
			345		
			$\bar{x}$ 310		
		S191/S811	1343	160:1	54%
			1312		
			$\bar{x}$ 1328		

Cont.

APPENDIX 3.2 CONT. (9)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	% CR^{51} release
		S191/S821	1690	160:1	77%
			1805		
			$\bar{x}$ 1748		
A5	S819	Freeze/thaw	2610		
			2912		
			2811		
			$\bar{x}$ 2778		
		Medium release	398		
			370		
			431		
			$\bar{x}$ 400		
		S191/S811	1912	160:1	64%
			1945		
			$\bar{x}$ 1929		
		S191/S821	2485	160:1	87%
			2443		
			$\bar{x}$ 2464		
A5	S834	Freeze/thaw	2258		
			2732		
			2174		
			$\bar{x}$ 2388		
		Medium release	308		
			328		
			283		
			$\bar{x}$ 306		
		S191/S811	1709	160:1	69%
			1761		
			$\bar{x}$ 1735		
		S191/S821	2322	160:1	100%
			2526		
			$\bar{x}$ 2424		
A5	S823	Freeze/thaw	3043		
			3428		
			3618		
			$\bar{x}$ 3363		
		Medium release	345		
			425		
			439		
			$\bar{x}$ 403		
		S191/S811	701	160:1	9%
			663		
			$\bar{x}$ 682		
		S191/S821	679	160:1	11%
			779		
			$\bar{x}$ 729		

Cont.

APPENDIX 3.2 CONT. (10)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	%CR^{51} release
A5	S829	Freeze/thaw	3039		
			3510		
			3364		
			$\bar{x}$ 3304		
		Medium release	351		
			300		
			-		
			$\bar{x}$ 326		
		S191/S811	485	160:1	6%
			530		
			$\bar{x}$ 508		
		S191/S821	546	160:1	8%
			573		
			$\bar{x}$ 560		
A5	B166	Freeze/thaw	1374		
			1202		
			$\bar{x}$ 1311		
		Medium release	230		
			220		
			205		
			$\bar{x}$ 218		
		S191/S811	543	160:1	28%
			510		
			$\bar{x}$ 527		
		S191/S821	534	160:1	25%
			451		
			$\bar{x}$ 493		
A6	S811	Freeze/thae	3180		
			3502		
			3376		
			$\bar{x}$ 3353		
		Medium release	528		
			351		
			482		
			$\bar{x}$ 454		
		B470/S811	2692	160:1	80%
			2837		
			$\bar{x}$ 2765		
		B470/S821	2948	160:1	83%
			2798		
			$\bar{x}$ 2873		
A6	S821	Freeze/thaw	3070		
			3125		
			3007		
			$\bar{x}$ 3067		
		Freeze release	466		
			409		
			373		

Cont.

APPENDIX 3.2 CONT. (11)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	%CR^{51} release
			$\bar{x}$ 416		
		B470/S811	3267	160:1	100%
			3390		
			$\bar{x}$ 3329		
		B470/S821	3118	160:1	100%
			3201		
			$\bar{x}$ 3160		
A6	S816	Freeze/thaw	2059		
			2056		
			2130		
			$\bar{x}$ 2082		
		Medium release	391		
			403		
			371		
			$\bar{x}$ 388		
		B470/S811	2013	160:1	93%
			1917		
			1965		
		B470/S821	1896	160:1	87%
			1819		
			$\bar{x}$ 1858		
A6	S817	Freeze/thaw	5714		
			5516		
			5867		
			$\bar{x}$ 5699		
		Medium release	403		
			356		
			429		
			$\bar{x}$ 396		
		B470/S811	4878	160:1	80%
			4737		
			$\bar{x}$ 4658		
		B470/S821	4618	100:1	77%
			4332		
			$\bar{x}$ 4475		
A6	S828	Freeze/thaw	3898		
			3648		
			3652		
			$\bar{x}$ 3733		
		Medium release	296		
			366		
			330		
			$\bar{x}$ 331		
		B470/S811	2916	160:1	80%
			3218		
			$\bar{x}$ 3067		
		B470/S821	2848	160:1	75%
			2903		
			$\bar{x}$ 2876		

APPENDIX 3.2 CONT. (12)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	$\%CR^{51}$ release
A6	S803	Freeze/thaw	3828		
			4000		
			3939		
			$\bar{x}$ 3922		
		Medium release	484		
			550		
			597		
			$\bar{x}$ 544		
		B470/S811	2176	160:1	47%
			2115		
			$\bar{x}$ 2146		
		B470/S821	1885	160:1	44%
			2184		
			$\bar{x}$ 2035		
A6	S815	Freeze/thaw	5576		
			5651		
			6028		
			$\bar{x}$ 5752		
		Medium release	404		
			493		
			-		
		B470/S811	$\bar{x}$ 2313	160:1	35%
			2259		
			$\bar{x}$ 2286		
		B470/S821	2197	160:1	33%
			2239		
			$\bar{x}$ 2218		
A6	S818	Freeze/thaw	2816		
			2891		
			2634		
			$\bar{x}$ 2780		
		Medium release	485		
			434		
			433		
			$\bar{x}$ 451		
		B470/S811	2217	160:1	76%
			2226		
			$\bar{x}$ 2222		
		B470/S821	1723	160:1	58%
			1864		
			$\bar{x}$ 1794		
A6	S836	Freeze/thaw	3783		
			4050		
			4117		
			$\bar{x}$ 3983		
		Medium release	538		

Cont.

APPENDIX 3.2 CONT. (13)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	%CR^{51} release
			626		
			400		
			$\bar{x}$ 521		
		B470/S811	2836	160:1	68%
			2911		
			$\bar{x}$ 2874		
		N470/S821	2554	160:1	56%
			2398		
			$\bar{x}$ 2476		
A6	S804	Freeze/thaw	4417		
			4737		
			4955		
			$\bar{x}$ 4703		
		Medium release	548		
			560		
			588		
			$\bar{x}$ 555		
		B470/S811	3393	160:1	70%
			3548		
			$\bar{x}$ 3471		
		B470/S821	3215	160:1	62%
			3019		
			$\bar{x}$ 3117		
A6	S807	Freeze/thaw	3519		
			3873		
			3799		
			$\bar{x}$ 3732		
		Medium release	749		
			672		
			$\bar{x}$ 704		
		B470/S811	2085	160:1	43%
			1940		
			$\bar{x}$ 2013		
		B470/S821	1836	160:1	40%
			1985		
			$\bar{x}$ 1911		
A6	S819	Freeze/thaw	3211		
			3396		
			3487		
			$\bar{x}$ 3365		
		Medium release	751		
			903		
			1137		
			$\bar{x}$ 930		
		B470/S811	2282	160:1	57%
			2375		
			$\bar{x}$ 2329		

Cont.

APPENDIX 3.2 CONT. (14)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	%CR^{51} release
		B470/S821	2040	160:1	48%
			2144		
			$\bar{x}$ 2092		
A6	S834	Freeze/thaw	3977		
			4256		
			4009		
			$\bar{x}$ 4101		
		Medium release	497		
			584		
			$\bar{x}$ 541		
		B470/S811	3213	160:1	72%
			2992		
			$\bar{x}$ 3103		
		B470/S821	2847	160:1	66%
			2913		
			$\bar{x}$ 2880		
A6	S814	Freeze/thaw	5906		
			5930		
			5823		
			$\bar{x}$ 5888		
		Medium release	517		
			549		
			564		
			$\bar{x}$ 543		
		B470/S811	1561	160:1	19%
			1557		
			$\bar{x}$ 1559		
		B470/S821	1839	160:1	26%
			2035		
			$\bar{x}$ 1937		
A6	S823	Freeze/thaw	6164		
			5852		
			6565		
			$\bar{x}$ 6194		
		Medium release	413		
			505		
			547		
			$\bar{x}$ 488		
		B470/S811	1173	160:1	13%
			1338		
			$\bar{x}$ 1256		
		B470/S821	1510	160:1	19%
			1593		
			$\bar{x}$ 1552		

Cont.

APPENDIX 3.2 CONT. (15)

Experiment	TArget	Responder/ stimulator	1 minute gamma counts	E/T	%CR^{51} release
A6	S829	Freeze/thaw	3514		
			3535		
			3738		
			$\bar{x}$ 3596		
		Medium release	943		
			778		
			883		
			$\bar{x}$ 868		
		B470/S811	468	160:1	0%
			551		
			$\bar{x}$ 510		
		B470/S821	510	100:1	0%
			500		
			$\bar{x}$ 505		
A6	S191	Freeze/thaw	5210		
			5948		
			5633		
			$\bar{x}$ 5597		
		Medium release	991		
			624		
			1069		
			$\bar{x}$ 895		
		B470/S811	1052	160:1	5%
			1165		
			$\bar{x}$ 1109		
		B470/S821	1355	160:1	11%
			1515		
			$\bar{x}$ 1435		
A6	B166	Freeze/thaw	1808		
			1961		
			1880		
			$\bar{x}$ 1883		
		Medium release	291		
			243		
			259		
			$\bar{x}$ 264		
		B470/S811	373	160:1	5%
			317		
			$\bar{x}$ 345		
		B470/S821	324	160:1	6%
			390		
			$\bar{x}$ 357		
B1	811	Freeze/thaw	7335		
			7518		
			7054		
			$\bar{x}$ 7302		
		Medium release	656		

Cont.

APPENDIX 3.2 CONT. (16)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	%CR^{51} release
			582		
			667		
			$\bar{x}$ 635		
		S995/S811	4136	80:1	51%
			3911		
			$\bar{x}$ 4024		
		S995/S817	2872	80:1	34%
			2879		
			$\bar{x}$ 2876		
B1	S817	Freeze/thaw	8293		
			8459		
			8326		
			$\bar{x}$ 8359		
		Medium release	901		
			1076		
			1071		
			$\bar{x}$ 998		
		S995/S811	5380	80:1	58%
			5119		
			$\bar{x}$ 5250		
		S995/S817	3824	80:1	40%
			4050		
			$\bar{x}$ 3937		
B1	S821	Freeze/thaw	4494		
			4344		
			4450		
			$\bar{x}$ 4429		
		Medium release	494		
			409		
			480		
			$\bar{x}$ 461		
		S995/S811	467	80:1	0%
			382		
			$\bar{x}$ 425		
		S995/S817	382	80:1	0%
			408		
			$\bar{x}$ 395		
B1	S816	Freeze thaw	9288		
			10103		
			11093		
			$\bar{x}$ 10161		
		Medium release	586		
			734		
			588		
			$\bar{x}$ 636		
		S995/S811	787	80:1	2%

Cont.

APPENDIX 3.2 CONT. (17)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	%CR^{51} release
			819		
			$\bar{x}$ 803		
		S996/S817	855	80:1	2%
			892		
			$\bar{x}$ 874		
B1	S828	Freeze/thaw	4689		
			4999		
			5724		
			$\bar{x}$ 4937		
		Medium release	470		
			495		
			575		
			$\bar{x}$ 493		
		S995/S811	3928	80:1	74%
			3660		
			$\bar{x}$ 3794		
		S995/S817	3066	80:1	56%
			2898		
			$\bar{x}$ 2982		
B1	S829	Freeze thaw	4839		
			5421		
			5474		
			$\bar{x}$ 5245		
			343		
			380		
			473		
			$\bar{x}$ 399		
		S995/S811	409	80:1	0%
			367		
			$\bar{x}$ 388		
		S995/S817	421	80:1	0%
			353		
			$\bar{x}$ 387		
B1	S191	Freeze/thaw	7289		
			7174		
			-		
			$\bar{x}$ 7232		
		Medium release	1092		
			1174		
			1060		
			$\bar{x}$ 1109		
		S995/S811	818	80:1	0%
			835		
			$\bar{x}$ 827		
B1	B487	Freeze/thaw	8763		

Cont.

APPENDIX 3.2 CONT. (18)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	%CR^{51} release
		Freeze/thaw	7938		
			8063		
			$\bar{x}$ 8255		
		Medium release	563		
			628		
			497		
			$\bar{x}$ 563		
		S995/S811	1784	80:1	16%
			1840		
			$\bar{x}$ 1812		
B2	S811	Freeze/thaw	3673		
			-		
			-		
			$\bar{x}$ 3673		
		Medium release	301		
			301		
			348		
			$\bar{x}$ 317		
		S816/S811	3193	160:1	88%
			3349		
			$\bar{x}$ 3271		
		S816/S821	776	160:1	14%
			773		
			$\bar{x}$ 775		
B2	S821	Freeze/thaw	5473		
			5848		
			5550		
			$\bar{x}$ 5623		
		Medium release	720		
			773		
			743		
			$\bar{x}$ 745		
		S816/S811	680	160:1	0%
			754		
			$\bar{x}$ 717		
		S816/S821	885	160:1	2%
			831		
			$\bar{x}$ 858		
B2	S817	Freeze/thaw	5828		
			5517		
			5285		
			$\bar{x}$ 5543		
		Medium release	669		
			718		
			625		
			$\bar{x}$ 671		
		S816/S811	5800	160:1	98%

Cont.

APPENDIX 3.2 CONT. (19)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	$\%CR^{51}$ release
			5043		
			$\bar{x}$ 5422		
		S816/S821	1216	160:1	11%
			1216		
			$\bar{x}$ 1216		
B2	S828	Freeze/thaw	2865		
			3256		
			3681		
			$\bar{x}$ 3268		
		Medium release	1118		
			1085		
			1026		
			$\bar{x}$ 1076		
		S816/S811	3858	160:1	100%
			3731		
			$\bar{x}$ 3795		
		S816/S821	836	160:1	0%
			836		
			$\bar{x}$ 836		
B2	S814	Freeze/thaw	4802		
			5265		
			5501		
			$\bar{x}$ 5189		
		Medium release	516		
			660		
			660		
			$\bar{x}$ 612		
		S816/S811	1360	160:1	18%
			1501		
			$\bar{x}$ 1431		
		S816/S821	1493	160:1	19%
			1450		
			$\bar{x}$ 1472		
		S816/S191	1348	160:1	17%
			1458		
			$\bar{x}$ 1403		
B2	B470	Freeze/thaw	4938		
			4876		
			5786		
			$\bar{x}$ 5000		
		Medium release	369		
			425		
			478		
			$\bar{x}$ 424		
		S816/8811	1162	160:1	16%
			1157		
			$\bar{x}$ 1160		

Cont.

APPENDIX 3.2 CONT. (20)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	%CR^{51} release
		S816/S821	1053	160:1	14%
			1064		
			$\bar{x}$ 1059		
B2	B487	Freeze/thaw	3634		
			3890		
			4012		
			$\bar{x}$ 3845		
		Medium release	547		
			658		
			586		
			$\bar{x}$ 597		
		S816/S811	3299	160:1	85%
			3421		
			$\bar{x}$ 3360		
		S816/S821	1395	160:1	23%
			1313		
			$\bar{x}$ 1354		
B2	B807	Freeze/thaw	2439		
			2721		
			2351		
			$\bar{x}$ 2504		
		Medium release	438		
			406		
			363		
			$\bar{x}$ 402		
		S816/S811	930	160:1	22%
			789		
			$\bar{x}$ 860		
		S816/S821	678	160:1	13%
			686		
			$\bar{x}$ 682		
B2	S191	Freeze/thaw	3195		
			3306		
			2763		
			$\bar{x}$ 3088		
		Medium release	1613		
			1503		
			1645		
			$\bar{x}$ 1587		
		S816/S191	2488	160:1	60%
			2498		
			$\bar{x}$ 2493		
B3	S811	Freeze/thaw	1917		
			2079		
			1724		
			$\bar{x}$ 1906		
		Medium release	260		
			182		

APPENDIX 3.2 CONT. (21)

Experiment	Target	Responder stimulator	1 minute gamma counts	E/T	%CR^{51} release
			206		
			$\bar{x}$ 216		
		S995//S811	524	80:1	16%
			458		
			$\bar{x}$ 491		
		S995/S821	357	80:1	7%
			320		
			$\bar{x}$ 339		
B3	S821	Freeze/thaw	2239		
			2347		
			2317		
			$\bar{x}$ 2301		
		Medium release	543		
			536		
			471		
			$\bar{x}$ 517		
	S995/S811		315	80:1	0%
			302		
			$\bar{x}$ 309		
			772		
			825		
			$\bar{x}$ 799		
B3	S816	Freeze/thaw	2242		
			2437		
			2373		
			$\bar{x}$ 2351		
		Medium release	397		
			383		
			-		
			$\bar{x}$ 390		
		S995/S811	178	80:1	0%
			214		
			$\bar{x}$ 196		
		S995/S821	225	80:1	0%
			319		
			$\bar{x}$ 272		
B3	S817	Freeze/thaw	2802		
			2959		
			2721		
			$\bar{x}$ 2827		
		Medium release	688		
			621		
			584		
			$\bar{x}$ 631		
		S995/S811	977	80:1	16%
			949		
			$\bar{x}$ 973		

APPENDIX 3.2 CONT. (22)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	%CR[21] release
		S995/S821	449	80:1	0%
			472		
			$\bar{x}$ 461		
B3	S828	Freeze/thaw	2090		
			2158		
			2048		
			$\bar{x}$ 2099		
		Medium release	295		
			228		
			210		
			$\bar{x}$ 244		
		S995/S811	608	80:1	22%
			712		
			$\bar{x}$ 660		
		S995/S821	310	80:1	3%
			271		
			$\bar{x}$ 291		
		S995/S191	336	80:1	3%
			226		
			$\bar{x}$ 301		
B3	S823	Freeze/thaw	2459		
			2722		
			2319		
			$\bar{x}$ 2500		
		Medium release	272		
			205		
			221		
			$\bar{x}$ 232		
		S995/S811	268	80:1	3%
			324		
			$\bar{x}$ 296		
		S995/S821	428	80:1	7%
			374		
			$\bar{x}$ 401		
B3	S829	Freeze/thaw	2873		
			2630		
			2750		
			$\bar{x}$ 2751		
		Medium release	375		
			393		
			-		
			$\bar{x}$ 384		
		S995/S811	188	80:1	0%
			247		
			$\bar{x}$ 216		
		S995/S821	338	80:1	0%

Cont.

APPENDIX 3.2 CONT. (23)

Experiment	Target	Responder/ stimulator	1 minute gamma counts	E/T	%CR^{51} release
			364		
			$\bar{x}$ 351		
		S995/S191	468	80:1	3%
			420		
			444		
B3	S191	Freeze/thaw	3708		
			3684		
			3217		
			$\bar{x}$ 3536		
		Medium release	472		
			577		
			570		
			$\bar{x}$ 520		
		S995/S811	557	80:1	2%
			617		
			$\bar{x}$ 587		
		S995/S821	817	80:1	14%
			101		
			$\bar{x}$ 929		
		S995/S191	3310	80:1	93%
			3313		
			$\bar{x}$ 3312		

APPENDIX 3.2 CONT. (24)

Expt.	Target	Cold target	Cold/ hot	Responder/Stimulator	1 minute gamma counts	E/T	% CR^{51} release
C1	S814	-	-	Freeze/thaw	5938 5660 4765 $\bar{x}$ 5454		
				Medium release	416 359 327 $\bar{x}$ 367		
					619 623 $\bar{x}$ 621	40:1	5%
	S816	-	-	Freeze/thaw	3223 3317 3240 $\bar{x}$ 3260		
				Medium release	412 281 537 $\bar{x}$ 410		
				S191/S816	1530 1436 $\bar{x}$ 1483	40:1	38%
	S816	S816	10:1	S191/S816	368 468 $\bar{x}$ 418	40:1	3%
	S816	S816	5:1	S191/S816	601 676 $\bar{x}$ 639	40:1	9%
	S816	S816	2.6:1	S191/S816	1018 888 $\bar{x}$ 953	40:1	19%
	S816	S816	1.25:1	S191/S816	1116 1182 $\bar{x}$ 1149	40:1	26%
	S816	S821	10:1	S191/S816	380 458	40:1	3%
	S816	S821	5:1	S191/S816	592 617	40:1	9%
	S816	S821	2.5:1	S191/S816	756 815 $\bar{x}$ 781	40:1	13%

Cont.

APPENDIX 3.2 CONT. (25)

Expt.	Target	Cold Target	Cold/ hot	Responder/Stimulator	1 minute gamma counts	E/T	%CR^{51} release
	S816	S821	5:1	S191/S816	975 974 $\bar{x}$ 975	40:1	20%
	S816	S836	10:1	S191/S816	900 951 $\bar{x}$ 926	40:1	18%
	S816	S836	5:1	S191/S816	1141 1077 $\bar{x}$ 1109	40:1	26%
	S816	S836	2.5:1	S191/S816	1221 1200 $\bar{x}$ 1211	40:1	28%
	S816	S836	1:25:1	S191/S816	1295 1494 $\bar{x}$ 1395	40:1	35%
	S816	S834	10:1	S191/S816	763 811 $\bar{x}$ 787	40:1	13%
	S816	S834	5:1	S191/S816	706 827 $\bar{x}$ 767	40:1	13%
	S816	S834	2.5:1	S191/S816	1101 911 $\bar{x}$ 1001	40:1	21%
	S816	S834	1.25:1	S191/S816	1241 1157 $\bar{x}$ 1199	40:1	28%
	S816	S829	10:1	S191/S816	1424 1458 $\bar{x}$ 1441	40:1	36%
	S816	S829	5:1	S191/S816	1624 1683 $\bar{x}$ 1654	40:1	44%
	S816	S829	2.5:1	S191/S816	1623 1496 $\bar{x}$ 1560	40:1	40%
	S816	S829	1.25:1	S191/S816	1604 1615 $\bar{x}$ 1610	40:1	42%
C2	S829	-	-	Freeze/thaw	4854 4823 4898		

APPENDIX 3.2 CONT. (26)

Expt.	Target	Cold Target	Cold/ hot	Responder/Stimulator	1 minute gamma counts	E/T	%CR^{51} release
					$\bar{x}$ 4858		
				Medium release	260		
					223		
					232		
					$\bar{x}$ 238		
				S191/S821	745	80:1	11%
					722		
					$\bar{x}$ 734		
	S821	-	-	Freeze/thaw	5180		
					5247		
					5389		
					$\bar{x}$ 5272		
				Medium releaee	498		
					453		
					392		
					$\bar{x}$ 448		
				S191/S821	2632	80:1	46%
					2695		
					$\bar{x}$ 2664		
	S821	S821	20:1	S191/S821	768	80:1	9%
					998		
					$\bar{x}$ 883		
	S821	S821	10:1	S191/S821	1290	80:1	17%
					1224		
					$\bar{x}$ 1254		
	S821	S821	5:1	S191/S821	1012	80:1	13%
					1100		
					$\bar{x}$ 1056		
	S821	S821	2.5:1	S191/S821	1953	80:1	30%
					1877		
					$\bar{x}$ 1915		
	S821	S811	20:1	S191/S821	1271	80:1	17%
					1261		
					$\bar{x}$ 1266		
	S821	S811	10:1	S191/S821	1585	80:1	21%
					1304		
					$\bar{x}$ 1445		
	S821	S811	5:1	S191/S821	1745	80:1	24%
					1462		
					$\bar{x}$ 1604		
	S821	S811	2.5:1	S191/S821	1498	80:1	24%

Cont.

APPENDIX 3.2 CONT. (27)

Expt	Target	Cold Target	Cold/ hot	Responder/Stimulator	1 minute gamma counts	E/T	%CR^{51} Release
					1689 $\bar{x}$ 1594		
	S821	S816	20:1	S191/S821	1243 1505 $\bar{x}$ 1374	80:1	19%
	S821	S816	10:1	S191/S821	1744 1788 $\bar{x}$ 1766	80:1	27%
	S821	S816	5:1	S191/S821	1812 1760 $\bar{x}$ 1786	80:1	28%
	S821	S816	2.5:1	S191/S821	2075 1997 $\bar{x}$ 2036	80:1	33%
	S821	S817	20:1	S1o1/S821	1452 1284 $\bar{x}$ 1368	80:1	19%
	S821	S817	10:1	S191/S821	1402 1276 $\bar{x}$ 1340	80:1	18%
	S821	S817	5:1	S191/S821	1654 1402 $\bar{x}$ 1528	80:1	22%
	S821	S817	2.5:1	S191/S821	1901 1840 $\bar{x}$ 1871	80:1	29%
	S821	S828	20:1	S191/S821	998 837 $\bar{x}$ 918	80:1	10%
	S821	S828	10:1	S191/S821	1122 1160 $\bar{x}$ 1141	80:1	14%
	S821	S828	5:1	S191/S821	1279 1392 $\bar{x}$ 1336	80:1	18%
	S821	S828	2.5:1	S191/S821	1789 1663 $\bar{x}$ 1726	80:1	26%

APPENDIX 3.2 CONT. (28)

Expt.	Target	Cold Target	Cold/ hot	Responder/Stimulator	1 minute gamma counts	E/T	%CR^{51} Release
	S821	S807	20:1	S191/S821	1186 - $\bar{x}$ 1186	80:1	15%
	S821	S807	10:1	S191/S821	1517 1387 $\bar{x}$ 1452	80:1	21%
	S821	S807	5:1	S191/S821	1710 1578 $\bar{x}$ 1644	80:1	25%
	S821	S807	2.5:1	S191/S821	1860 1445 $\bar{x}$ 1653	80:1	25%
	S821	S836	20:1	S191/S821	1583 1578 $\bar{x}$ 1581	80:1	23%
	S821	S836	10:1	S191/S821	2191 2218 $\bar{x}$ 2205	80:1	36%
	S821	S836	5:1	S191/S821	2416 2499 $\bar{x}$ 2456	80:1	42%
	S821	S836	2.5:1	S191/S821	2248 2084 $\bar{x}$ 2166	80:1	36%
	S821	S818	20:1	S191/S821	1983 1926 $\bar{x}$ 1955	80:1	31%
	S821	S818	10:1	S191/S821	2062 2312 $\bar{x}$ 2188	80:1	36%
	S821	S818	5:1	S191/S821	1994 2178 $\bar{x}$ 2086	80:1	34%
	S821	S818	2.5:1	S191/S821	2296 2347 $\bar{x}$ 2332	80:1	39%
	S821	S834	20:1	S191/S821	1193	80:1	18%

Cont.

APPENDIX 3.2 CONT. (29)

Expt.	Target	Cold Target	Cold/ hot	Responder/Stimulator	1 minute gamma counts	E/T	%CR^{51} Release
				1449 1321			
	S821	S834	10:1	S191/S821	1648 1667 $\bar{x}$ 1658	80:1	25%
	S821	S834	5:1	S191/S821	1942 2155 $\bar{x}$ 2049	80:1	33%
	S821	S834	2.5:1	S191/S821	2106 2163 $\bar{x}$ 2135	80:1	35%
	S821	S814	20:1	S191/S821	1895 2160 $\bar{x}$ 2028	80:1	33%
	S821	S814	10:1	S191/S821	2309 2269 $\bar{x}$ 2289	80:1	38%
	S821	8814	5:1	S191/S821	2171 2233 $\bar{x}$ 2202	80:1	36%
	S821	S814	2.5:1	S191/S821	2443 2588 $\bar{x}$ 2516	80:1	43%
	S821	S829	20:1	S191/S821	2397 2359 $\bar{x}$ 2378	80:1	40%
	S821	S829	10:1	S191/S821	2652 2697 $\bar{x}$ 2675	80:1	46%
	S821	S829	5:1	S191/S821	2471 2550 $\bar{x}$ 2511	80:1	43%
	S821	S829	2.5:1	S191/S821	2476 2175 $\bar{x}$ 2326	80:1	39%

Cont.

APPENDIX 3.2 CONT. (30)

Expt.	Target	Cold Target	Cold/ hot	Responder/Stimulator	1 minute gamma counts	E/T	%CR^{51} Release
	S821	S807 S818	20:1	S191/S821	992 1040 $\bar{x}$ 1016	80:1	12%
	S821	S807 S818	10:1	S191/S821	1340 1364 $\bar{x}$ 1352	80:1	10%
	S821	S807 S818	5:1	S191/S821	1911 1860 $\bar{x}$ 1886		
	S821	S807 S818	2.5:1	S191/S821	2186 1945 $\bar{x}$ 2066	80:1	34%
	S821	S834 S836	20:1	S191/S821	1035 1035 $\bar{x}$ 1035	80:1	12%
	S821	S834	10:1	S191/S821	1458 1532 $\bar{x}$ 1495	80:1	22%
	S821	S934 S836	5:1	S191/S821	1688 1748 $\bar{x}$ 1718	80:1	26%
	S821	S834 S836	2.5:1	S191/S821	2174 1918 $\bar{x}$ 2046	80:1	33%