

H-2 ANTIGENIC REQUIREMENTS FOR T CELL RECOGNITION

by

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S T A T E M E N T

Except for the experiments represented by Figures 1 and 4 in the Appendix which were done by Dr C.R. Parish, all experiments described in this thesis represent my own work and were carried out by me. Dr T.J. Higgins prepared the glycolipid extracts which were used for experiments described in Chapter 7 and Dr T.J. O'Neill did some of the statistical analyses described in Chapters 2, 4 and 5.

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A C K N O W L E D G E M E N T S

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A B B R E V I A T I O N S

bis-acrylamide	N, N'-bis-methylene acrylamide
BSA	bovine serum albumin
cpm	counts per minute
F15	Eagle's minimal essential medium with nonessential amino acids
FCS	fetal calf serum
FITC-avidin	avidin conjugated with fluorescein isothiocyanate
FITC-GAMIG	goat anti-mouse Ig conjugated with fluorescein isothiocyanate
H	histocompatibility
H chain	immunoglobulin heavy chain
HY	male specific antigen
Ia	<i>I</i> region-associated
Ig	immunoglobulin
<i>Ir</i> gene	immune response gene
i.v.	intravenously
L chain	immunoglobulin light chain
LCMV	lymphocytic choriomeningitis virus
LRT	likelihood ratio test
<i>MHC</i>	major histocompatibility complex
MLR	mixed lymphocyte reaction
NA	neuraminidase
NP-40	nonidet P-40
OV	ovalbumin
P1/P2	parent 1/parent 2
PBS	phosphate buffered saline

PFU	plaque-forming unit
PMSF	phenylmethanesulphonyl fluoride
RFC	rosette forming cell
RIA	radioimmunoassay
SDS	sodium dodecyl sulphate
SE	standard error of the mean
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SV40	simian virus 40
TEMED	N,N,N',N'-tetramethylethylenediamine
T cell	thymus-derived lymphocyte
Tc	cytotoxic T cell
Th	helper T cell
Td	delayed type hypersensitivity T cell
TNP	trinitrophenyl
TRITC-GAMIG	goat anti-mouse Ig conjugated with tetramethylrhodamine isothiocyanate
V gene	variable region gene
V _H gene	Ig heavy chain variable region gene
X	foreign antigen

A B S T R A C T

The material presented in this thesis covers various aspects of the nature and expression of the major histocompatibility antigens in mouse, the H-2 antigens, and the experimental work has involved serological analysis of antigen expression in both homozygous and F_1 hybrid mice using both alloantiserum preparations and several monoclonal anti-H-2 antibodies.

Quantitative absorption analysis has revealed variability in the relative expression of individual K and D antigens in F_1 hybrid and parental strain spleen cells, and this result has been confirmed using several different F_1 hybrids and different anti-H-2 alloantiserum preparations. Genetic studies have shown that the expression of individual K and D antigens is dependent on the K and D allelic composition of the F_1 hybrid in that some alleles tend to dominate others.

A quantitative role for H-2 antigens in cytotoxic T cell (Tc cell) responsiveness was subsequently demonstrated when several F_1 hybrids, known to express certain parentally-derived H-2 antigens more weakly than parental strain cells, were found to be less effective as target cells for both alloreactive and H-2 restricted Tc cells. Furthermore, quantitative differences in H-2 antigen expression between F_1 hybrid and parental cells were also found to influence Tc cell responsiveness when these cells were used as stimulators at the induction phase of the response.

Since these results suggested a quantitative interpretation for H-2-linked differences in Tc cell responsiveness, a method was devised for measuring the expression of individual K and D antigens in different

homozygous strains of mice. Anti-H-2 sera were used in a two-stage radioimmunoassay, and antigen expression was calculated by a reciprocal plot estimation method. Small, but significant differences were detected in the expression of those antigens studied, and more variability was evident in the expression of different D than K antigens. These results have been discussed in the light of current evidence for H-2-linked differences in Tc cell responsiveness.

Several aspects of H-2 antigen expression were analysed using monoclonal anti-H-2 antibodies. When two different anti-H-2K^k antibodies were used in a radioimmunoassay to compare parameters of antibody binding to F₁ hybrid and parental strain spleen cells, no differences in antigen-antibody dissociation constants were found, but differences in the number of antibody binding sites were detectable. The results are consistent with a quantitative difference, with no evidence for any qualitative change in the F₁ hybrid antigens studied.

The existence of two classes of H-2K^k molecules was also demonstrated with the use of monoclonal antibodies. Firstly, two monoclonal antibodies were found to bind to only half of the total number of H-2K^k molecules detectable with anti-H-2K^k alloantisera. Mutual exclusiveness between these two molecules was then demonstrated by cocapping and antibody blocking experiments, and was confirmed with the use of a modified immunoprecipitation method. This method involves the use of a rosette inhibition assay to measure absorption of antigen from a solubilised cell preparation, and several advantages of this method over previous immunoprecipitation procedures involving gel electrophoresis have been discussed.

Chemical characterisation of the antigenic determinants recognised by several different anti-H-2K^k monoclonal antibodies, has revealed the

existence of both protein- and carbohydrate-defined H-2 antigens. Since glycolipid extracts from cells were found to inhibit the binding of antibodies specific for carbohydrate determinants, this suggested that the carbohydrate determinants were carried on glycolipid molecules. This finding has been discussed in terms of similar evidence for the existence of two chemically different types of Ia antigens, and of the involvement of the different H-2 molecules in T cell recognition.

During studies involving anti-H-2 sera to immunoprecipitate H-2 molecules, weak antibody activity was detected in two different anti-H-2D sera which was specific for new antigenic determinants mapping between the *S* and *D* regions of the *H-2* gene complex. Since these determinants were detectable on molecules which resembled Ia antigens by virtue of their molecular weight and cellular distribution, this new region has been called *I'*.

Results obtained during the course of this study have been discussed in terms of the genetic control of H-2 antigen expression, and of the involvement of these antigens in T cell interactions. The culmination of this work has been the development of a new model for T cell recognition with predictions on the nature of the T cell receptor and mechanisms for generation of diversity in the T cell pool.

CHAPTER 1

GENERAL INTRODUCTION

1. Outline of Thesis

The generation of cellular and humoral immune responses is made possible by complex interplay between various types of cells. In the past few months, evidence has accumulated which indicates a basic requirement for cell surface molecules encoded by the major histocompatibility complex (MHC) in some cellular interactions which involve thymus-derived lymphocytes, or T cells. These evidence were originally derived by their role as transplantation antigens, but are now known to be fundamentally involved in T cell mediated immune responses.

H-2 ANTIGENIC REQUIREMENTS

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This thesis presents various aspects of responses controlled by the

1. Outline of thesis.
2. Current interpretation of the *H-2* gene complex in mice.
3. The nature and expression of H-2 antigens.
4. *H-2* restricted T cell functions.
5. H-2 antigenic requirements for cytotoxic T cell recognition.
6. T cell receptors.

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Before submission of the final version of this thesis, relevant genetic information on the *H-2* gene complex is reviewed by way of introduction to the material covered by the following chapters. The present information on the role of H-2 antigens in T cell recognition is also discussed.

1. Outline of Thesis

The generation of cellular and humoral immune responses in mammals involves complex interplay between various types of cells. In the past two decades, evidence has accumulated which indicates a basic requirement for cell surface antigens encoded by the major histocompatibility complex (*MHC*) in those cellular interactions which involve thymus-derived lymphocytes, or T cells. These antigens were originally defined by their role as 'transplantation' antigens, but are now known to be fundamentally involved in self recognition required for T cell-mediated immune responses.

This thesis concerns various aspects of antigens controlled by the *MHC* in mice, the *H-2* complex, with a view to understanding more about their role in T cell recognition. While the original objective of this study was to analyse various *H-2*-linked differences in cytotoxic T cell (*Tc* cell) function to different viruses and foreign cell surface antigens, this work revealed previously undetected variability in the expression of particular *H-2* antigens. As a result, the experimental work presented here comprises a series of papers covering different aspects of the nature and expression of these antigens, and is followed by a discussion of the results in terms of a new model for T cell recognition.

Before embarking on the original work of this thesis, relevant genetic information on the *H-2* gene complex is reviewed by way of introduction to the material covered by the following chapters, and current information on the role of *H-2* antigens in T cell recognition is also discussed.

2. Current Interpretation of the H-2 Gene Complex in Mice

Progress in understanding the function of major histocompatibility antigens has been dependent on knowledge of the genetic organisation of the gene complex which controls their synthesis and expression. Because of the role of these antigens in skin graft rejection, the *MHC* has become one of the more readily studied mammalian genetic regions and, as a result, a similar region has been defined for all vertebrate species so far studied. The *HLA* complex in man and the *H-2* complex in mice appear to be genetically and functionally homologous (reviewed by McKenzie 1977). However, with the availability of recombinant inbred strains of mice, analysis of the function of *H-2* controlled antigens has been experimentally more accessible.

The *H-2* gene complex is located on chromosome 17 and has currently been divided into several regions, namely *K*, *I*, *S*, *G*, *D*, and *L*, although genetic interpretation of the *H-2* complex is continually changing. Except for the *S* region, all regions control the expression of different cell surface antigens involved in a variety of immune functions (reviewed by Klein 1975, 1976, 1978, Vitetta and Capra 1978). By way of reference for the remainder of this thesis, a genetic map of the *H-2* region derived from information recently cited by Klein in a number of review articles (1975, 1976, 1978) is shown in Figure 1. This map also includes the location of the *T* region, proximal to *H-2*, which controls the expression of cell surface antigens involved in differentiation and embryogenesis (reviewed by Klein and Hammerberg 1977) as well as the *T1a* region, distal to *H-2*, which controls the expression of a number of different antigens, namely *TL*, *Qa-1*, *Qa-2*, *Qa-3*, *Qat-4* and *Qat-5*, which are expressed on T lymphocytes during different stages of differentiation (Boyse *et al.* 1968, Stanton and Boyse 1976, Flaherty *et al.* 1978,

Hämmerling *et al.* 1979a). Serological evidence for the division of the *H-2* complex into separate regions is extensive, as too is information on the functional role of antigens which map to each of the individual regions (reviewed by Klein 1975, 1976, Snell 1978).

The *K* and *D* regions each control the expression of antigenic determinants present on serologically distinct though functionally similar molecules. In brief, these antigens are responsible for the activation of T cells, e.g. in graft-versus-host reactions, mixed lymphocyte reactions, and in the induction of cell-mediated lymphocytotoxicity. More specifically, they are known to be target antigens for both alloreactive Tc cells (reviewed in Klein 1975), as well as for Tc cells specifically sensitised to viruses or foreign cell surface antigens (Zinkernagel and Doherty 1974, Shearer 1974, Bevan 1975a, Gordon *et al.* 1975). More recently, genetic complexity has become evident within the *D* region and currently three serologically distinct molecules are known to map to this one region (reviewed by Démant and Néauport-Sautès 1978, see also Iványi and Démant 1979). The *L* locus was assigned to account for the existence of a second *D*-region molecule (Démant *et al.* 1977). Similar genetic complexity now appears to exist in the *K* region and evidence in favour of the existence of at least two different classes of molecules encoded by *K* is presented in Chapter 6.

The *I* region appears to be the most genetically complex region and has currently been divided into six separate subregions. The *I-A*, *I-B*, and *I-C* subregions were initially located on the basis of immune response genes (*I_r* genes) (reviewed by Benacerraf and Germain 1978), and when *I* region-associated (*I_a*) antigens were identified, the existence of two further subregions, *I-E* and *I-J*, was confirmed (reviewed by David 1976). More recently, a sixth subregion *I-N* has been assigned on the

basis of Ia antigen involvement in mixed lymphocyte reactions (Hayes and Bach, 1980). While Ia antigens from different subregions differ somewhat in their immunogenicity, as well as function, antigens from the whole *I*-region have basic lymphocyte activation properties similar to the antigens controlled by the *K* and *D* regions (see above). However, Ia-antigen-specific functions also exist and are, in general, related to the function of only certain classes of T cells (see Section 4).

The existence of a separate *G* region within the *H-2* complex is now in doubt (Huang and Klein 1979a) and the single H-2.7 antigenic determinant, originally mapped to this region, is thought to be related to a product of the *S* locus (Huang and Klein 1979b). The *S* region which now divides the *H-2* region, codes for the Ss and Slp serum proteins, one of which has been shown to be functionally the fourth component of complement (Démant *et al.* 1973, Lachman *et al.* 1975, Curman *et al.* 1975), and recent immunochemical evidence now suggests that this region may contain two separate structural loci (Parker *et al.* 1979). A New *I'* region has also been assigned to the gene map in Figure 1, and evidence for its map location, as well as the similarity of its antigens to the conventional Ia antigens, is presented in Chapter 8.

In this study, those molecules which carry the *K*- and *D*-controlled antigenic determinants are of prime importance, but *I*-region associated molecules are also considered, generally by way of comparison with respect to their function and the chemical nature of their antigenic determinants.

3. The Nature and Expression of H-2 Antigens

3.1 The K and D antigens

A large number of antigenic specificities mapping to the two separate *K* and *D* regions of the *H-2* complex have been defined serologically using many different alloantiserum preparations (Klein 1975), and this number has increased since monoclonal anti-*H-2* antibodies have become available for typing new determinants (Klein *et al.*, 1979, Hämmerling *et al.* 1979b). While some antigenic specificities are shared between the two regions, reflecting their close structural relationship, many specificities are specific for *K* or *D*. The existence of a large number of determinants on the *K* and *D* gene products of a variety of mouse strains is indicative of extensive polymorphism associated with these genes. In general, antigens encoded by these regions are referred to as 'H-2 antigens', or more specifically, as 'K' and 'D' antigens.

H-2 antigens appear to be expressed on cells of most tissues (Klein 1975). However, since the original typing studies have involved relatively insensitive serological techniques, it remains uncertain how widely these antigens are expressed on normal tissue components other than lymphomyeloid cells, such as T and B cells and macrophages, which have high concentrations. Recent histochemical studies on the intact thymus have demonstrated localisation of those cells which express H-2 antigens (Rouse *et al.* 1979), and electron microscopic studies of dissociated cells using an immunoferritin labelling method have also shown that these antigens are not ubiquitous and are only detectable on particular types of cells isolated from a variety of tissues (Parr 1979, Parr and Kirby 1979). Variability in the expression of different H-2 antigens is further discussed in Chapters 2, 3, 4 and 5.

While studies on the chemical composition and biochemical properties of the *K* and *D* gene products suggest that the antigenic determinants involved are protein in nature, there was a time when carbohydrates were thought to determine antigenicity (reviewed by Klein 1975). Evidence presented in Chapter 7 bears on this original controversy and now confirms the existence of both types of antigenic determinants. Despite this new finding, the evidence for glycoprotein carriers of H-2 antigenicity has been well documented, and probably the best demonstration has been obtained by immunoprecipitation of radiolabelled molecules from cell extracts solubilised with nonionic detergents (see, for example, Nathenson *et al.* 1976, Cullen *et al.* 1976). Gel electrophoresis studies of immunoprecipitates have led to characterisation of 45,000 dalton glycoprotein molecules specific for each of the *K* or *D* region alleles. H-2 antigenicity resides in the protein portion of these molecules, and the carbohydrate portion, which is relatively small (approximately 3,000 daltons) and associated with the amino-terminal region of the polypeptide chain, appears to be antigenically silent (Nathenson and Muramatsu 1971) and is probably involved in the orientation of the molecule in the cell membrane. A smaller 12,000 dalton β_2 -microglobulin molecule which immunoprecipitates with H-2K and H-2D glycoproteins, also appears to be associated in the cell membrane with several other H-2-controlled antigens which are biochemically similar to the H-2K and H-2D molecules (reviewed by Vitetta and Capra 1978). Since its structure and amino acid sequence appears to be conserved between species, the exact function of β_2 -microglobulin can not be easily determined.

Analysis of radiolabelled H-2K and H-2D molecules from a number of different mouse haplotypes by comparative peptide mapping techniques

has revealed as much diversity between allelic products of a single region (*K* or *D*) as between the *K* or *D* region products of a single haplotype (Brown *et al.* 1974, Nathenson *et al.* 1976). Furthermore, conventional amino acid sequencing analysis has indicated remarkable conservation amongst the first 25-30 amino-terminal residues of molecules encoded by different *K* and *D* genes (Vitetta *et al.* 1976, Capra *et al.* 1976, Silver and Hood 1976), and more recent studies involving sequencing of cyanogen bromide-induced peptide fragments from different H-2K and H-2D molecules (Coligan *et al.* 1980) has shown that this structural homology extends over the entire molecule. Apart from their characteristic primary structure, different H-2K and H-2D molecules also appear to have a small number of unique residues which account for only about 20% of sites so far analysed (Coligan *et al.* 1980).

While extensive homology could account for the existence of many serologically crossreactive *K* and *D* determinants, i.e. the 'public' specificities, the unique sites could reflect the existence of determinants which are specific for each of the different H-2K and H-2D molecules, i.e. the 'private' specificities. In order to account for the unique functional specificity associated with different *K* and *D* gene products (see Section 5), these small sequence differences would have to contribute to modifications which affect the function of H-2 molecules in T cell recognition. Such a proposition is exemplified by studies involving several mutant strains of mice which are known to carry only minor changes in the sequence of their respective H-2K and H-2D molecules (Brown and Nathenson 1977, Brown *et al.* 1978). Although small, these changes result in remarkable differences in T cell specificity between the mutant strains and their wild type counterparts (McKenzie *et al.* 1977a, Morgan *et al.* 1978).

One interesting characteristic of mutations in the *K* and *D* region is that they generally remain serologically silent, despite their strong functional distinctiveness in terms of T cell recognition (reviewed by Klein 1978). When this information is considered together with evidence for the high degree of serological crossreactivity between different H-2K and H-2D molecules, and the small number of unique sites associated with each molecule, it is easy to imagine that a lot of functional heterogeneity could exist amongst a given population of H-2K and H-2D molecules yet go unnoticed by serological analysis, or for that matter, by amino acid sequence analysis. This proposition is interesting in the light of recent evidence for such heterogeneity amongst H-2K, H-2D and H-2L molecules of a given haplotype (Hess and Davies 1974, Jones 1977, Krakauer *et al.* 1980). When separated by two-dimensional gel electrophoresis, H-2 molecules encoded by a given gene appear to represent a heterogeneous mixture of molecules, differing only slightly by their charge and size. Possible explanations for this phenomenon include either posttranslational modification of *H-2* gene products, or the existence of a multigene complex in the *K* and *D* regions. This latter proposition suggests that the *K* and *D* regions are genetically more complex than previously thought and could encode a large number of H-2 molecules which are functionally distinct though very similar in primary sequence. This topic is the subject of further discussion in Chapter 9.

One mutant mouse strain which has been valuable in analysis of the genetic complexity associated with the *D* region (see Section 2) is the BALB/c-*H-2*^{dm2} strain. Such mice are unusual in that they contain a mutation in the *D*^d region which results in a 'loss' with no concurrent gain of new antigenic specificities, as measured by skin graft rejection

(McKenzie *et al.* 1977b). This mutation has been serologically defined as a loss of the H-2L^d molecule, a *D* region-controlled molecule, known to be serologically distinct from the classical H-2D^d molecule (Morgan *et al.* 1978, see also review by Démant and Néauport-Sautès 1978). Using antiserum raised in mutant mice primed with wild type cells, Hansen and Sachs (1978) were able to demonstrate that these mutant mice lack a 45,000 dalton H-2-like glycoprotein molecule, yet retain the H-2D^d molecule in common with wild type mice. In general, the H-2L molecule has also been shown to be similar in function to the H-2K and H-2D molecules (reviewed by Levy and Hansen 1980), and also carries unique antigenic determinants (Hansen and Sachs 1978). While recent peptide map analysis of H-2D^d molecules in mutant and wild type strains suggests that the site of mutation lies outside the *D*^d locus (Nairn and Nathenson 1978), amino acid sequencing analysis is consistent with the H-2L^d molecule being a distinct molecular species, though homologous with other H-2K and H-2D molecules (Coligan *et al.* 1980). The combined evidence suggests division of the *D* region into at least two genes encoding similar molecules.

3.2 The Ia Antigens

Like the *K* and *D* regions, the *I* region is also highly polymorphic and a large number of antigenic specificities have been defined, most of which map in the *I-A* subregion (reviewed by David 1976). Unlike the H-2 antigens, Ia antigens have restricted cellular distribution indicative of their different functional role. They are expressed in highest concentration on B cells and macrophages, but can also be detected on T cells especially when these cells have become activated by either non-specific mitogens, or in the presence of a foreign antigen (reviewed by McKenzie and Potter 1979).

While the general consensus is that Ia antigenic specificities are protein in nature, considerable evidence has now been obtained which suggests that Ia antigenicity can also reside in carbohydrate determinants (reviewed by Parish and McKenzie 1980). The carriers of these antigens appear to be gangliosides (Parish *et al.* 1976a,b,c), and have been detected both in serum, on the cell surface, as well as on many soluble T cell factors with immunoregulatory functions (reviewed by Parish and McKenzie 1980). The existence of two different types of Ia antigens has recently been confirmed by the finding that monoclonal anti-Ia antibodies fall into two classes, those specific for protein determinants, and those specific for carbohydrate-defined antigens (Higgins *et al.* 1980).

Protein-defined Ia antigens have been well characterised by many laboratories. Most recent studies indicate that Ia antigens exist on the surface of B cells as two non-covalently associated glycoproteins of slightly different size (reviewed by Cullen *et al.* 1976). The molecular weight of these chains is variable for antigens encoded by different haplotypes and subregions of *I*, but is in the range 25-30,000 daltons for the smaller (β) chain, and 30-35,000 daltons for the larger (α) chain (Cook *et al.* 1978). While only Ia antigens controlled by the *I-A* and *I-E* subregions have been detectable by immunoprecipitation and gel electrophoresis, protein molecules resembling Ia antigens, have also been detected by this method which map to a new *I*-like region between *S* and *D*. This evidence is discussed in Chapter 8.

Genetic control of the expression of Ia protein-defined antigens appears to be more complex than for H-2 antigens, and can involve interactions between more than one *I* subregion. Recent two-dimensional gel electrophoresis studies by Jones *et al.* (1978) suggest that the Ia molecules controlled by the *I-E* subregion arise by complementation

between genes mapping in both the *I-A* and *I-E* subregions. Subsequently, Cook *et al.* (1979) have shown considerable heterogeneity among the smaller β chains of *I-E* molecules encoded by recombinant strains differing only at the *I-A* subregion. Since no heterogeneity was found among the α -chains, these results together suggest that the *I-E*-controlled molecules are hybrids, and while the α chains appear to be under the control of this locus, the β chains map to the *I-A* subregion. Interaction between products encoded by these two gene regions has been used as an explanation for the existence of F_1 hybrid-specific Ia antigens recently reported by Lafuse *et al.* (1980), as well as for complementation observed between the *I-A* and *I-E* subregion genes in immune responsiveness to the synthetic peptide antigen poly (GL ϕ) (Schwartz *et al.* 1978).

Despite obvious differences in the biochemical nature and genetic control of H-2 and Ia antigen expression, molecules carrying these different antigenic determinants appear to play a similar functional role in T cell recognition and this is outlined in the following section.

Although genetic information on the *H-2* gene complex is extensive and readily obtained, and the requirement for *H-2*-controlled antigens in T cell recognition is well documented, no exact biochemical role has yet been assigned to molecules which carry these antigens. As a result, there is no way of telling whether the *H-2* gene complex actually encodes the antigens themselves, or is a bank of regulator genes controlling the expression of other structural genes.

4. *H-2* Restricted T Cell Functions

The *H-2* gene complex is now known to affect a spectrum of T cell functions including cell-mediated immunity towards virally infected or

altered-self cells, collaborative interactions between T and B cells for antibody production, proliferative responses to antigen pulsed macrophages, and the expression of delayed type hypersensitivity. A more definitive list of currently described functions associated with the *H-2* complex, is presented in Table 1. These various functions involve different classes of T cells, including those which elicit delayed type hypersensitivity (Td), those with helper functions (Th), as well as the cytotoxic T (Tc) cells. While different antigens, encoded by different regions of the *H-2* gene complex, e.g. the *K*, *D* and *I* regions, are involved in the different responses, a common requirement exists such that T cells only recognise these antigens on cells which carry self *H-2*-coded markers, i.e. syngeneic cells. Not included in Table 1 is the function of several Ia-bearing T cell factors involved in either help or suppression of various classes of T cells, and which are also *I*-region restricted in their action (Tada *et al.* 1976, Munro and Taussig 1975, Greene *et al.* 1977, Thèze *et al.* 1977, Delovitch and McDevitt 1977, Rich *et al.* 1977, Moorhead 1977, Howie and Feldman 1977). In sum, all of this information suggests a common operational role for both *H-2* and Ia antigens in T cell recognition apart from their obvious functional and molecular distinction.

Despite extensive demonstrations of 'MHC restriction' of T cell functions in several different species, the exact functional role of *MHC* antigens is still unknown, and as a result, the process of T cell recognition, involving a receptor site on the effector cell and an *MHC*-controlled antigen on the target cell, is poorly understood. A major limitation in many analyses of T cell specificity has been the need to rely on ancillary T cell activities, i.e. the capacity of T cells to help or suppress humoral responses, or inhibit delayed-type hypersensitivity in order to assess T cell recognition of antigen. In this

respect, Tc cell responses prove to be superior, since antigen recognition can be directly measured by the lysis of targets carrying appropriate antigens. It was probably this feature of Tc cell responses which triggered the tremendous interest in T cell recognition following the discovery that Tc cell responses to virally infected cells were restricted by the *MHC*.

This initial observation was made by Zinkernagel and Doherty in 1974, using mice infected with lymphocytic choriomeningitis virus (LCMV). They showed that Tc cells from infected mice would lyse only LCMV-infected target cells which shared *H-2* genes with the Tc cell donors. The relevant genes were mapped to the *K* and *D* regions of the *H-2* complex by Blanden and coworkers in 1975, and the phenomenon has since been referred to as '*H-2* restriction'. This finding probably established the first biologically important role for *MHC* antigens, and its *in vivo* importance was later demonstrated in adoptive transfer experiments (Kees and Blanden 1976) which showed that clearance of virus by transferred Tc cells also required *K* or *D* matching of donor and recipient strains of mice.

MHC restricted Tc cell responses have now been demonstrated in many different species, including man, and involve responses to many different viruses, minor histocompatibility (H) antigens, as well as to chemically-modified cells. The experimental evidence is extensive and will not be discussed here, since it has been the subject of many recent reviews (for example, Zinkernagel and Doherty 1979, Zinkernagel 1978a, Shearer and Schmitt-Verhulst 1977, Simpson and Gordon 1977, Doherty *et al.* 1976). Despite extensive studies on Tc cell responses, especially in mice, where the antigens of the *H-2* complex have been well characterised, the nature of the recognition process is still the subject of many ongoing studies.

5. H-2 Antigenic Requirements for Cytotoxic T Cell Recognition

The observation that the *H-2* gene complex imposes constraints on Tc cell recognition of foreign cell surface antigens via the K and D antigens on the cells involved has been well documented (reviewed by Zinkernagel and Doherty 1979, Shearer and Schmitt-Verhulst 1977). As a result, many generalisations have now been made about the *H-2* restriction phenomenon. While these seem to hold true for Tc cell responses to most viruses and foreign cell surface antigens, a few exceptions have been noted and some of these will be considered further in Chapter 9. The following five points seem to summarise basic findings on the requirement for H-2 antigens in Tc cell recognition:

1. Tc cells can be generated either, *in vivo* by priming mice with virus or cells expressing foreign (X) antigens, or *in vitro*, by coculturing normal or pre-primed T cells with syngeneic stimulator cells infected with the same virus, or carrying the same X antigen.
2. Such Tc cells are highly specific for self H-2 determinants, as well as for the viral or X antigens present on the stimulating cell used to induce the response. They therefore recognise and lyse only target cells carrying these antigens together with appropriate K or D antigens. The specificity for self H-2 is exquisite in most viral systems, but much less specific in the case of trinitrophenyl (TNP) - modified cells (Shearer and Schmitt-Verhulst 1977).

3. *H-2* restricted Tc cells are clonally expressed, i.e. upon stimulation with foreign cell surface antigens, different subsets of cells become activated which are specific for either K or D antigens present on target cells bearing the same foreign antigen. Evidence in favour of clonality has been obtained by many different experimental approaches including cold target competition studies and antiserum blocking experiments (reviewed by Zinkernagel and Doherty 1979), as well as by specific depletion of effector Tc cells by either adsorption on target cell monolayers (Kees *et al.* 1978), or antigen-induced Tc cell suicide (Janeway *et al.* 1978). More recently, von Boehmer *et al.* (1979) have used cloning experiments to verify this finding.
4. While alloreactive Tc cells tend to be crossreactive, *H-2* restricted Tc cells are highly specific for self K or D antigens. This finding has been exemplified by studies using various *H-2* mutant strains of mice carrying minor alterations in their *K*- or *D*-controlled gene products. Anti-viral Tc cells generated in such mice are highly specific for infected mutant strain targets and do not crossreact significantly on infected wild type targets (Blanden *et al.* 1976, Zinkernagel 1976b).
5. While foreign (X) cell surface antigens characteristically stimulate a Tc cell response restricted to K and D antigens shared by effector and target cells,

responses specific for allogeneic X-bearing targets can also be induced. Manipulation is necessary to detect such responses and can involve the following conditions:

(i) Removal of the alloreactive component of the response which masks detection of the X-specific response is essential. This can be achieved either by the filtration of alloreactive cells through an irradiated host (Wilson *et al.* 1977, Doherty and Bennink 1979), by suiciding alloreactive T cells with radioactive nucleic acid analogues (see, for example, Janeway *et al.* 1978), or by the induction of tolerance (Forman *et al.* 1977a, Forman and Streilein, 1979).

(ii) Such responses can be generated in irradiation chimeras when the stimulator cells are syngeneic with some, as yet undefined, radioresistant cell resident in the thymus (reviewed by Zinkernagel 1978b, Bevan and Fink 1978).

In summary, *H-2* restriction seems to be imposed at two levels of a Tc cell response, at the induction phase as well as in the thymus during T cell differentiation.

5.1 Antigenic Requirements for T Cell Induction

Apart from the dual requirement for both K and D antigens presented together with X antigens in the induction of H-2 restricted Tc cell responses, there is no definitive information on whether H-2 and X antigens have to be associated in some way to stimulate a response. While H-2 antigens have been shown to cocap with vaccinia-virus induced antigens (Senik and Néauport-Sautés 1979), TNP-modified target antigens (Forman *et al.* 1979b), oncornovirus-induced antigens (Schrader *et al.* 1975, Bubbers *et al.* 1976), and vesicular stomatis virus-induced antigens (Geiger *et al.* 1979), there are nearly as many reports showing no association of H-2 with X antigens, e.g. the male specific HY antigen does not cocap with H-2 antigens (Geib *et al.* 1977), and Moloney virus-induced cell surface antigens do not coprecipitate with H-2 antigens under detergent solubilisation conditions (Fox and Weissman 1979). In general, the role of X antigens in the induction of H-2 restricted Tc cell responses is poorly understood, and evidence for X antigen involvement seems to rest mainly on the anti-X specificity associated with H-2 restricted Tc cell recognition and the fact that X antigens must be present on the same cell as H-2 antigens in order to stimulate a response.

While the role of K and D antigens as targets for both allo-reactive and H-2 restricted Tc cells can be readily demonstrated by blocking target cell lysis with specific anti-H-2 serum (Lindahl and Lemke 1979, Blanden *et al.* 1979, also reviewed by Zinkernagel and Doherty 1979), similar attempts to inhibit Tc cell recognition of target cells with antiserum specific for the infecting virus or foreign antigen have generally been unsuccessful. Very few workers have achieved good blocking (Mullbacher and Blanden 1979a, Effros *et al.*

1979), and many seem to have failed (for example, Ertl, O'Neill and Ada - unpublished results).

H-2-linked differences in Tc cell responsiveness have been reported which have been interpreted in terms of the ability of different K and D antigens to associate with a foreign antigen in a manner appropriate for stimulation of a Tc cell response (Simpson and Gordon 1977, Shearer and Schmitt-Verhulst 1975, Mullbacher and Blanden 1978, Zinkernagel *et al.* 1978d, Kurrle *et al.* 1978). Material presented in Chapters 2 and 3 also indicates a quantitative requirement for antigen in the stimulation of a Tc cell response.

5.2 A Role for H-2 Antigens in T Cell Differentiation

The possibility that the thymus might be involved in determining *H-2* restriction specificity became very evident as a result of the original findings by Bevan (1977) and Zinkernagel and coworkers (1978a,b). They showed that chimeric mice, made by transferring immature F₁ hybrid stem cells into lethally irradiated parental strain mice, attained *H-2* restriction specificity preferential for the *H-2* type of the recipient. Experiments involving thymus grafting prior to irradiation and reconstitution of chimeric mice indicated a role for some radioresistant thymic cell in determining the *H-2* restriction 'phenotype'. This has now been shown to hold true for Tc cell responses to viruses (Zinkernagel *et al.* 1978a,b) as well as minor H antigens (Fink and Bevan 1978) which are restricted by the K and D regions, and for Th and Td cell responses (Waldmann *et al.* 1979, Miller *et al.* 1979) which are restricted by the I region. Similar experiments with chimeric mice have shown that the thymic *H-2* environment can also determine *Ir* gene effects on T cell responsiveness (Press and McDevitt 1977, Warner

et al. 1978, von Boehmer *et al.* 1978, Billings *et al.* 1978, Zinkernagel *et al.* 1978e, Kappler and Marrack 1978, Miller *et al.* 1979). In these experiments, chimeras made between responder and nonresponder mice adopted responsiveness typical of the *H-2* type of the recipient strain.

More recent experimental results from Zinkernagel and coworkers indicate that *H-2* antigens in the thymic environment are essential but not sufficient for selection of the restriction specificity. These workers found that parent 1 \rightarrow F₁ hybrid chimeras failed to express significant levels of Tc cell activity for targets of parent 2 type (Zinkernagel *et al.* 1978c). Such experiments, and subsequent ones involving reconstitution of nude mice with allogeneic or semi-allogeneic thymus grafts, have been interpreted to mean that lymphohemopoietic cells of the same *H-2* type as the thymus are necessary for maturation of immunocompetent T cells (Zinkernagel *et al.* 1979, 1980a). At this point it is still unclear whether this step occurs during thymic development, or by a postthymic maturation step.

In general, each of these studies indicates a functional role for *H-2* antigens in the thymic selection of precursor T cells. Although it is not known how this occurs, one possible mechanism is discussed in Chapter 9. While antiserum blocking experiments offer the best evidence for target cell *H-2* antigen involvement in T cell recognition, such experiments do not exclude the possibility that *H-2* antigens on both effector and target cells are concurrently involved in T cell recognition. This proposition is also considered in Chapter 9.

6. T Cell Receptors

Since T cells are activated by antigen and are specifically reactive for that antigen, it has been necessary to invoke an antigen receptor on T cells. While the antigen receptor on B cells, the immunoglobulin molecule, has been well characterised, there is very little definitive evidence for the nature of the antigen receptor on T cells. Both functional and chemical evidence has been emerging but many questions are still unanswered. Most workers involved in T cell research are hopeful that just as the 1970s heralded a major upheaval in immunological thinking with the discovery of the *H-2* restriction phenomenon, that the 1980s will bring elucidation of the T cell receptor puzzle.

6.1 Biochemical studies

Ramseier and Lindemann (1972) first proposed that T cells like B cells should carry idiotype determinants present on antigen-specific receptor molecules. Following their initial observation, considerable evidence has accumulated in the past few years which suggests that T cells have receptor molecules which bear idiotypes identical to those present on antibodies specific for the same antigen (reviewed by Eichmann 1978, Binz and Wigzell 1977a). Analysis of this phenomenon has involved the use of antibodies raised against either antigen-specific antibodies, or against alloantigen reactive T cells. The binding of such 'anti-idiotypic' antibodies to both B as well as T cells has been taken as evidence that receptors on the two cell types share idiotypes.

While the existence of an Ig-like molecule on T cells has been disputed for many years (reviewed by Marchalonis 1980), it now seems clear that T cells can express a molecule which shares variable (V)

region determinants with serum Ig. In biochemical studies, using anti-idiotypic antiserum raised against alloreactive T cells, Binz and Wigzell (1977b) demonstrated that the isolated receptor was a dimer comprising two 70,000 dalton subunits. By testing with various reagents, they showed that the only known antigen present on this molecule was the V_H idiotype. In similar experiments, Krawinkel *et al.* (1976) have isolated antigen-binding receptors from T cells and these molecules appear to resemble those characterised by Binz and Wigzell (1977b).

Studies on idiotypic molecules isolated from T cells suggest that these cells must use V_H genes to generate antigen-binding sites, and that a heavy chain Ig molecule probably constitutes at least a part of the T cell receptor (Janeway *et al.* 1976). However, it is not clear from these studies whether such V gene coded receptors are used for recognition of self H-2 antigens in H-2 restricted responses, or are used only for alloantigen and foreign antigen recognition. While many workers feel that anti-idiotypic reagents may provide a handle with which to investigate the nature of Tc cell receptors, the success obtained by only a few workers now appears to contrast with the difficulty reported by others in generating anti-idiotypic responses (see, for example, Bellgrau and Wilson 1979). Any other methods for isolation of receptor molecules face problems of obtaining enriched effector cell populations. New techniques for producing T cell hybridomas, and for maintaining specific T cell lines (Dennert and Raschke 1977, Hämmerling 1977; Nabholz *et al.* 1978; Baker *et al.* 1979, Watson 1979, von Boehmer *et al.* 1979) may provide another possible means.

7. Functional Studies

The very phenomenon of *H-2* restriction suggests that a restricted repertoire of recognition structures must exist on the peripheral pool of immunocompetent T cells. While the thymic environment appears to play a role in selection of the repertoire of the receptors (see *Immunological Reviews* 42, 1978, whole volume), there has been only speculation about how this occurs. Some form of clonal deletion or selection within the thymus is already well accepted (Jerne 1971, Blanden and Ada 1978, Langman 1978, Schwartz 1978), and predicts that only those cells expressing appropriate receptors will be allowed to emigrate and enter the T cell pool. As a result, traffic of immature lymphocytes through the thymus seems to be a critical step, not only for T cell differentiation, but also for regulation of the spectrum of T cell reactivity. How the thymus functions in this capacity is unknown, but an explanation would appear to relate to the need for surplus production of thymocytes as well as the extensive cell death of thymocytes *in situ* (reviewed by Stutman 1978).

In the past few years, speculation on whether *MHC* restricted Tc cells have one or two receptors involved in recognising target antigens has been rife (reviewed by Zinkernagel and Doherty 1979). 'Altered self' models predict that one receptor exists which is specific for a neoantigenic determinant formed by some interaction between self *MHC* antigens and the foreign (X) antigen (Bevan 1975a,b), while 'dual recognition' models require two receptors on the T cell, one specific for X, the other specific for self *MHC* antigens (Zinkernagel and Doherty 1975, Shearer *et al.* 1975). As a result, there are two main unknown issues concerning T cell recognition: the nature of the target antigen recognised, and the nature (and number) of the recognition structures on T cells.

Previous attempts to invoke mechanisms for T cell recognition, including the organisation of T cell receptors, have been fraught with the difficulty of explaining all of the following features of T cell function:

1. T cell recognition of foreign antigens must involve at least one receptor with specificity for self *MHC* antigens.
2. Functionally different T cells with diverse roles such as help, suppression, cytotoxicity etc., all have receptors which recognise *MHC* gene products.
3. A much larger proportion of the T cell pool is potentially capable of responding to foreign *MHC* antigens than to unrelated foreign antigens.
4. *MHC*-linked *I_r* gene effects can influence responsiveness to foreign (X) antigens.
5. Receptors on T cells appear to share idiotypic determinants with B cell receptors. This result can be interpreted to mean that either or both anti-*MHC* and anti-X receptors originate by the somatic mutation of germline *V* genes.

Subsequent to the finding that selection of the *H-2* restriction specificity of T cells was dependent on the *H-2* antigens expressed on a radioresistant thymic cell (Bevan 1977, Zinkernagel *et al.* 1978a,b) and was independent of foreign (X) antigens, models for T cell recognition involving separate anti-*H-2* and anti-X receptors became more favourable. Adoption of such models, however, raised the problem of how to invoke a two receptor model yet prevent anti-self reactivity,

as well as how to explain the generation of a pool of T cells with self recognition capacity which can function in both alloreactive as well as *H-2* restricted recognition. Different models have been proposed based on whether positive (Jerne 1971, Langman 1978, Miller 1978, Zinkernagel 1978b) or negative (Janeway *et al.* 1976, Blanden and Ada 1978) selection mechanisms are invoked to account for the generation of the T cell pool. Proponents of positive selection models have attempted different explanations for the derivation of *I_r* gene effects based on how the selection of an anti-self receptor prevents expression of a particular anti-X receptor (Langman 1978, Cohn and Epstein 1978, von Boehmer *et al.* 1978), and according to the different models, alloreactive recognition can include either anti-X receptors (Langman 1978, Cohn and Epstein 1978), or anti-self receptors (Janeway *et al.* 1976, von Boehmer *et al.* 1978b).

While each of the models has been debated at length, none has been exactly refuted. Structural information about T cell receptors appears to be a necessary next step.

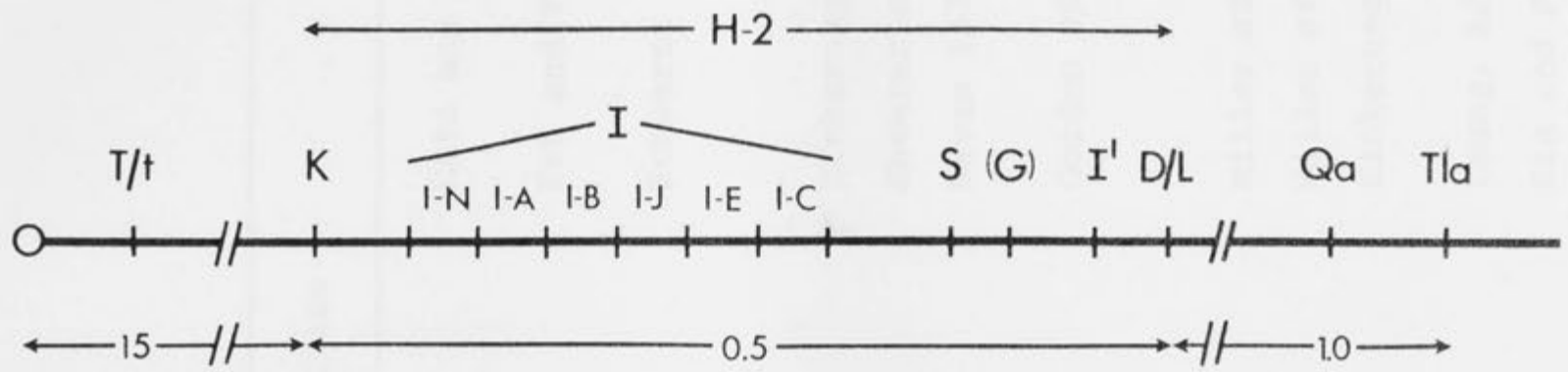


Fig. 1 - Genetic map of the H-2 gene complex
(map distance in centimorgans)

TABLE 1
H-2 RESTRICTED T CELL RESPONSES

Experimental System	H-2 Region Involved	T Cell Class	References
Cooperation between primed T and B cells for <i>in vivo</i> antibody responses	I-A	Th	Katz and Benacerraf 1975
Induction of Th cells by macrophage-associated antigens <i>in vivo</i>	I-A	Th	Erb and Feldman 1975
Antigen-induced proliferation of sensitised lymphocytes <i>in vitro</i>	I	-	Schwartz <i>et al.</i> 1976
Cytotoxic T cell responses to:			
1. Virus infected cells	K,D	Tc	Zinkernagel and Doherty 1974
2. Chemically modified cells	K,D	Tc	Shearer <i>et al.</i> 1975
3. Minor histocompatibility antigens	K,D	Tc	Bevan 1975a,b
4. HY antigen	K,D	Tc	Gordon <i>et al.</i> 1975
Transfer of delayed-type hypersensitivity to:			
1. Proteins and polypeptides	I-A	Td	Miller <i>et al.</i> 1975; 1977
2. Contact chemicals	I,K or D	Td	Miller <i>et al.</i> 1976
3. Lymphocytic choriomeningitis virus	K,D	-	Zinkernagel 1976a
4. Influenza viruses	I	Td	Leung, Ada and McKenzie 1980
Autorosetting of thymocytes	D(L)	-	Sia and Parish 1980a
Honing of lymphocytes	K,D	-	Degos <i>et al.</i> 1980

The relative products of the major histocompatibility complex play a crucial role in the regulation of immune responses. Parentally-derived antigens are processed and presented by H-2 antigens to T cells. The H-2 system is highly polymorphic, and the expression of H-2 antigens is controlled by a complex set of regulatory genes. The H-2 system is essential for the development of a T cell response to foreign antigens. It is generally accepted that the H-2 system is a major determinant of the magnitude and quality of the T cell response. In the mouse, H-2 or H-2D antigens are involved in regulating T cell (Th) cell responses (see review by Sherman et al., 1978; Klein et al., 1978; Sherman et al., 1978). The H-2 system is also involved in the regulation of B cell responses (see review by Sherman et al., 1978).

CHAPTER 2

The role of H-2 antigens in the regulation of T cell responses which determines the magnitude and quality of the T cell response.

QUANTITATIVE DIFFERENCES IN THE EXPRESSION OF PARENTALLY-DERIVED H-2 ANTIGENS IN F₁ HYBRID MICE AFFECT T CELL RESPONSES

It has been shown that quantitative differences in the expression of H-2 antigens in F₁ hybrid mice affect T cell responses. The H-2 system is highly polymorphic, and the expression of H-2 antigens is controlled by a complex set of regulatory genes. The H-2 system is essential for the development of a T cell response to foreign antigens. It is generally accepted that the H-2 system is a major determinant of the magnitude and quality of the T cell response. In the mouse, H-2 or H-2D antigens are involved in regulating T cell (Th) cell responses (see review by Sherman et al., 1978; Klein et al., 1978; Sherman et al., 1978). The H-2 system is also involved in the regulation of B cell responses (see review by Sherman et al., 1978). In this report, quantitative differences in the expression of H-2 antigens in F₁ hybrid mice affect T cell responses. The H-2 system is highly polymorphic, and the expression of H-2 antigens is controlled by a complex set of regulatory genes. The H-2 system is essential for the development of a T cell response to foreign antigens. It is generally accepted that the H-2 system is a major determinant of the magnitude and quality of the T cell response. In the mouse, H-2 or H-2D antigens are involved in regulating T cell (Th) cell responses (see review by Sherman et al., 1978; Klein et al., 1978; Sherman et al., 1978). The H-2 system is also involved in the regulation of B cell responses (see review by Sherman et al., 1978).

INTRODUCTION

The antigenic products of the major histocompatibility complex (*MHC*) play a crucial role in the stimulation of immune responses of thymus-derived lymphocytes (T cells) (for reviews see Doherty *et al.* 1976, Shearer and Schmitt-Verhulst 1977, Shearer *et al.* 1976, Snell 1978). A T cell response to foreign antigen (X) is apparently stimulated not by X alone, but by an antigenic pattern dependent upon both X and an appropriate *MHC*-coded antigen. In the mouse, H-2K or H-2D antigens are involved in cytotoxic T cell (Tc cell) responses (Bevan 1975, Doherty *et al.* 1976, Gordon *et al.* 1975, Shearer *et al.* 1976) and *I*-region-dependent antigens are involved in helper (Erb and Feldman 1975, Katz and Benacerraf 1975) and delayed hypersensitivity responses (Miller *et al.* 1976).

Thus far, *MHC*-linked control of T cell responses which determines high- or low-responder status of a particular mouse strain for a particular antigen, has been interpreted in terms of qualitative differences between different *K*, *D*, or *I* gene products (Blank and Lilly 1977, von Boehmer *et al.* 1977, 1978, Hurme *et al.* 1978, Simpson and Gordon 1977). For example, Tc cells of female inbred mice may or may not respond to the male-specific HY antigen of otherwise identical inbred males depending upon whether or not the *K*, *D*, or *I* region gene products are 'permissive' for the Tc cell or helper T cell responses (von Boehmer *et al.* 1977, 1978, Hurme *et al.* 1978, Gardner and Blanden 1976). In this report, quantitative differences in the expression of the same K and D antigens between cells of parental strain and F₁ hybrid mice have been demonstrated, and these differences have been shown to markedly influence the ability of the cells to stimulate Tc cell responses, and to act as targets for Tc cell-mediated lysis.

MATERIALS AND METHODS

Animals

Mice were bred in the John Curtin School of Medical Research and used when 6-10 weeks old. Mice of the same age and sex were used in each experiment.

Priming of mice

Mice were primed by i.v. injection of 10^5 PFU of ectromelia virus (attenuated Hampstead egg strain) and used from 2 weeks to 6 months post-priming.

Antisera

All antisera were generously provided by Dr Ian McKenzie (University of Melbourne, Australia). They were raised by multiple immunizations of lymphoid cells and thereafter bleeding at regular intervals (McKenzie and Snell 1973). They were produced in the following strain combinations: anti-D^b (H-2.2) in (B10.D2xA)₁F₁ anti-B10.A(2R); anti-K^b (H-2.33) in (B10.D2xA) anti-B10.A(5R); anti-D^d (H-2.4) in (B10.AKMx129)₁F₁ anti-B10.A; anti-K^k (H-2.23) in (AQRxB10) anti-B10.A.

In vitro generation of cytotoxic T cells

(i) Alloreactive Tc cells. One-way mixed lymphocyte reactions (MLR), utilising spleen cell responders and ⁶⁰Co γ-irradiated (2000 rads) spleen stimulators were set up in tissue culture flasks (Falcon Plastics, Becton Dickinson and Co., Oxnard, Ca.) at a concentration of 2.5×10^6 responder cells/ml. Usually 10^8 responder cells were cultured with 2.5×10^7 stimulator cells for 5 days in 40 ml of Eagle's minimal essential medium (F15, Grand Island Biological Co., Grand Island, NY) containing 10^{-4} M 2-mercaptoethanol and supplemented with antibiotics and 10% fetal calf serum (FCS) in an atmosphere of 10% CO₂, 7% O₂ and 83% N₂ for 5 days at 37°C. When a mixture of stimulators was used,

they were irradiated before mixing. Responder:stimulator ratio was always 4:1.

(ii) Anti-ectromelia Tc cells. The method used for generating an *in vitro* secondary response to ectromelia virus is similar to that described previously by Gardner and Blanden (1976). The procedure involved culturing spleen cells from mice previously immunised with attenuated ectromelia virus (responder cells) with normal syngeneic infected spleen cells (stimulator cells). Stimulator cells were infected with 5 PFU/cell of the virulent Moscow strain of ectromelia virus and a responder to stimulator ratio of 10:1 was used routinely. Cells were cultured for 5 days at 39°C (a temperature, non-permissive for ectromelia virus) at a concentration of 2×10^6 responder cells/ml in F15 medium supplemented with 10% FCS, 10^{-4} M 2-mercaptoethanol and antibiotics in an atmosphere of 10% CO₂, 7% O₂ and 83% N₂.

Each experiment involving the generation of Tc cells was repeated at least twice, and included cells from individual mice, and single cultures. Either a male was used as responder, or females were used throughout each experiment to eliminate possible effects due to the HY antigen.

Cytotoxicity assays

A ⁵¹Cr release assay using macrophage targets was employed. This method was originally described by Gardner and Blanden in 1976. Briefly, 10^5 ⁵¹Cr-labelled (sodium chromate, C.A.E., Gif sur Yvette, France) peritoneal macrophages were used as targets, and were incubated at 37°C for 6 hours with effector cells at a wide range of killer:target ratios, in an atmosphere of 10% CO₂, 7% O₂ and 83% N₂.

Effector cells were cleared of dead cells by centrifugation through Isopaque/Ficoll as described by Davidson and Parish (1975) before

addition to the target cells. The assay was carried out in triplicate in 96 well (6 mm diameter) plastic tissue culture trays (Linbro Chemical Co., New Haven, Conn.) containing cells in a final volume of 0.2 ml F15 medium with 10% FCS. The target cells for anti-ectromelia responses were infected with 10 PFU/cell virulent Moscow strain of virus for 1 hour at 37°C, and effector cells were added 1 hour after unabsorbed virus was washed away. Percent maximum releasable ^{51}Cr was calculated by water lysis of cells. Spontaneous release was about 3% per hour. The corrected percent lysis was calculated by the formula:

$$\% \text{ specific } ^{51}\text{Cr} \text{ release} = \frac{\% \text{ releasable by killers} - \% \text{ medium release}}{\% \text{ maximum releasable} - \% \text{ medium release}}$$

Two-stage microcytotoxicity assay

The method used is similar to that described previously by McKenzie *et al.* (1976). In short, spleen cells were cleared of red and dead cells by centrifugation through Isopaque/Ficoll (Davidson and Parish 1975) and then resuspended to a concentration of 5×10^6 cells/ml in F15 medium supplemented with 5% FCS. Two-fold diluting concentrations of antiserum (50 μl) were then absorbed to 50 μl of cells for 30 minutes at 4°C in the wells of a U-bottomed Linbro microtitre plate. Unabsorbed antiserum was washed away by three additions of medium and centrifugation of the plate. Preabsorbed rabbit serum, diluted 1 in 4 (50 μl), was used as a complement source, and cells were incubated in the presence of complement for 30 minutes at 37°C. The percentage of dead cells was measured by trypan blue staining.

Quantitative absorption analysis

The method used is essentially similar to that documented by McKenzie *et al.* in 1976. It involves preabsorption of antiserum onto diluting numbers of cells, followed by measurement of unbound antibody

in a routine two-stage microcytotoxicity assay. Spleen cells which had been cleared of red and dead cells by centrifugation through Isopaque/Ficoll (Davidson and Parish 1975) were used both for absorption and titration. Firstly, antisera were titred at least twice on control cells. For quantitative absorption, 50 μ l of cells (doubling dilutions) was added to 50 μ l of antiserum (used at a dilution which gives 80% lysis of control cells in a routine titration) for 30 minutes at room temperature. The dilutions used are listed in the legend to Figure 1. Following centrifugation, each sample of absorbed antiserum was removed for titration in the two-stage microcytotoxicity assay. Unabsorbed antiserum and rabbit complement were titred as controls and results were plotted as percent dead cells versus number of absorbing cells (log 2 scale). The likelihood ratio test (LRT) was used to compare data sets in each experiment, and maximum likelihood estimates of the 50% endpoints were calculated.

RESULTS

Absorption of anti-H-2 sera by spleen cells from parental strain and F_1 hybrid mice

Spleen cells from B10.A(5R) (K^b, D^d) and B10.A(2R) (K^k, D^b) mice were compared with cells from the [B10.A(5R)xB10.A(2R)] F_1 hybrid for their ability to absorb out activity from specific anti-H-2 serum. The results (Figure 1) show that B10.A(2R) and F_1 cells were indistinguishable in their ability to absorb anti- K^k and anti- D^b sera, but F_1 cells were significantly inferior to B10.A(5R) in absorbing anti- K^b and anti- D^d sera. The data suggested that F_1 cells expressed approximately 4-fold less K^b and D^d surface antigen than B10.A(5R) cells, and that the difference was possibly more pronounced with D^d than K^b .

Sensitivity of macrophages from parental strain and F_1 hybrid mice to lysis by alloreactive and $H-2$ restricted Tc cells

Tc cells from B10.G($H-2^d$) mice were stimulated in MLR by either B10.A(2R) or B10.A(5R) cells. The effector Tc cells thus generated were assayed on macrophage target cells from B10.A(2R), B10.A(5R) and F_1 hybrid mice (Table 1). The results show that lysis of B10.A(2R) and F_1 targets by anti-B10.A(2R) Tc cells was similar, but that B10.A(5R) targets were lysed significantly more than F_1 targets by anti-B10.A(5R) Tc cells. This latter difference was of the order of 10-fold, i.e. up to 10 times more Tc cells were required to lyse F_1 cells to the same extent as B10.A(5R) cells.

Four other MLR were set up to generate Tc cells specific for the K^b , D^d , K^k or D^b antigens and were assayed against B10.A(5R), B10.A(2R) and F_1 macrophage targets (Table 2). Again, B10.A(2R) and F_1 macrophages were lysed similarly by anti- K^k and anti- D^b Tc cells, while B10.A(5R) targets were lysed more efficiently than F_1 cells by anti- K^b and anti- D^d Tc cells. However, the difference between B10.A(5R) and F_1 targets was less pronounced than in Table 1, and was seen most prominently at high killer:target ratios. Also the difference between B10.A(5R) and F_1 was greater with anti- D^d than with anti- K^b Tc cells, a result which conforms with the antiserum absorption results (Figure 1).

Differences between F_1 and parental strain macrophage were also investigated using $H-2$ restricted Tc cells (Table 3). Secondary anti-ectromelia Tc cell responses were generated *in vitro* using B10.A(2R) and B10.A(5R) responders and their reactivity tested on infected and uninfected targets. B10.A(2R) Tc cells which recognise K^k and D^b plus viral antigens killed B10.A(2R) infected cells as efficiently as F_1 cells over a 10-fold range of killer:target ratios. In contrast,

there was approximately 3 times less lysis of F_1 than B10.A(5R) infected targets by B10.A(5R) Tc cells. Similar results have been obtained using B10.A(5R) and B10.A(2R) Tc cells recognising Sendai virus-infected cells (data not shown).

Taken together, the data in Tables 1, 2 and 3 support the conclusion that the concentration of K^b and D^d antigens on F_1 macrophages is lower than on B10.A(5R) macrophages, as reflected in the lower efficiency of recognition and lysis by alloreactive or *H-2* restricted Tc cells.

Ability of spleen cells from parental strain and F_1 hybrid mice to stimulate production of Tc cells in MLR

Irradiated spleen cells from B10.A(2R), B10.A(5R) and the [B10.A(5R)xB10.A(2R)] F_1 hybrid were used as stimulator cells in an MLR with either B10.A(2R) or B10.A(5R) responders (Table 4). F_1 cells were compared with either B10.A(2R) or B10.A(5R) cells, for their ability to stimulate a response in the opposite parent. The stimulating ability of limiting numbers of cells was investigated in terms of the effector Tc cell activity generated for a given initial responder cell population. F_1 cells were as efficient as B10.A(2R) cells in producing an anti-B10.A(2R) response, but they were significantly inferior to B10.A(5R) cells in stimulating an anti-B10.A(5R) Tc cell response.

DISCUSSION

The results of this investigation indicate that less B10.A(5R)-derived H-2K and H-2D antigenic determinants are expressed on the surfaces of peritoneal macrophages and spleen cells of heterozygous [B10.A(5R)xB10.A(2R)] F_1 hybrid mice than similar cells of B10.A(5R) homozygotes. The difference is selective and not due to heterozygosity

per se, since H-2K and H-2D antigens coded by the B10.A(2R) haplotype were apparently expressed in similar concentrations on cells from [B10.A(5R)xB10.A(2R)]F₁ and B10.A(2R) mice. Examination of other F₁ hybrids (see Chapter 5) has shown that some mouse strain combinations in F₁ hybrids exhibit this differential expression of parentally-derived H-2 antigens, while others show even-handed expression. Galfre and co-workers (1977) have seen a similar difference in MHC antigen expression between DA and (HOxDA)F₁ hybrid rats. In their case, the two strains of rats had different genetic backgrounds, so that the location or nature of the genes which regulate expression of MHC genes is unknown. In this study, two congenic mouse strains which differ only in the region of chromosome 17 carrying the H-2 gene complex have been used, which raises the possibility of H-2-linked regulation of H-2 gene expression.

Several workers have reported selective depression of MHC-coded antigen expression on the surfaces of cell lines cultured in the presence of anti-MHC antibody (Pious *et al.* 1973, Rajan 1977). It is interesting that antibody specific for one out of four H-2K and H-2D antigens expressed on murine F₁ tumour cells depressed the expression not only of that antigen, but in some cases also depressed the antigen coded by the K or D gene in the *cis* position. This observation is similar in that it involves reduced expression of the K and D genes of one parental chromosome of the F₁ hybrid, but whether this reflects a common mechanism is unknown. One obvious area under investigation is the expression of maternal versus paternal antigens.

Apart from its intrinsic interest as a case study for the regulation of H-2 antigen expression, the phenomenon reported here has implications for the induction and expression of T cell responses in

which specific recognition of antigens coded by the *MHC* is crucial. For example, experiments in which macrophages were used as targets for lysis by alloreactive or *H-2* restricted Tc cells showed that there was less lysis of the F_1 hybrid targets than of B10.A(5R) targets by anti-B10.A(5R) effector Tc cells, but similar lysis of F_1 and B10.A(2R) targets by anti-B10.A(2R) Tc cells. The differences between B10.A(5R) and F_1 were variable, but generally conformed with the differences in $H-2K^b$ and $H-2D^d$ antigen concentration between spleen cells of B10.A(5R) and F_1 mice, which appeared to be about 4-fold by quantitative absorption experiments with specific anti-*H-2* sera.

There was also a clear effect on the ability of spleen cells to stimulate Tc cell responses. The data in Table 4 show that alloreactive Tc cells specific for *H-2* antigens coded by the B10.A(5R) haplotype were less efficiently stimulated by the F_1 cells than by B10.A(5R) cells, whereas B10.A(2R) and the F_1 hybrid cells were of similar efficiency in stimulating Tc cells specific for the *H-2* antigens coded by the B10.A(2R) haplotype. The ability of F_1 hybrid cells to stimulate *H-2* restricted Tc cell responses against various minor histocompatibility antigens and viruses appears to be more difficult to analyse and may be influenced by differences in the nature and density of the foreign antigen stimulating the response.

F_1 hybrids may not necessarily be at a selective advantage as proposed by Doherty and Zinkernagel (1975a,b) simply because they express a wider repertoire of *H-2* antigens than homozygotes. An F_1 hybrid with some weakly expressed *H-2* antigens could be a poor responder with respect to *H-2* restricted Tc cells which recognise these antigens.

The concept that H-2 antigen concentration on stimulator cell surfaces is a crucial variable determining the strength of T cell responses is relevant to two other phenomena. First, Egorov *et al.* (1977) have reported several examples of graft versus-host reactions against H-2 antigens coded by a given haplotype that were weaker when the reaction was stimulated by a semi-histocompatible heterozygote rather than a homozygote. This otherwise puzzling result is readily explained by a decreased concentration of parentally-derived H-2 antigens on the cells of F_1 hybrids. Second, Lafferty and colleagues (1976) have observed that survival of allografted thyroid tissue can be spectacularly prolonged if measures, such as organ culture, are taken to remove passenger lymphomyeloid cells from the tissue prior to grafting. Since histocompatibility antigens are generally expressed in higher concentrations on lymphomyeloid cells than other cell types (Klein 1975), the results in Table 4 may partly explain why lymphomyeloid cells are potent stimulators of allogeneic T cell responses (Greineder and Rosenthal 1975), and why the response to pre-cultured thyroid grafts is weak or absent.

SUMMARY

Quantitative absorption with specific anti-H-2 sera has shown that the H-2K^b and H-2D^d antigens coded by the B10.A(5R) haplotype are expressed in about 4-fold lower amount on the spleen cells of [B10.A(5R) x B10.A(2R)] F_1 hybrids than on parental B10.A(5R) cells. In contrast, the H-2K^k and H-2D^b antigens of B10.A(2R) are expressed equally on parental and F_1 hybrid cells. These quantitative differences are reflected in Tc cell function. Macrophage target cells from F_1 hybrid mice are killed less efficiently than B10.A(5R) targets

by alloreactive or *H-2* restricted Tc cells specific for H-2K^b or H-2D^d, and spleen cells of F₁ hybrid mice are less efficient stimulators of alloreactive Tc cells specific for B10.A(5R) H-2 antigens, whereas the F₁ hybrid and B10.A(2R) cells are equal as targets and stimulators for Tc cells recognising B10.A(2R) H-2 antigens.

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Fig. 1 Quantitative absorption of anti H-2 serum by spleen cells of the F_1 hybrid [B10.A(5R)xB10.A(2R)] in comparison with B10.A(5R) and B10.A(2R) cells. The F_1 hybrid was compared with B10.A(5R) for absorption of anti- K^b and anti- D^d serum and with B10.A(2R) for anti- K^k and anti- D^b serum. F_1 (●); 2R (■); 5R (▲). Lysis by unabsorbed antiserum plus rabbit complement (AS) and by rabbit complement alone (RC) are shown as controls. Each experiment was repeated thrice, but one representative result is shown. The number of cells absorbing was plotted on a \log_2 scale.

The likelihood ratio test was used to compare data sets in each experiment. The 50% end point of titration was calculated from the curve of best fit (maximum likelihood estimation) and the results are summarised below.

Antiserum specificity	Dilution used for absorption	LRT	Cell number required to reduce cytotoxicity of antiserum by 50%		
			(5Rx2R) F_1	5R	2R
K^b (H-2.33)	$\frac{1}{300}$	$\chi_{10}^2 = 24.36$	11.3×10^5	3.5×10^5	
D^d (H-2.4)	$\frac{1}{120}$	$\chi_{10}^2 = 44.12$	19.1×10^5	4.0×10^5	
K^k (H-2.23)	$\frac{1}{16}$	$\chi_8^2 = 8.48^a$	6.0×10^5		8.8×10^5
D^b (H-2.2)	$\frac{1}{50}$	$\chi_8^2 = 12.57^a$	4.6×10^5		4.6×10^5

a Not significantly different at the 95% level.

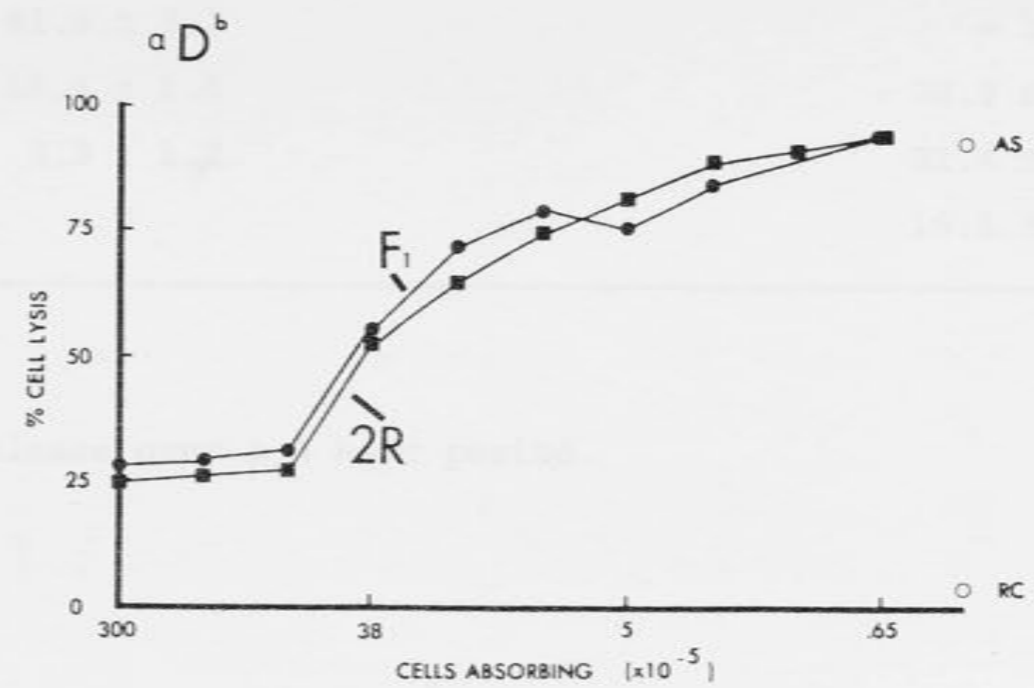
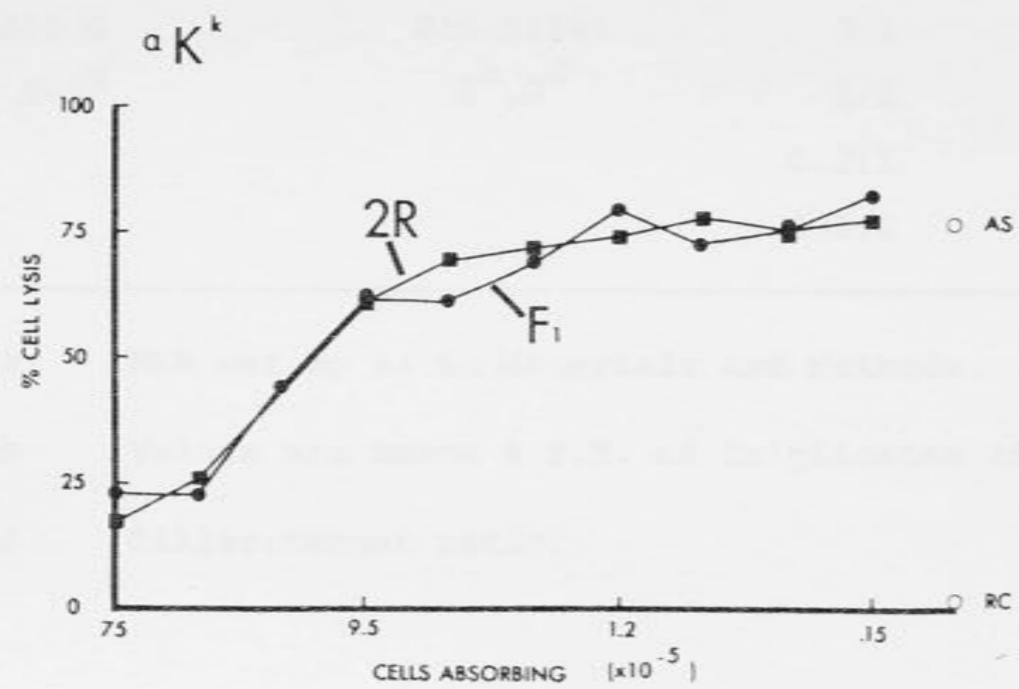
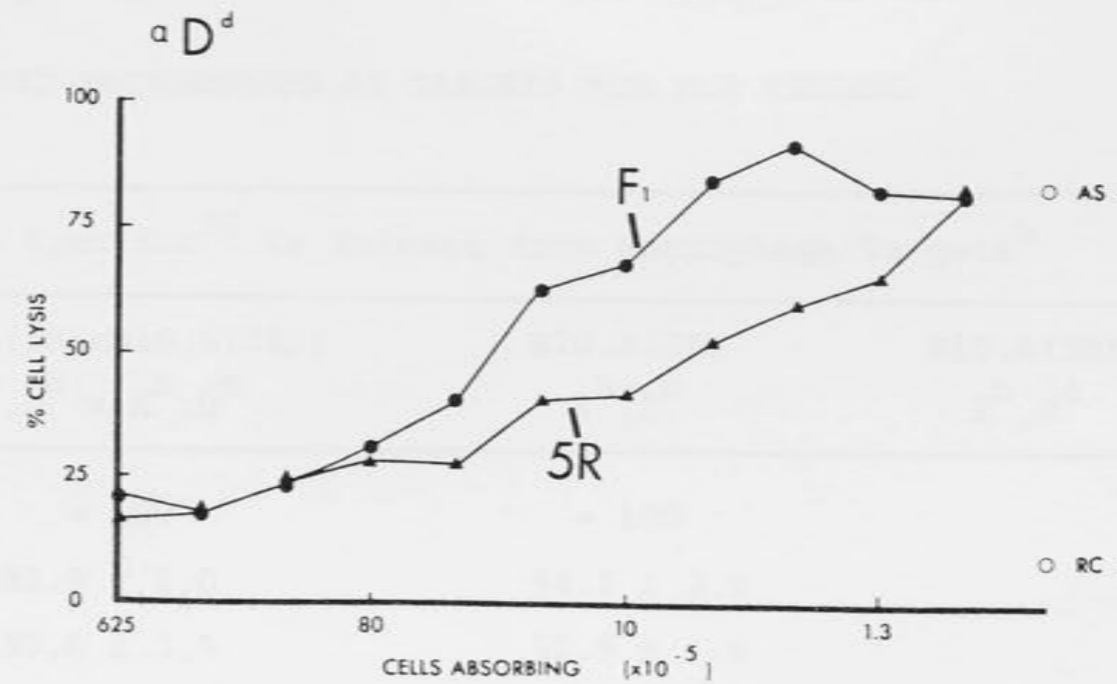
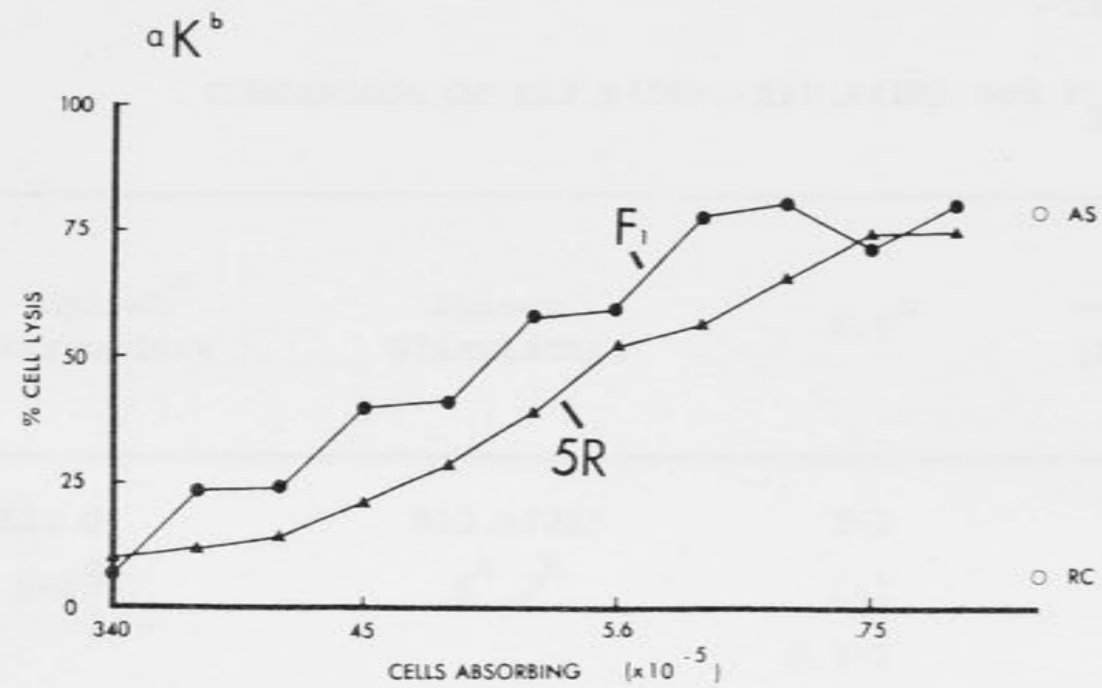


TABLE 1

COMPARISON OF B10.A(5R), B10.A(2R) and F₁ HYBRID MACROPHAGES AS TARGETS FOR MLR KILLERS

Spleen ^a Responders	Spleen Stimulators	K:T ^c	% Specific ⁵¹ Cr Release from Macrophage Targets ^b		
			[B10.A(5R) x B10.A(2R)] K ^b ,D ^d x K ^k ,D ^b	B10.A(2R) K ^k ,D ^b	B10.A(5R) K ^b ,D ^d
B10.G H-2 ^q	B10.A(2R) K ^k ,D ^b	3:1	= 100	= 100	
		1:1	82.9 ± 1.0	54.2 ± 3.9	
		0.3:1	37.0 ± 3.6	32.5 ± 1.6	
		0.1:1	6.1 ± 0.7	6.0 ± 0.6	
B10.G H-2 ^q	B10.A(5R) K ^b ,D ^d	3:1	42.9 ± 3.3		= 100
		1:1	16.4 ± 1.0		78.3 ± 1.2
		0.3:1	3.3 ± 1.2		31.4 ± 3.8
		0.1:1	0		16.1 ± 2.1

a MLR set up as in Materials and Methods.

b Values are means ± S.E. of triplicates of ⁵¹Cr release over a 6 hour period.

c Killer:target ratio.

TABLE 2

DIFFERENTIAL H-2 ANTIGEN EXPRESSION ON [B10.A(5R)xB10.A(2R)]F₁ HYBRID MACROPHAGE TARGETS

Spleen ^a Responders	Spleen Stimulators	K:T ^c	% Specific ⁵¹ Cr Release from Macrophage Targets ^b				
			[B10.A(5R)xB10.A(2R)] K ^b ,D ^d x K ^k ,D ^b	B10.A(2R) K ^k ,D ^b	B10.A(5R) K ^b ,D ^d	B10 H-2 ^b	B10.BR H-2 ^k
B10 H-2 ^b (anti-K ^k)	B10.A(2R) K ^k ,D ^b	9:1	96.4 ± 1.1	89.2 ± 0.4		14.0 ± 1.0	
		3:1	85.8 ± 1.6	84.0 ± 1.3		8.6 ± 0.4	
		1:1	68.5 ± 3.2	61.2 ± 3.7		4.8 ± 1.2	
		0.3:1	19.3 ± 3.9	30.9 ± 0.8		0	
B10.BR H-2 ^k (anti-D ^b)	B10.A(2R) K ^k ,D ^b	9:1	= 100	= 100			19.2 ± 1.4
		3:1	97.8 ± 1.9	90.6 ± 0.7		7.2 ± 2.3	
		1:1	46.8 ± 1.1	43.3 ± 2.0		1.4 ± 2.1	
		0.3:1	28.8 ± 1.9	17.5 ± 1.0		0	
B10.D2 H-2 ^d (anti-K ^b)	B10.A(5R) K ^b ,D ^d	9:1	59.8 ± 0.6		76.5 ± 1.0		6.2 ± 0.2
		3:1	53.8 ± 1.1		63.3 ± 2.6		5.5 ± 0.8
		1:1	26.4 ± 0.6		30.4 ± 0.4		2.3 ± 0.7
		0.3:1	9.7 ± 1.5		17.2 ± 1.5		0
B10 H-2 ^b (anti-D ^d)	B10.A(5R) K ^b ,D ^d	9:1	63.0 ± 0.5		82.8 ± 1.2	9.3 ± 0.9	
		3:1	60.6 ± 1.3		75.2 ± 2.7	2.8 ± 0.6	
		1:1	35.7 ± 1.3		54.0 ± 4.3	4.8 ± 1.0	
		0.3:1	7.2 ± 1.8		15.8 ± 1.9	2.1 ± 0.9	

a, b, c As for Table 1.

TABLE 3

RECOGNITION OF H-2 ANTIGENS IN (5R x 2R)F₁ TARGETS BY H-2-RESTRICTED ANTI-ECTROMELIA Tc CELLS

Secondary ^a Anti-Ectromelia Tc Cells	K:T ^c	% Specific ⁵¹ Cr Release from Macrophage Targets ^b					
		B10.A(2R) <i>K^k,D^b</i>		B10.A(5R) <i>K^b,D^d</i>		[B10.A(5R) x B10.A(2R)] <i>K^b,D^d x K^k,D^b</i>	
		Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
B10.A(2R) <i>K^k,D^b</i>	3:1	57.1±0.2	12.3±0.8			61.4±1.1	22.5±1.6
	1:1	43.0±0.2	8.0±1.7			45.8±0.7	11.4±1.4
	0.3:1	34.0±1.0	0			37.8±1.4	3.3±1.0
B10.A(5R) <i>K^b,D^d</i>	1:1			68.2±2.1	12.9±1.7	62.9±2.0	25.6±1.6
	0.3:1			62.5±2.3	6.6±0.5	47.1±1.7	8.1±1.5
	0.1:1			48.2±1.8	4.1±1.6	36.4±1.5	4.0±0.7

a 10^7 ectromelia-infected spleen stimulators were cultured for 5 days with 10^8 syngeneic spleen responders from mice preprimed intravenously with 10^5 PFU hamstead egg strain virus.

b, c As for Table 1.

TABLE 4

Tc CELL STIMULATION BY H-2 ANTIGENS OF THE [B10.A(5R)xB10.A(2R)]F₁ HYBRID

Spleen ^a Responders	Spleen Stimulators	Fraction of ^d culture assayed	% Specific ⁵¹ Cr Release from Macrophage Targets ^b					
			B10.A(5R) K ^b ,D ^d			B10.A(2R) K ^k ,D ^b		
			4:1 ^c	8:1 ^c	16:1 ^c	4:1 ^c	8:1 ^c	16:1 ^c
B10.A(2R) K ^k ,D ^b	[B10.A(5R)xB10.A(2R)] [K ^b ,D ^d x K ^k ,D ^b]	0.15	22.0±3.2	6.5±1.6	0	5.1±1.6	1.9±0.7	0.8±1.3
		0.08	10.0±1.7	1.7±2.1	2.8±2.9	1.9±0.3	0	0
		0.04	9.5±1.1	2.0±0.8	0.5±0.7	2.4±1.9	0	1.2±1.2
B10.A(2R) K ^k ,D ^b	B10.A(5R) K ^b ,D ^d	0.15	58.4±0.6	39.0±1.8	1.8±2.1	5.0±1.7	6.0±0.9	0
		0.08	32.6±2.7	18.6±1.8	0	3.9±1.5	0	0
		0.04	16.9±2.3	5.1±0.6	0	0	0	0
B10.A(5R) K ^b ,D ^d	[B10.A(5R)xB10.A(2R)] [K ^b ,D ^d x K ^k ,D ^b]	0.15	0	0	0	31.2±2.4	12.0±1.6	9.3±2.2
		0.08	0	0	0	14.6±0.7	6.5±4.0	3.7±1.5
		0.04	0	0	0	7.1±1.5	6.9±1.2	0
B10.A(5R) K ^b ,D ^d	B10.A(2R) K ^k ,D ^b	0.15	0	0	0	30.3±0.5	13.9±1.6	7.7±2.3
		0.08	0	0	0	29.5±3.2	13.1±1.0	4.2±1.2
		0.04	0	0	0	9.2±0.9	3.3±2.6	2.2±2.3

a, b As for Table 1. Cells from a pool of 2 mice were used as both responders and stimulators in MLR.

c Ratio of responder to stimulator cells used in MLR.

d Cultures employ splenic responders at a concentration of 2×10^6 cells/ml and diluting numbers of γ -irradiated spleen stimulators. Triplicate 2ml cultures were pooled after 5 days and given fraction of input cells assayed.

INTRODUCTION

The first F₁ hybrid mice appear to express on their surface both H-2K and H-2D antigens. It is possible that the H-2D antigen is expressed on a separate H-2D molecule, but the possibility of a non-independent expression of H-2 antigens (Palmer and Hildreth 1977) has been suggested. The H-2D antigen has been shown to be a separate gene product (Palmer and Hildreth 1977) and is not a variant of H-2K. The H-2D antigen has been shown to be a separate gene product (Palmer and Hildreth 1977) and is not a variant of H-2K. The H-2D antigen has been shown to be a separate gene product (Palmer and Hildreth 1977) and is not a variant of H-2K.

CHAPTER 3

VARIATION IN H-2 ANTIGEN EXPRESSION IN F₁

HYBRID MICE: ANALYSIS USING MONOCLONAL ANTIBODIES

In this investigation, a simple technique for the analysis of H-2 antigen expression on the surface of cells was used. The method is described here and has been used to analyze the relative number of H-2 antigens expressed on the surface of cells. The method is described here and has been used to analyze the relative number of H-2 antigens expressed on the surface of cells. The method is described here and has been used to analyze the relative number of H-2 antigens expressed on the surface of cells.

INTRODUCTION

Cells from F_1 hybrid mice appear to express on their surface membranes less of some H-2K and H-2D antigens than cells of parental strain mice, while other H-2 antigens are expressed in equal amounts (Chapter 2). So far, these findings have been discussed in purely quantitative terms, but the possible existence of H-2-dependent antigens unique to F_1 hybrids (Fathman and Nabholz 1977), the demonstration of F_1 anti-parent responses (Ishikawa and Dutton 1979, Warner and Cudkowicz 1979, Cudkowicz *et al.* 1979), and evidence for multi-genic control of Ia antigens (Fathman and Nabholz 1977, Jones *et al.* 1978) raise the possibility of a qualitative explanation. Thus, if a significant proportion of H-2 molecules coded by a structural gene from one parent was modified by interaction with gene product(s) from the other parent they may bind with reduced affinity to antibodies or T cells (thymus-derived lymphocytes) raised against the parental type antigen. This would give the false impression of reduced expression of the parentally-derived structural gene.

In this investigation, a simple two-stage radioimmunoassay is described which has been used to estimate the relative numbers of H-2K^k antigen molecules expressed on the surface of CBA/H and (BALB/c x CBA/H) F_1 cells, as well as the approximate affinity with which these molecules bind two different monoclonal anti-H-2K^k antibodies. The assay involves the use of ¹²⁵I-labelled protein A to measure antibody binding (Goding 1978), together with a simple reciprocal plot estimation method commonly used to measure enzyme kinetics (Klotz 1953) in order to estimate parameters of antibody binding (Ada and Yap 1979). For each of the monoclonal antibodies, the results showed a quantitative

reduction in H-2K^k expression in the F₁ hybrid, with no indication of any qualitative difference.

MATERIALS AND METHODS

Mice

Mice were bred in the John Curtin School of Medical Research and used when 6-10 weeks old. Mice of the same age and sex were used in each experiment.

Immunization

Mice were primed with ectromelia virus by i.v. inoculation of 10⁵ plaque-forming units (PFU) of the attenuated Hampstead egg strain and used from 2 weeks to 6 months post priming.

Antisera

Anti H-2K^k serum (#433), produced by repeated inoculation of (AQRxB10)F₁ mice with B10.A lymphoid cells, was donated by Dr Ian McKenzie (University of Melbourne, Australia).

The monoclonal antibodies 27R9 and 30R3 were produced and described in detail by Lemke and co-workers (Lemke *et al.* 1978). They were obtained as freeze-dried ascitic fluid, produced by hybridomas derived from fusion of the myeloma P3-X63-Ag8 with BALB/c (H-2^d) spleen cells obtained from mice immunized against CBA/H spleen cells. 27R9 is IgG_{2a} and 30R3 is IgG_{2b}; thus both of them bind protein A (Goding 1978).

Quantitative absorption analysis

The method used is that of McKenzie *et al.* (1976) and has been described in detail in Chapter 2. Briefly, diluting numbers of F₁ hybrid and parental strain spleen cells, separated from dead cells and red cells by centrifugation through Isopaque/Ficoll, were compared for their ability to absorb activity from a given amount of anti-H-2

antibody. Remaining antibody was titred on parental strain cells in a two-stage complement-mediated microcytotoxicity assay using trypan blue staining of dead cells. This assay has also been described in Chapter 2.

In vitro generation of cytotoxic T cells

The methods for the generation of cytotoxic T cells (Tc cells) in secondary anti-ectromelia responses *in vitro* and primary mixed lymphocyte reactions (MLR) have been previously described (Gardner and Blanden 1976, Chapter 2).

Briefly, secondary virus-specific Tc cells were generated by culturing splenic responders from virus-primed mice for 5 days at 39°C (nonpermissive for ectromelia replication) with virus-infected syngeneic splenic stimulators at a responder to stimulator ratio of 10:1. The virulent Moscow strain was used to infect both stimulators and target cells.

One-way MLR utilising splenic responders and ^{60}Co γ -irradiated (2000 rads) splenic stimulators at a 4:1 ratio were cultured for 5 days at 37°C.

Each experiment was repeated several times and involved cells from a pool of two mice. Either males were used as responders or female mice were used throughout each experiment to eliminate possible effects due to the HY antigen.

Cytotoxic assay

The ^{51}Cr release assay using macrophage targets has been described previously (Gardner and Blanden 1976, Chapter 2). Briefly, 10^5 ^{51}Cr -labelled peritoneal macrophages, collected from a pool of at least 4 mice, were used as targets, and were incubated for 6 hours with effector cells at various killer:target ratios. Percent maximum

releasable ^{51}Cr was calculated by water lysis of cells and spontaneous release was about 3% per hour. The corrected percent lysis was calculated by the formula:

$$\% \text{ specific } ^{51}\text{Cr} \text{ release} = \frac{\% \text{ releasable by killers} - \% \text{ medium release}}{\% \text{ maximum releasable} - \% \text{ medium release}}$$

Results are expressed as mean percentage of targets lysed in a triplicate assay.

^{125}I -labelling of protein A

Radioiodination of protein A (*Staphylococcus aureus*, Pharmacia, Uppsala, Sweden) was performed by the Iodogen method (Fraker and Speck 1978) using carrier-free iodide-125 (IMS3, Radiochemical Centre, Amersham, Bucks, U.K.). Briefly, 0.025 ml (5 μg m) of Iodogen solution (1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril) was air dried in the bottom of a 1 cm glass tube. To this was added 0.02 ml of 0.1M borate buffer (pH 8.5) followed by 0.02 ml (10 μg m) Protein A solution in phosphate buffered saline (PBS) and 2 μl of carrier-free ^{125}I . After a 5 minute reaction time, with constant mixing, the labelled reagent in 250 μl PBS was dialysed against PBS until equilibrium labelling was reached.

Radioimmunoassay using ^{125}I -protein A

The assay involved absorption of monoclonal antibody (30R3 or 27R9) to spleen cells followed by washing and binding of ^{125}I -protein A to cell-bound antibody (Goding 1978). It was carried out at 4°C in flexible polyvinyl chloride microtitre plates (Dynatech Laboratories Inc., Alexandria, Virginia, U.S.A.) which were incubated with complete medium at room temperature for 30 minutes prior to use to prevent non-specific binding of protein to the wells. Eagle's minimal

essential medium (F15, Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) was used at pH 7.4 until addition of ^{125}I -protein A (see below). Spleen cell preparations from a pool of at least 2 mice were purified by 14% Isopaque/Ficoll separation to remove red and dead cells and then washed thrice. Three separate cell counts were made using a Coulter counter (Coulter Electronics Limited, Bedfordshire, U.K.) and cell concentrations were adjusted to give 8×10^7 cells/ml. The Coulter counter was also used to measure cell size distribution of spleen cell preparations, and both CBA/H and (BALB/c x CBA/H) F_1 cells gave a similar distribution. To 25 μl aliquots of 2-fold dilutions of antibody was added 25 μl of cells (i.e. finally 2×10^6 cells/well in 50 μl). Following a 60 minute absorption with continuous agitation to ensure saturation binding, the unbound antibody was removed by 4 washes. A known excess of ^{125}I -protein A (60 ηgm ; 33 μl of 1/200 dilution) was added to each well in PBS at pH 7.3. This was absorbed for 30 minutes with continuous agitation and then removed by 5 washes of complete medium. Mouse ascitic fluid was used as a control for non-specific binding. The wells were cut from the tray and the radioactivity was counted in a Packard Auto Gamma Spectrometer. The relative amounts of H-2 antigen on different cell populations, and the relative dissociation constants were estimated by plotting the reciprocal of bound ^{125}I -protein A against the reciprocal of antibody dilution (Ada and Yap 1979, Klotz 1953).

Use of the reciprocal plot

Several assumptions had to be made in using the reciprocal plot to estimate relative H-2 expression and the dissociation constant for antibody binding. Firstly, it is necessary that all binding sites are equivalent and independent, and that only one antibody molecule binds to one H-2 molecule (Ada and Yap 1979). Secondly, the ^{125}I -protein A must saturate all sites on the cell-associated antibody regardless of antibody dilution, and finally, there must be no loss of antibody or ^{125}I -protein A during washing. The first assumption is justified since monoclonal antibodies were used, and because multivalent binding of antibody to H-2 antigens would appear to be sterically impossible. The latter assumptions seem valid since antibody was absorbed under continuous agitation and since a large excess of ^{125}I -protein A was used. They were further validated since straight lines were obtained. Estimates of H-2 expression obtained by this method are independent of the antibody concentration or affinity, as well as its Ig composition.

RESULTS

Expression of H-2^k antigens on cells from CBA/H and (BALB/c x CBA/H)F₁ mice

Less H-2^k antigens were apparently expressed on (BALB/c x CBA/H)F₁ cells than on CBA/H cells, as detected by Tc cells or by antibodies. First, F₁ hybrid macrophages were about 3-fold less susceptible than CBA/H macrophages as targets for lysis by alloreactive or H-2^k restricted virus-immune Tc cells (Table 1). In contrast, BALB/c and F₁ macrophages behaved similarly as targets for Tc cells recognising H-2^d antigens.

Second, quantitative absorption of anti-H-2K^k serum by spleen cells from F₁ hybrid or CBA/H mice also indicated about 3-fold less H-2K^k antigen on F₁ cells (Figure 1).

Detection of H-2K^k using monoclonal antibodies and ¹²⁵I-protein A

Monoclonal antibodies 27R9 and 30R3 are known to bind strongly to H-2K^k antigens and very weakly to H-2D^k antigens (Lemke *et al.* 1978, Lindahl and Lemke 1979). Differences in their pattern of reactivity for cells from different strains of mice and in their ability to block Tc cell-mediated lysis suggest that these antibodies bind to different antigenic sites (Lemke *et al.* 1978, Lindahl and Lemke 1979, Blanden *et al.* 1979). They were used in attempts to determine whether the apparent reduced expression of the H-2K^k antigen on F₁ hybrid cells was a quantitative or a qualitative phenomenon.

More antibody bound to CBA/H than to the F₁ cells (Figure 2A) and, as expected, a small amount bound to C3H.OH via H-2D^k; this latter binding was more pronounced with 27R9 than 30R3 as indicated by previous work (Lindahl and Lemke 1979, Blanden *et al.* 1979). The reciprocal of ¹²⁵I-protein A bound (an approximation of the amount of antibody bound) was then plotted against the reciprocal of antibody dilution (Figure 2B) using data from the linear portions of the curves in Figure 2A. The intercept on the vertical axis of the line of best fit through these points gives an estimate of the reciprocal of the number of H-2 sites to which antibody would bind at infinite antibody concentration (Ada and Yap 1979). The intercepts of 4.1 (F₁) and 2.2 (CBA/H) for 27R9 and 3.8 (F₁) and 1.9 (CBA/H) for 30R3 (see Figure 2B), indicate that there were approximately 2-fold less H-2K^k sites on F₁ hybrid cells than on CBA/H cells. In a second similar experiment these intercepts were 4.2 (F₁) and 2.3 (CBA/H) for 27R9 and 4.0 (F₁) and 1.8 (CBA/H) for 30R3.

The intercept on the horizontal axis in Figure 2A is an estimate of the dissociation constant of the antibody-antigen interaction (Ada and Yap 1979). There was no difference between the intercepts for F_1 hybrid and CBA/H cells with 30R3 and virtually no difference in the case of 27R9. In sum, the results indicate a quantitative reduction in H-2 expression in the F_1 hybrid, but do not reveal any qualitative difference.

DISCUSSION

This investigation adds new information about expression of certain H-2 antigens on F_1 hybrid cells compared with parental strain cells. First, in the (BALB/c x CBA/H) F_1 hybrid studied here, the paternally-derived CBA/H ($H-2^k$) antigens are expressed in lower amounts than on CBA/H cells, whereas the maternally-derived BALB/c ($H-2^d$) antigens seem to be expressed in similar amounts on the F_1 hybrid and parental cells. In the case of the [B10.A(5R)xB10.A(2R)] F_1 described previously in Chapter 2, the maternally derived antigens were expressed weakly. Thus a consistent maternal influence on antigen expression in the F_1 offspring is excluded. Further evidence to support this result is presented in Chapter 5.

Second, the observations that some H-2 antigens are expressed in similar amounts on F_1 hybrid and parental cells argues against a simple gene dose effect. Furthermore, the same antigen, $H-2K^k$, was expressed in similar amounts by cells of the [B10.A(5R)xB10.A(2R)] and B10.A(2R) mice, but was expressed less by the (BALB/c x CBA/H) F_1 hybrid than by CBA/H cells. These results suggest that regulation of expression of a given H-2 antigen depends upon particular combinations of genes present in the F_1 hybrid. Further investigation of this aspect is continued in Chapter 5.

Third, the use of two different monoclonal anti-H-2^k antibodies in a two-stage radioimmunoassay showed quantitatively reduced expression of H-2K^k on (BALB/c x CBA/H)F₁ hybrid cells in comparison with CBA/H. This conclusion is based on findings that less of either antibody bound to F₁ hybrid than to CBA/H cells, but the dissociation constants of the antibody-antigen reactions were similar with F₁ and CBA/H cells, thus suggesting no qualitative change in the sites on the H-2K^k antigen to which the two different antibodies bound. These results do not exclude the possibilities that some H-2K^k molecules expressed on F₁ hybrid cells are changed qualitatively to such an extent that they do not bind the antibodies at all, or that qualitative differences exist in areas of the H-2K^k molecule not bound by 30R3 and 27R9.

The present finding that H-2K^k is expressed less on (BALB/c x CBA/H) F₁ hybrid cells than on CBA/H cells is of particular interest in the context of recent results of Ishikawa and Dutton (1979) who showed that several (H-2^k x H-2^d)F₁ hybrids give Tc cell responses against H-2^k parental cells. The immunologically conventional explanation for this phenomenon is that a minor histocompatibility (H) antigen is expressed on cells of the homozygous parent, but not on F₁ hybrid cells (Fathman and Nabholz 1977, Ishikawa and Dutton 1979). However, the recent results of Ishikawa and Dutton (1979), Cudkowicz *et al.* (1979) and Warner and Cudkowicz (1979) make this explanation almost untenable. First, primary F₁ anti-parent Tc cell responses were obtained *in vitro*, but such responses do not occur against minor H antigens (Bevan 1975). Second, the use of congenic and H-2 recombinant mice established that the target antigen(s) were dependent on gene(s) in or near the H-2K^k region and were apparently not dependent on background genes (Warner and Cudkowicz 1979, Ishikawa and Dutton 1979). Third, Cudkowicz and

co-workers (1979) have shown in another F_1 hybrid anti-parent Tc cell response, that F_1 cells act as cold competitors for the lysis of parental targets but with less efficiency than parental competitors. This is consistent with the idea that F_1 hybrid cells express the same target antigen as the parental cells, but in lower concentration.

When these results are viewed together with the present findings, they raise the intriguing possibility that Tc cells in F_1 hybrids may possess specific receptors for self H-2K (or H-2D) antigens, but are only stimulated by parental cells that deploy larger numbers of these same H-2 antigen molecules on their surface membranes than F_1 hybrid cells. Thus, self tolerance in T cells may be quantitatively dependent upon the level of self H-2 antigen expression. However further work is required to definitively exclude qualitative explanations.

SUMMARY

Cells of (CBA/H x BALB/c) F_1 hybrid mice express CBA/H-derived H-2^k antigens more weakly than do CBA/H cells, but H-2^d antigens are similarly expressed by F_1 hybrid and BALB/c cells. This was evident when F_1 hybrid macrophages were compared with CBA/H and BALB/c macrophage as targets for both alloreactive and H-2 restricted antiviral Tc cells. Quantitative absorption of anti-H-2K^k serum by spleen cells of F_1 hybrid or CBA/H mice also suggested about 3-fold less H-2K^k antigen on the F_1 cells. With the use of two anti-H-2K^k monoclonal antibodies, 30R3 and 27R9, the reduced expression of H-2K^k on this F_1 hybrid was further analysed in a two-stage radioimmunoassay employing the uptake of ¹²⁵I-protein A to measure antibody binding. By a thermodynamic approach, estimates were made of the dissociation constant for antibody binding, and of the relative numbers of H-2 molecules expressed by both F_1 hybrid and CBA/H spleen cells. The results indicate that there is a

two-fold reduction in the number of H-2K^k molecules expressed on the surface of (BALB/c x CBA/H)F₁ cells. Similar dissociation constants for F₁ hybrid and CBA/H cells indicated no detectable qualitative difference in their H-2K^k antigens with respect to sites recognised by 30R3 and 27R9.

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Fig. 1 Quantitative absorption of anti-H-2K^k serum (1/20 dilution) by spleen cells of the F₁ hybrid (BALB/c x CBA/H) (■) in comparison with CBA/H cells (▲). Remaining lytic activity was titred on CBA/H spleen cells. Lysis by unabsorbed antiserum plus rabbit complement (AS), and by rabbit complement alone (RC) are included as controls. The number of cells absorbing was plotted on a log₂ scale. The 50% end point of titration was calculated from the curve of best fit. This shows that 1.9×10^6 F₁ hybrid cells were needed to absorb out 50% antiserum activity, compared with 6.8×10^5 CBA/H cells.

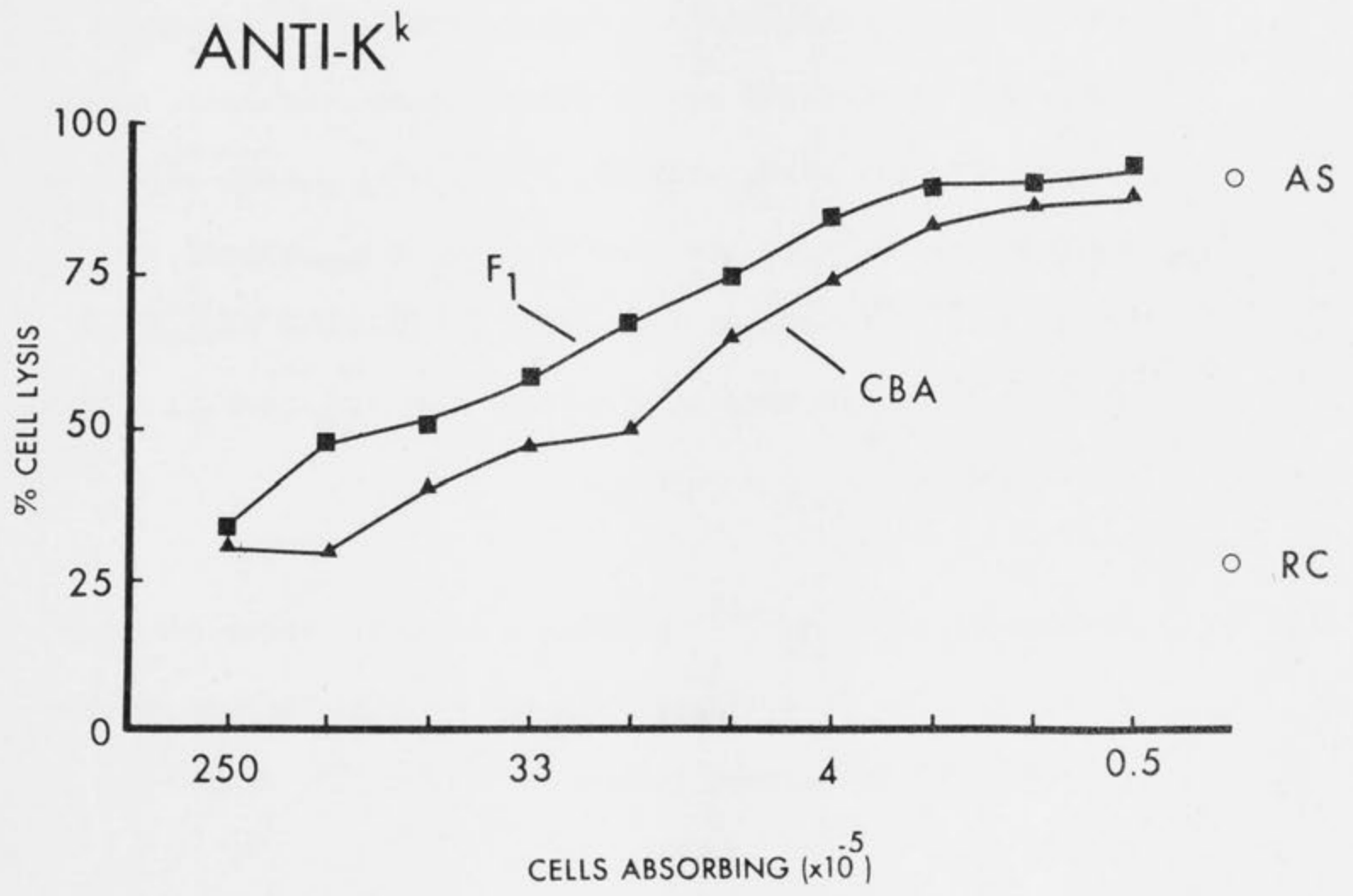


Fig. 2A Spleen cells of F_1 hybrid (BALB/c x CBA/H) (■) and CBA/H (●) mice were compared for their uptake of ^{125}I -protein A following exposure to serial dilutions of monoclonal antibodies 30R3 and 27R9. C3H.OH (K^d, D^k) (▲) spleen cells were included to indicate the weak binding to D^k antigen. Binding of ^{125}I -protein A to cells preabsorbed with mouse ascitic fluid was used as a control for non-specific binding (open circles).

Fig. 2B Reciprocal plots of ^{125}I -protein A binding versus antibody dilution. The line of best-fit was plotted through points representing the linear region of the binding curves in Figure 2A.

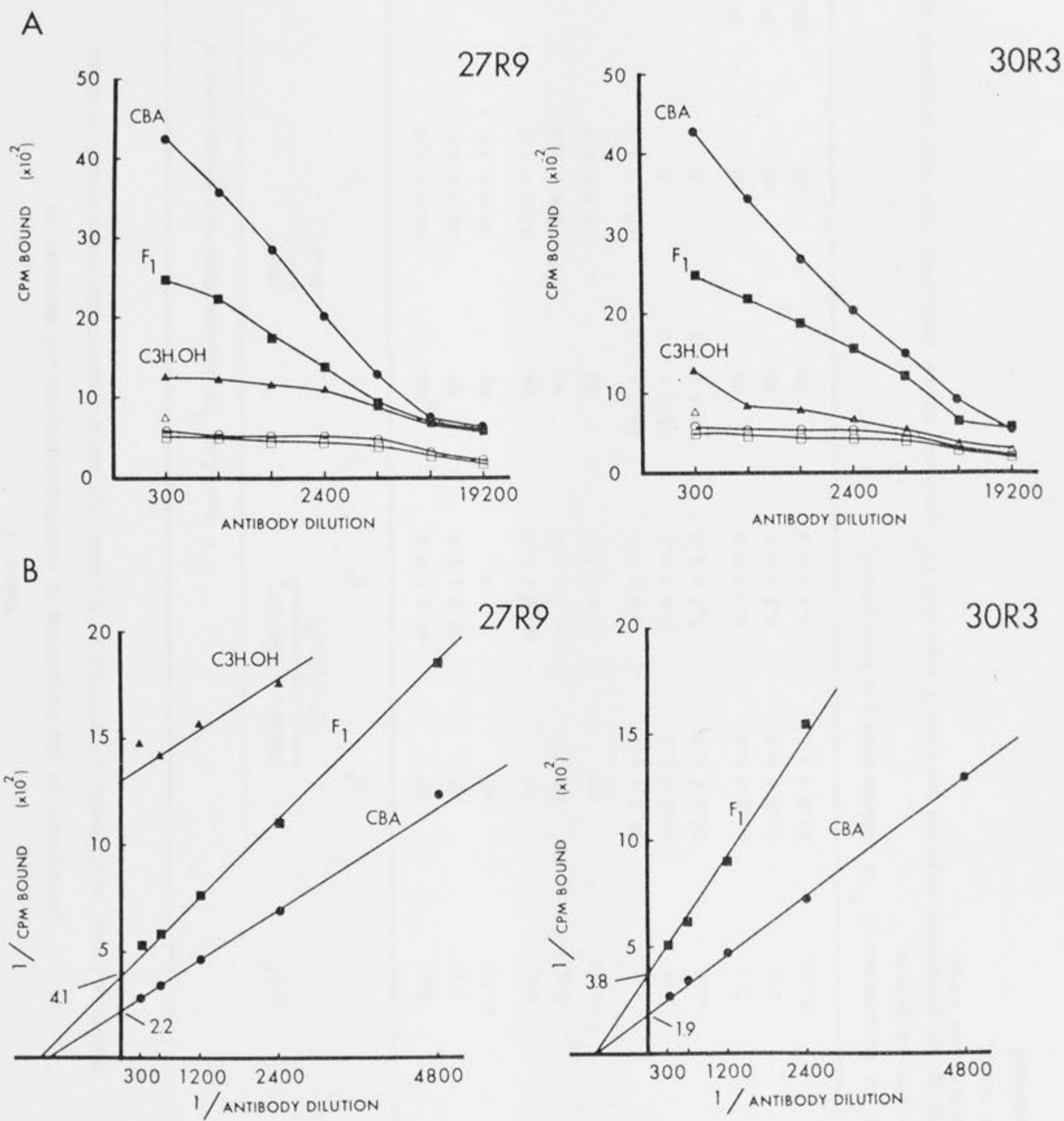


TABLE 1

DIFFERENCES IN THE EXPRESSION OF PARENTALLY-DERIVED H-2 ANTIGENS ON
(BALB/c x CBA/H) MACROPHAGES AS DETECTED BY ALLOREACTIVE AND H-2 RESTRICTED VIRUS-IMMUNE Tc CELLS

Source of Tc cells	K:T ^d	% Specific ⁵¹ Cr release from macrophage targets ^a					
		(BALB/c x CBA/H)F ₁ (H-2 ^d x H-2 ^k)		CBA/H (H-2 ^k)		BALB/c (H-2 ^d)	
		I ^e	U ^e	I	U	I	U
^b BALB/c anti-CBA/H	3:1	ND ^f	44.0 ± 0.8	ND	86.4 ± 0.6	ND	0
	1:1	ND	33.8 ± 1.8	ND	46.7 ± 0.6	ND	4.6 ± 1.7
	0.3:1	ND	0	ND	15.0 ± 2.0	ND	0
^b CBA/H anti-BALB/c	3:1	ND	68.8 ± 0.8	ND	13.2 ± 1.0	ND	76.2 ± 1.0
	1:1	ND	36.3 ± 0.4	ND	4.5 ± 0.3	ND	46.3 ± 2.7
	0.3:1	ND	18.6 ± 4.2	ND	1.5 ± 1.2	ND	24.0 ± 0.6
^c CBA/H anti-ectromelia	3:1	46.2 ± 0.7	1.6 ± 2.5	84.7 ± 2.3	2.2 ± 1.0	ND	ND
	1:1	27.6 ± 1.7	6.6 ± 0.1	65.8 ± 2.1	0	ND	ND
	0.3:1	20.1 ± 1.0	7.7 ± 1.5	37.9 ± 2.5	0	ND	ND
^c BALB/c anti-ectromelia	3:1	63.5 ± 1.1	1.5 ± 0.2	ND	ND	69.2 ± 1.8	11.2 ± 1.9
	1:1	51.5 ± 3.0	0.5 ± 0.1	ND	ND	50.1 ± 1.4	5.2 ± 1.6
	0.3:1	29.8 ± 2.5	1.1 ± 0.2	ND	ND	20.5 ± 1.8	0

a Values are means ± S.E. of triplicates of ⁵¹Cr release over a 6 hour period.

b MLR set up as in Materials and Methods.

c 10⁷ ectromelia-infected spleen stimulators were cultured for 5 days with 10⁸ syngeneic spleen responders from mice preprimed intravenously with 10⁴ PFU hampstead egg strain ectromelia virus.

d K:T = killer to target ratio.

e I = infected; U = uninfected.

f ND = not done.

INTRODUCTION

The H-2 and H-3 regions of the H-2 gene complex, the major histocompatibility (MHC) complex in mice, have been the classical immunogenetically defined gene loci for transplantation antigens which are expressed in high polymorphic lymphocyte cell lines (reviewed by Klein 1974). These genes are highly polymorphic and many alleles have been identified and are being identified with various techniques such as hybridization and polymerase chain reaction (PCR). They are located on chromosome 17 and encode proteins which are associated with the cell surface with a major role in the immune response to antigens. A major role is also played by H-2 antigens in the regulation of T cell responses to antigen presented by H-2 antigens on the surface of T cell presenting cells.

CHAPTER 4

QUANTITATIVE VARIATION IN H-2 ANTIGENIC EXPRESSION

I. ESTIMATION OF H-2K and H-2D EXPRESSION

IN DIFFERENT STRAINS OF MICE

The H-2K and H-2D antigens are the most polymorphic of the H-2 antigens and are the most immunogenic. They are encoded by the H-2K and H-2D genes, respectively, and are expressed on the cell surface as part of the H-2 antigenic complex. The H-2K and H-2D antigens are highly polymorphic and many alleles have been identified and are being identified with various techniques such as hybridization and PCR. They are located on chromosome 17 and encode proteins which are associated with the cell surface with a major role in the immune response to antigens. A major role is also played by H-2 antigens in the regulation of T cell responses to antigen presented by H-2 antigens on the surface of T cell presenting cells.

INTRODUCTION

The *K* and *D* regions of the *H-2* gene complex, the major histocompatibility (H) complex in mice, code for the classical serologically defined major transplantation antigens which are expressed in highest amount on lymphomyeloid cells (reviewed by Klein 1975). These genes are highly polymorphic and many allelic forms of H-2K and H-2D molecules can be identified with antisera recognising both private (unique) and public (cross reactive) determinants. They are both 45,000 molecular weight glycoproteins and are associated in the cell membrane with a smaller 12,000 molecular weight β_2 -microglobulin polypeptide. A major biological role for H-2 antigens in the induction of T cell responses to viruses became evident after the discovery of *H-2* restriction. Cytotoxic T cell (Tc cell) recognition of virus-infected cells was found to involve recognition of viral antigens only in combination with self H-2K and H-2D antigens (reviewed by Doherty *et al.* 1976). More recently, a functional role for H-2 antigens expressed on radioresistant cells of the thymus is implicated by evidence that the thymus determines the *H-2* restriction phenotype of chimeric mice (reviewed by Zinkernagel 1978).

The exclusive role of different H-2K and H-2D antigens in *H-2* restricted responses is evident from many examples of *K*- and *D*-linked differences in Tc cell responsiveness to several viruses (Zinkernagel *et al.* 1978, Gomard *et al.* 1977, Bubbers *et al.* 1977, Doherty *et al.* 1978, Blanden *et al.* 1975, Mullbacher and Blanden 1978, Pfizenmaier *et al.* 1978), trinitrophenyl (TNP)-modified cells (Levy and Shearer 1979, Shearer *et al.* 1976), and to the male-specific HY antigen (Simpson and Gordon 1977, von Boehmer *et al.* 1977). A quantitative requirement

for H-2 antigens in influencing T cell responsiveness was established in Chapters 2 and 3 where it was shown that F_1 hybrids which express certain H-2 antigens more weakly than parental strain cells were less effective as target cells in H-2 restricted responses. Thus far, no attempt has been made to compare the expression of different K and D antigens on the same cell. This information would now appear to be relevant to any explanation for H-2 linked differences in T cell responsiveness. By using a simple radioimmunoassay (RIA) previously described in Chapter 3 to measure the uptake of ^{125}I -labelled protein A to cells preabsorbed with anti-H-2 serum, we have found small but previously undetected differences in the number of antibody binding sites, reflecting differences in the expression of K and D antigens.

MATERIALS AND METHODS

Mice

Mice were bred in the John Curtin School of Medical Research and used when 6 to 12 weeks old. Mice of the same age and sex were used in each experiment. The H-2 haplotypes of mice used in this study are listed in Table 1.

Antisera

Highly specific antisera were obtained from Dr Ian McKenzie, University of Melbourne, Australia, and were raised by multiple immunizations of lymphoid cells, and thereafter bleeding at regular intervals (McKenzie and Snell 1973). The antisera used in this study are listed in Table 2. AS30 was obtained from the Transplantation and Immunology Division, N.I.H., Bethesda, Maryland. C57BL/6J anti CBA/H serum was also used.

Absorption of antisera

50 μ l of undiluted antiserum was absorbed for 30 minutes at 4°C on the cells of one thymus. Similarly, for absorption on spleen cells, 100 μ l of undiluted antiserum was absorbed for 30 minutes at 4°C on the cells of one spleen.

Complement-mediated lysis

The two-stage microcytotoxicity assay for measuring complement-mediated lysis has been previously described (McKenzie *et al.* 1976, Chapter 2). Each of the antisera reacted with approximately 90% of spleen cells.

Radioimmunoassay using 125 I-protein A

Methods for cell preparation, iodination of protein A, and the radioimmunoassay have been outlined in Chapter 3. Just briefly, the assay involves absorption of diluting amounts of anti-H-2 serum (from a 1/4 or 1/8 dilution) to spleen cells, followed by washing and binding of 125 I-protein A to cell-bound antibody, i.e. the binding of 125 I-protein A is used as an approximation of antibody binding (Goding 1978). The relative number of antibody binding sites, or H-2 antigens, on different cell populations and the relative dissociation constants can be estimated by plotting the reciprocal of bound 125 I-protein A against the reciprocal of antibody dilution (Ada and Yap 1979, Klotz 1953). Points in the linear region of the binding curve were used to calculate the reciprocal plot, and for this, counts per minute (cpm) bound to cells preabsorbed with normal mouse serum were subtracted from the cpm incorporated by specific antiserum.

Statistical analysis of data

The two sample t-test was used to compare estimated for significance.

RESULTS

Use of the ^{125}I -protein A RIA for Estimating Relative H-2 Concentration on Spleen Cells

An example of the use of the ^{125}I -protein A RIA and the reciprocal plot to make estimates of the relative numbers of H-2K^q and H-2D^q antigen molecules on Bl0.G spleen cells is shown in Figure 1. By extrapolation of the line of best fit through points on the reciprocal plot to the Y axis relative estimates can be made of the reciprocal number of H-2 sites to which antibody can bind at infinite antibody concentration (see Chapter 2, Ada and Yap 1979). In this report, these estimates are given only in terms of the cpm ^{125}I -protein A bound to 2×10^6 cells. In Figure 1B, the estimates are 4.0×10^3 cpm for D^q and 3.0×10^3 cpm for K^q.

Estimates of H-2 expression obtained by this method are independent of the antibody concentration or affinity, as well as its Ig composition. Despite the fact that protein A does not bind to all subclasses of IgG molecules in mice (Goding 1978, Ey *et al.* 1978), it can be used in this assay to compare estimates of H-2 expression made with any preparation of heterogeneous, although highly specific, anti-H-2 serum.

The intercept on the X-axis is an estimate of the dissociation constant for antigen-antibody binding (Ada and Yap 1979). For any antiserum used, differences were detectable only in Y-axis intercepts with no evidence for X-axis differences or in antibody binding affinity in greater than 95% of the experiments. As a result, no data is presented for X-axis values.

Consistency in Estimation

In any one experiment several antisera were tested on cells from several different strains of mice. Estimates of relative H-2 antigen concentration were highly consistent for the same antiserum used in different experiments on the same number and strain of cells and were independent of the sex of the animal. Different antisera with activity for the same H-2 private specificity also gave very similar estimates of H-2 concentration. Several examples are shown in Table 3. C57BL/6 anti-CBA/H serum which had been preabsorbed on BALB/c spleen cells, and showed no crossreactive binding to B10 and B10.A(5R) cells, also gave similar estimates for K^k and D^k when tested on B10.A(2R) and C3H.OH, respectively, as did the allele-specific antisera. Because of the consistency of results, estimates from different experiments using different antisera were pooled for each strain.

Specificity of Antisera

Before different anti-H-2 sera could be used to compare H-2 antigen expression in different strains of mice, it was necessary to establish that antibody binding in the ^{125}I -protein A RIA was specific only for H-2 molecules carrying the private specificity. Each of the antisera was produced in mouse strain combinations which differed, where possible, by just the private specificity of the H-2K or H-2D molecule of the strain used for immunization. However, very few strain combinations produce such specific antisera. The antisera were therefore tested for possible antibodies to public H-2 specificities and Ia antigens as predicted from the known H-2 and Ia antigenic specificities of the donor and recipient strains used to produce the antisera (Klein 1975 and Klein *et al.* 1978). Antibodies to public specificities should not influence results unless the specificities were carried on an H-2L-like molecule not bearing the private specificity (see review, Démant

and Néauport-Sautés 1978). Antisera were tested initially by complement-mediated lysis, either directly on other strains known to carry cross-reactive specificities, or they were preabsorbed on thymocytes, which lack Ia antigens, and tested for residual anti-Ia activity on syngeneic spleen cells. Antisera were also tested for crossreactive binding in the ^{125}I -protein A RIA. Results of titrations are included in Table 2, and some examples of crossreactive binding in the RIA are shown in Figure 2. Estimates for H-2 expression in different strains are given in Table 4.

AS30, AS303 and AS946

These antisera are specific only for the H-2.30 or H-2.2 private specificities.

AS241

Anti-H-2 activity is specific for H-2.17. Possible anti-Ia activity was not detectable by titration after thymocyte absorption, nor did it contribute to estimates of K^{q} expression, since B10.AQR and B10.G gave similar estimates.

AS292

This antiserum had been preabsorbed with A.TL and BALB/c spleen cells prior to use. The absorbed serum appears to detect only H-2.32, the private D^{k} specificity, since it did not crossreact on B10.A and gave similar D^{k} estimates for C3H.OH and CBA/H.

AS508 and AS116E

These antisera detect H-2.23. AS508 has a low titre on B10.AQR (H-2.3,11) and no activity on BALB/c (H-2.3,8,47). Approximately 20% crossreactive binding to B10.AQR was detectable in the RIA, and less on BALB/c cells. Since similar K^{k} estimates were made using either

AS508 or AS116E (Table 3), possible antibodies to Ia antigens in AS116E or to the public specificities in AS508 do not influence the H-2 estimates in this test.

AS285 and AS954

These antisera have H-2.4 activity but no anti-Ia.6 activity. This was shown firstly by the absence of crossreactivity on B10.A(2R), and by the observation that H-2D^d estimates on B10.T(6R) and B10.D2 were the same (Table 4). Activity against public specificities cannot be fully tested, but weak binding to SJL(H-2.42) and B10.G (H-2.13,49) was seen with AS285 in the RIA. So far, there is no evidence that any of the H-2.13, 41, 42, 44, 49 specificities are carried by the H-2L molecule (Démant and Néauport-Sautès 1978).

AS43 and AS742

Both antisera detect H-2.31; weak activity in AS742 for some of the public specificities H-2.27, 28, 29, 46 present on B10 cells was detected both by complement-mediated lysis and in the RIA. AS43 had no activity for B10.G in the lytic test but bound strongly to B10.G in the RIA. This difference could be explained if these antibodies in AS43 were predominantly IgG. This activity appears to be directed against H-2.34 since the RIA gave an H-2 estimate of 3.5 equivalent to that for K^q by AS241 (see Table 4). It is unlikely that activity against public specificities influences H-2 estimates since the two antisera give similar estimates for K^d (Table 3) and since no H-2L-like molecule has been demonstrated in the K region (Néauport-Sautès *et al.* 1978). Neither antiserum had residual anti-Ia activity on BALB/c following absorption on BALB/c thymocytes.

AS924A

This antiserum detects H-2.33 and has very weak activity for the H-2.35, 36, 39 specificities detectable both by lysis and in the RIA. These antibodies do not influence H-2 estimates since similar estimates were obtained using antiserum which had been absorbed on BALB/c spleen cells. This serum has no residual anti-Ia activity on B10 following absorption on B10 thymocytes.

Estimates of H-2 Antigen Expression in Different Strains of Mice

Estimates of H-2 antigen expression were made in many independent and recombinant strains of mice carrying K or D antigens defined by the $H-2^k$, $H-2^d$, $H-2^b$ and $H-2^q$ haplotypes. The pooled results from many experiments are presented in Table 4. Estimates were very similar for the same antigen in different strains, eliminating any effect on H-2 expression due to the presence of other K, D or Ia antigens present on the same cell. Background genes influence H-2 antigen expression, since strains with the B10 background have about 25% reduced expression compared with other strains. The difference in the expression of K^k in CBA/H and other B10 background mice expressing K^k is significant ($p = 0.035$), as is the difference in expression of D^d in BALB/c and the B10 strains ($p = 0.000$). It is interesting that although K^q expression is less in B10.G strain mice than in DBA/1j mice, D^q is expressed equally: suggestive of a new H-2-linked background effect. The difference in the binding of AS285 to the $H-2D^d$ molecule in BALB/c and B10.A mice is shown in Figure 3. This result gave D^d estimates equivalent to 5.0×10^3 cpm for BALB/c and 4.0×10^3 cpm for B10.A.

H-2K- and H-2D-linked Differences in H-2 Antigen Expression

All results from C57BL/10 H-2 congenic mice were pooled and presented in summary form in Table 5. Comparison of expression of different H-2 antigens shows more variation in the number of H-2D than H-2K molecules. The difference in relative H-2 antigen concentration between D^b and D^q is significant ($p = 0.008$), as is the difference between D^q and D^d ($p = 0.001$). D^k is expressed noticeably less than all other D antigens, irrespective of the genetic background. This gives an order of binding of H-2 antigens such as $D^b > D^q > D^d >> D^k$. While K^k and K^b estimates are not significantly different ($p = 0.169$), both are expressed in significantly greater amount than K^q ($p = 0.000$ in both cases). Since few B10 strains carry K^d , statistical comparison was not made, but when compared with K^d expression averaged over all strains it appears to be expressed at least as strongly as K^b and K^k . Significant differences in expression were also found for D^b and K^k ($p = 0.025$), K^q and D^k ($p = 0.000$) and D^d and K^q ($p = 0.000$), but not for K^b and D^q ($p = 0.680$).

Hence a proposed order for magnitude of expression would be:

$$D^b > K^k = K^b = D^q > D^d > K^q >> D^k$$

(K^d)

As a result, different strains appear to express different total numbers of molecules, and in some strains, such as CBA/H and C3H.OH, there is a 2-3 fold difference in the expression of K versus D antigens.

Estimation of Absolute Numbers of H-2 Molecules by the ^{125}I -Protein A

Binding Assay

By estimating the number of molecules of ^{125}I -protein A of molecular weight 42,000 (Sjöquist *et al.* 1972) bound at saturation, estimates of between 4×10^5 molecules of D^k and 10^6 molecules of K^k

per spleen cell were obtained. These calculations assume that one molecule of protein A binds to one IgG molecule, and so these values could be overestimates by up to 2-fold.

DISCUSSION

The ^{125}I -protein A RIA, combined with the reciprocal estimation method, proved to be a sufficient test for measuring H-2K and H-2D antigen expression. The estimate of absolute number of H-2 molecules bearing private specificities achieved by this method is in general agreement with published estimates of 2 to 5×10^5 H-2 molecules per mouse lymphoblast (Liberti *et al.* 1979) and with estimates of $4-5 \times 10^5$ HLA molecules on human tonsil and lymphoid cells (Barnstable *et al.* 1978, Plesner 1976). The consistency of the estimates indicate that for such a blunt test it is sensitive enough to reveal small differences of $\pm 10\%$ in H-2 antigen expression. These differences were highly consistent over many experiments and in many cases were statistically significant. Differences in the expression of H-2K and H-2D antigens do not appear to be caused by antibodies to other cell surface antigens for two reasons. Firstly, each of the antisera is highly specific for H-2 molecules carrying the private specificity, although possible anti-Ia activity in AS924A, AS43 and AS742 cannot be excluded with the use of recombinant strain mice. Secondly, since saturation binding is achieved only for some sera at very high concentrations (see Figure 3), any weak antibody activity does not contribute to the estimates. Estimates of H-2 expression are independent of strain differences in cell populations and the presence of antibody to endogenous viruses, since many different strains give similar estimates. There was no suggestion of any qualitative differences in the same antigen expressed

by different strains since no differences could be detected in X axis intercepts or antibody dissociation constants. Any such differences may only be detected with appropriate monoclonal antibodies.

A background gene influence on K and D antigen expression is not unexpected, and has been previously reported for other cell surface antigens such as Ia antigens (David 1976), the Ly 6 antigen (McKenzie and Potter 1979), as well as H-2D antigens on thymocytes (Boyse *et al.* 1968). Variation in the number of molecules expressed by different K and D alleles is a new finding, and suggests a control over antigen expression which could operate anywhere from the gene to the level of antigen presentation in the cell membrane. This effect is functionally linked to K and D genes since level of expression is unique for individual antigens. Regulation at the gene level may involve H-2-linked regulator genes, while regulation at the cell membrane level could involve several processes in posttranslational modification of H-2 molecules, such as cleavage, glycosylation or H-2 binding affinity for β_2 -microglobulin. In the light of recent evidence for heterogeneity amongst H-2D region molecules bearing both private and public specificities (Iványi and Démant 1979), the differences in the number of molecules bearing the private specificity shown in this report could also reflect differences in the number of variants of K and D molecules coded by different K and D region genes.

In line with the effect that just a 60 percent difference in H-2 expression between F₁ hybrid and parental cells can have on Tc cell responsiveness, both at the level of stimulation and target cell recognition (Chapters 2 and 3), differences of between 10 and 70 percent seen in homozygotes, may in some instances influence the magnitude of H-2 restricted Tc cell responses. It is expected that this might be

most apparent at the induction of a response where the foreign (X) antigen is in more limiting amount, and the size of the response generated is then proportional to the number of H-2+X associations recognised by T cells. The results of Shearer *et al.* (1979) support this model. They found that H-2-linked differences in Tc cell responsiveness to TNP-modified cells, were only detectable under conditions of limiting TNP stimulation.

Despite the influence of antigen expression on Tc cell responsiveness, other factors such as requirements for helper T cells, the nature of the H-2+X association, immune response gene effects as well as the size of the potential T cell pool, could vary for responses to different viruses and minor H antigens. For this reason, the role of quantitative differences in H-2 antigen expression is difficult to dissect out using strains other than F_1 hybrids. However, quantitative differences in H-2 expression in homozygotes might be expected to result in some H-2-linked genetic effects on Tc cell responsiveness which are independent of the cytotoxic specificity contributed by the infecting virus or modifying agent. In support of this, are similarities in the response pattern of particular K and D antigens to several viruses and TNP-modified cells (Levy and Shearer 1979), as well as in the responsiveness to Sendai and ectromelia viruses (author, unpublished observations). The most remarkable example is the general weakness of Tc cell responses to D^k , the most weakly expressed H-2 antigen for TNP (Levy and Shearer 1979), Vaccinia (Zinkernagel *et al.* 1978, Doherty *et al.* 1978), Sendai (Zinkernagel *et al.* 1978), ectromelia (Blanden *et al.* 1975), and SV40-mediated responses (Pfizenmaier *et al.* 1978). In contrast, the weak expression of D^k does not correlate with the strong responsiveness of the D^k

antigen in responses to alphaviruses (Mullbacher and Blanden 1978) and in the anti-HY response (Simpson and Gordon 1977). However, these responses are unique amongst any described here, in that very few H-2 antigens can generate a T cell response, and their H-2 antigenic requirements for T cell responsiveness may differ in other ways.

Quantitative variation in the expression of different H-2 antigens may also explain why only the K^k and D^b antigens are detectable in Friend virus particles isolated from the serum of infected mice (Bubbers *et al.* 1978). The more strongly expressed H-2 antigens may be more likely to be encapsulated by the virus when it buds from the cell membrane.

SUMMARY

Minor differences in the expression of individual H-2K and H-2D antigens were detected on mouse spleen cells. The method involved the use of an ^{125}I -protein A radioimmunoassay using highly specific anti-H-2 sera to make estimates of the number of cell bound antibody molecules. The maximum number of antibody binding sites varied for each H-2 antigen reflecting differences of between 10 and 70 per cent in the expression of any 2 antigens. The order of magnitude of expression was $D^b > (K^d) = K^k = K^b = D^q > D^d > K^q > D^k$. Minor background differences were detectable, but antigen expression was allele-specific and independent of the expression of other K, D or Ia antigens expressed on the same cell.

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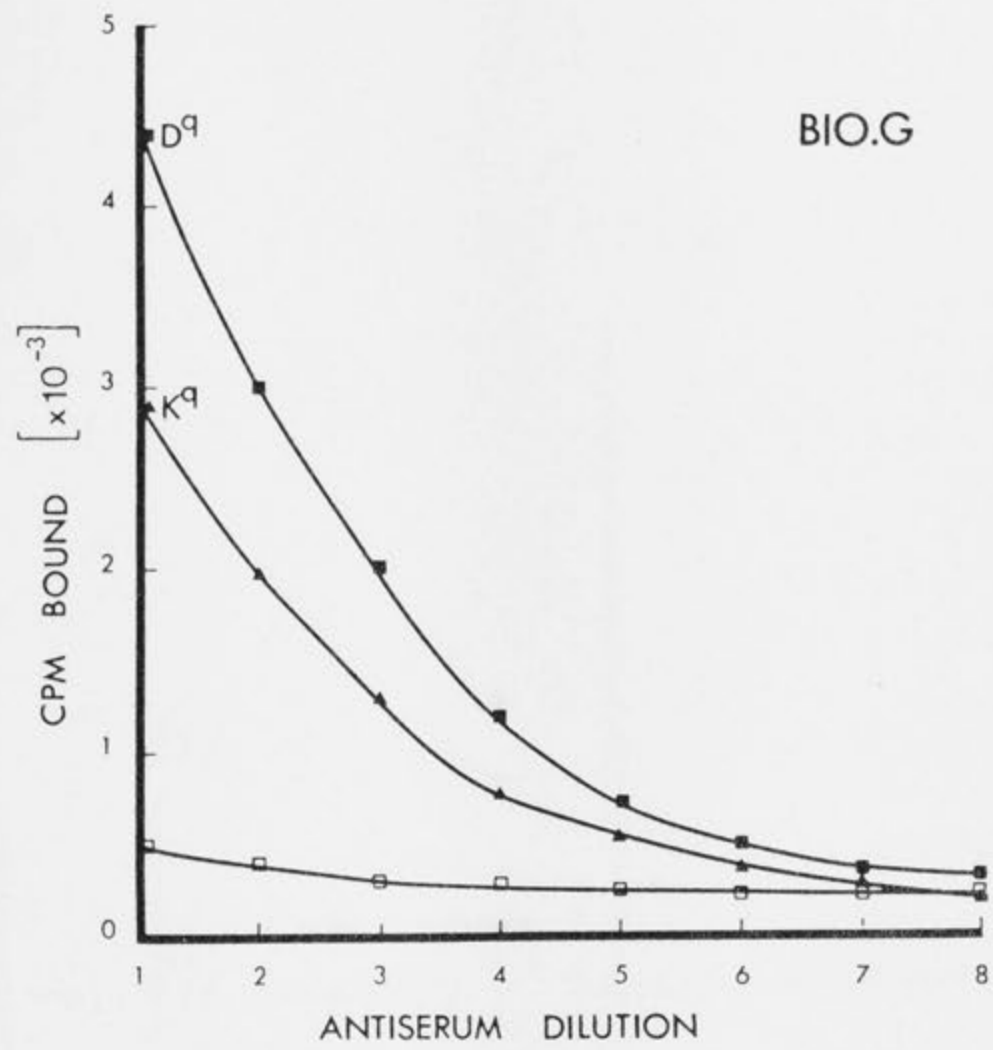
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Fig 1A The uptake of ^{125}I -protein A to B10.G spleen cells was measured following absorption of serial dilutions of anti-K^q (AS241) (\blacktriangle) and anti-D^q (AS30) (\blacksquare) sera. Binding of ^{125}I -protein A to cells preabsorbed with normal mouse serum (\square) was used as a control for non-specific binding.

Fig. 1B Reciprocal plot of ^{125}I -protein A binding versus antibody dilution. The line of best fit was plotted through points representing the linear region of the binding curves in Figure A.

A



B

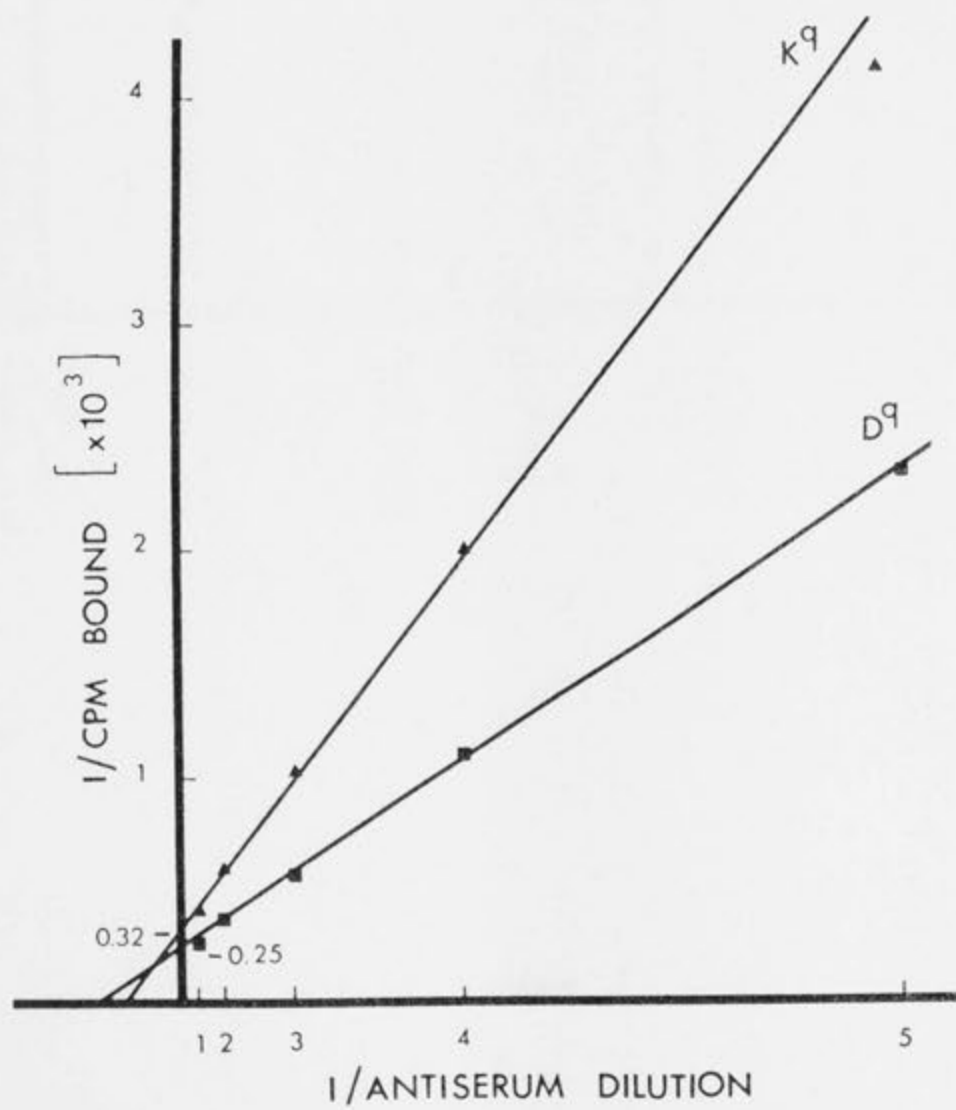


Fig. 2 Comparative uptake of ^{125}I -protein A by spleen cells from different strains of mice, following absorption of a 1/8 dilution of the antisera, AS43, 742, 285, 924A, 508 and 954.

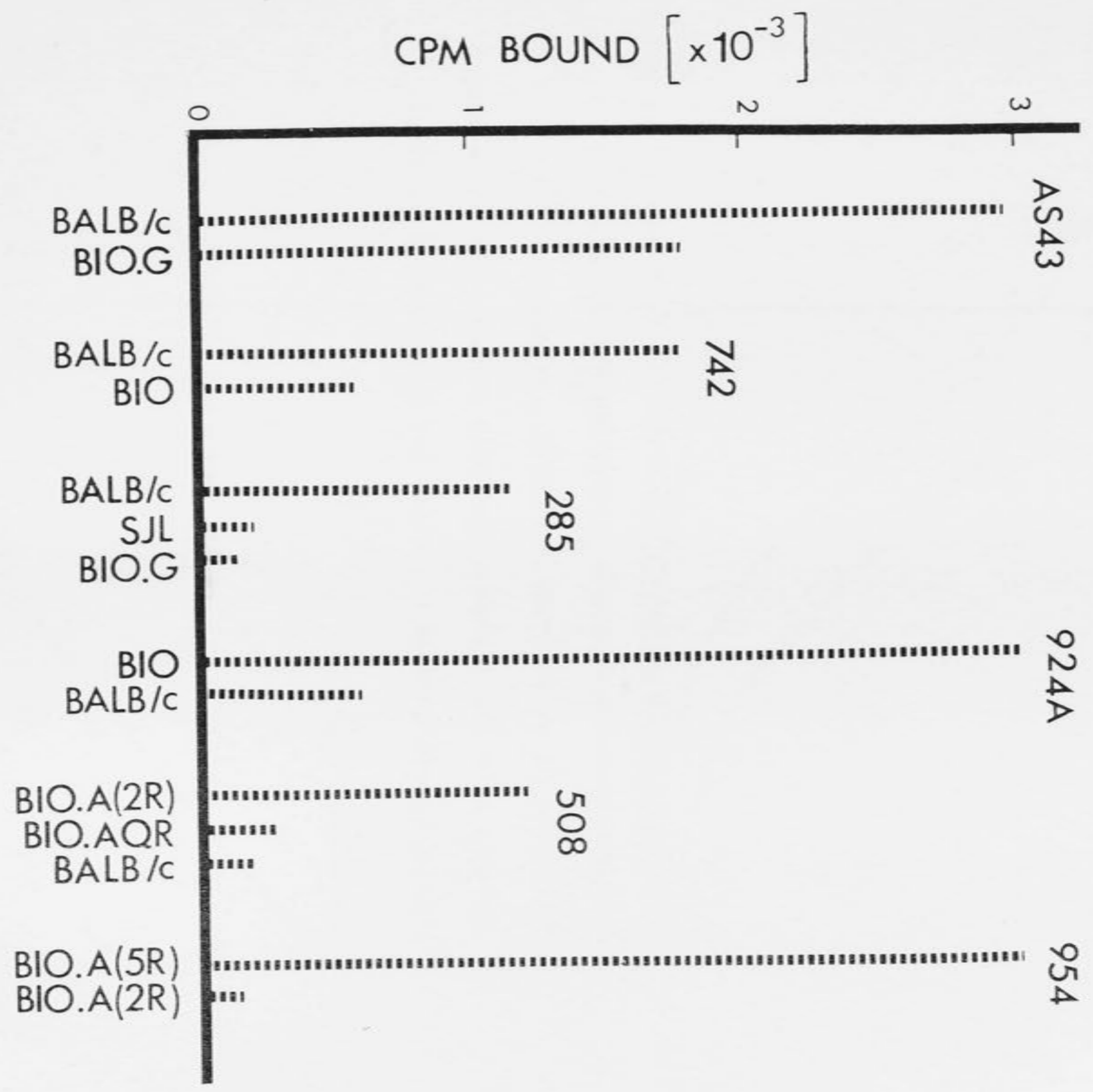
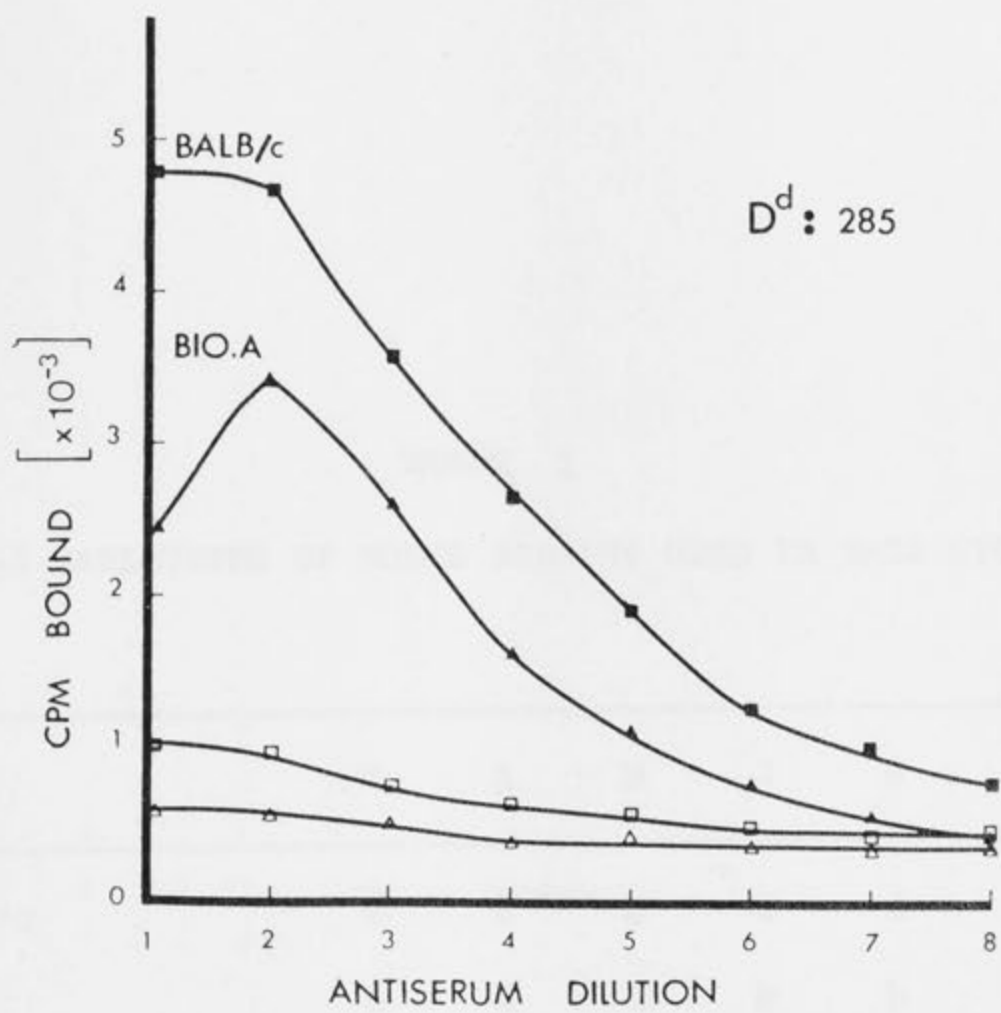


Fig. 3A Comparison of the uptake of ^{125}I -protein A to BALB/c (■) and B10.A (▲) spleen cells following absorption of serial dilutions of anti-D^d (AS285) serum. Binding of ^{125}I -protein A to cells preabsorbed with normal mouse serum (open symbols), was used as a control for non-specific binding.

Fig. 3B See Figure 3B.

A



B

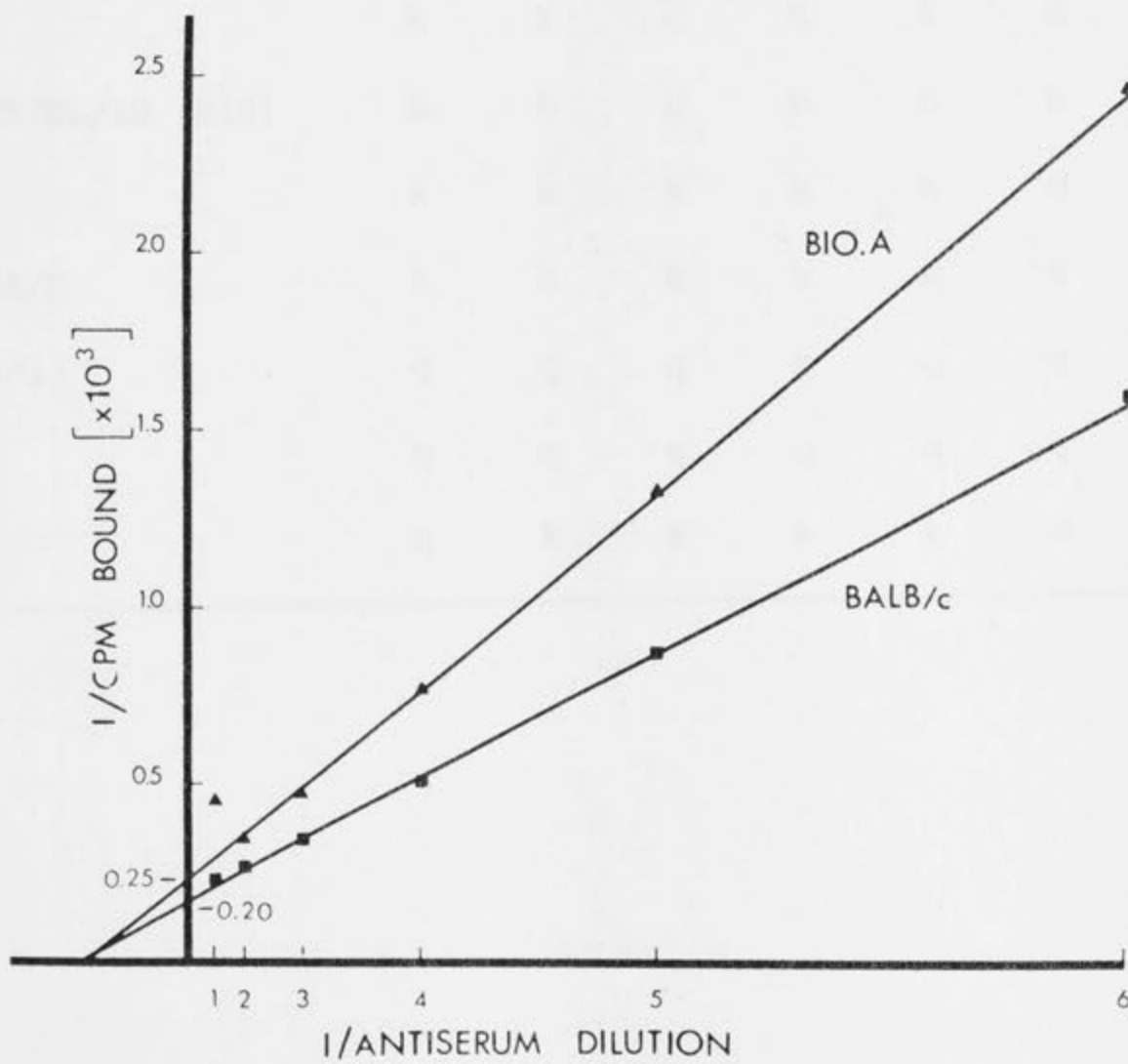


TABLE 1

H-2 HAPLOTYPES OF MOUSE STRAINS USED IN THIS STUDY

Strains	K	A	B	J	E	C	S	D
B10.D2, BALB/c	d	d	d	d	d	d	d	d
D2.GD	d	d	d	b	b	b	b	b
C3H.OH	d	d	d	d	d	d	d	k
B10.A(5R)	b	b	b	k	k	d	d	d
B10.A(2R)	k	k	k	k	k	d	d	b
C57BL/6, C57BL/10 (B10)	b	b	b	b	b	b	b	b
B10.A	k	k	k	k	k	d	d	d
B10.BR, CBA/H	k	k	k	k	k	k	k	k
B10.G, DBA/1j	q	q	q	q	q	q	q	q
B10.T(6R)	q	q	q	q	q	q	q	d
B10.AQR	q	k	k	k	k	d	d	d

TABLE 2
ANTI-H-2 SERA USED

Antiserum	Recipient Strain	Donor Strain	Major Specificity Detected	Other Possible Specificities	Titration ⁻¹ on Spleen Cells
AS43 (αK^d)	(B6 x A) ₁ F ₁	B10.D2	H-2.31	H-2.34 Ia.11,16,23	4096 ^a (BALB/c); <8 (B10.G) Absorbed ^b <8 (BALB/c)
AS742 (αK^d)	(B10.A x A) ₁ F ₁	B10.D2	H-2.31	H-2.27,28,29,34,46 Ia.8,11,16,23	160 (BALB/c); 8(B10) Absorbed ^b <8 (BALB/c)
AS285 (αD^d)	(B10.AKMx129) ₁ F ₁	B10.A	H-2.4	H-2.13,41,42,43,44,49 Ia.6	320 (BALB/c) <4 [B10.A(2R)]
AS954 (αD^d)	(B10.AKMx129) ₁ F ₁	B10.A	H-2.4	H-2.13,41,42,43,44,49 Ia.6	160 (BALB/c) <4 [B10.A(2R)]
AS924A (αK^b)	(B10.D2 x A) ₁ F ₁	B10.A(5R)	H-2.33	H-2.35,36,39 Ia.9,20	800 (B10); <10 (BALB/c) Absorbed ^b <8
AS303 (αD^b)	[B10.A(5R)xLP.RIII] ₁ F ₁	B10	H-2.2	-	640 (B10) -
AS946 (αD^b)	(B10.D2 x A) ₁ F ₁	B10.A(2R)	H-2.2	-	128 (B10) -
AS508 (αK^k)	A.TL	A.AL	H-2.23	H-2.3,8,11,25,47 -	1280 (B10.A); 40(B10.AQR); <8(BALB/c) -
AS116E (αK^k)	(C57BL/6 x LP.RIII) ₁ F ₁	B10.A(2R)	H-2.23	- Ia.2,6,18,22	40 (CBA/H) -
AS292 (αD^k)	[C3H.SWxB10.A(2R)] ₁ F ₁ (Absorbed on A.TL and BALB/c spleen cells prior to use.)	C3H	H-2.32	H-2.1,3,49 Ia.31,32,33	2560 (CBA/H); <4 (B10.A)
AS241 (αK^g)	(AKR.M x DI.C) ₁ F ₁	DBA/1j	H-2.17	- Ia.5,9,10,13	64 (B10.G) Absorbed ^b <8 (B10.G)
AS30 (αD^g)	(B10.AxLP.RIII) ₁ F ₁	B10.AKM	H-2.30	-	2000 (B10.G) -

a 50% end point of titration. Strain used for titration given in brackets.

b Antiserum was absorbed on thymocytes of the same strain as was used for titration by complement-mediated lysis.

TABLE 3

COMPARISON OF ESTIMATES MADE IN DIFFERENT EXPERIMENTS
USING DIFFERENT ANTISERA WITH SIMILAR ANTI-H-2 SPECIFICITY

H-2 Antigen	Strain	Relative H-2 Antigen Expression ^a		
		Antisera Used		
<u>K^k</u>	CBA/H	<u>AS508</u>	<u>AS116E</u>	C57BL/6 anti- CBA/H ^c
		4.8, 5.0 ^b	4.7	-
	B10.A(2R)	4.3, 4.3	4.0±0(3)	4.0
	(BALB/cxCBA/H)F ₁ ^d	3.3	3.0	-
	[B10.A(5R) x B10.A(2R)]F ₁ ^d	3.3	3.3	3.2
<u>D^d</u>		<u>AS954</u>	<u>AS285</u>	
	BALB/c	5.0±0(3)	5.0±0(5)	
	B10.A(5R)	3.8, 4.0	3.9±0.1(3)	
	B10.A	3.8	3.8±0.2(3)	
	(BALB/cxCBA/H)F ₁ ^d	2.9	3.2±0.1(3)	
	[B10.A(5R) x B10.A(2R)]F ₁ ^d	2.3	2.0	
<u>K^d</u>		<u>AS742</u>	<u>AS43</u>	
	BALB/c	6.2±0.2(3)	6.0±0.1(6)	
<u>D^k</u>		<u>AS292</u>	C57BL/6 anti- CBA/H ^c	
	C3H.OH	1.6	1.6	
<u>D^b</u>		<u>AS946</u>	<u>AS303</u>	
	B10	5.5, 5.0	4.5	

a Values are estimates of maximum ¹²⁵I-protein A bound (cpm x 10⁻³) by 2 x 10⁶ spleen cells absorbed with an infinite amount of antiserum.

b When more than 2 estimates were made, values are expressed as mean ± S.E., sample size bracketed.

c See text for specificity tests on this antiserum.

d Further analysis of H-2 expression in F₁ hybrids is presented in Chapter 5.

TABLE 4
 SUMMARY OF ESTIMATES OF H-2
 ANTIGEN EXPRESSION ON SPLEEN CELLS

Strain	Relative H-2 Antigen Expression ^a				Total
	K Antigen	K Antigen Estimate	D Antigen	D Antigen Estimate	
BALB/c	K ^d	6.0±0.1(9) ^b	D ^d	4.9±0.1(8)	10.9
B10.D2	K ^d	4.5±0.5(3)	D ^d	3.9±0.1(3)	8.4
C3H.OH	K ^d	5.7	D ^k	1.6,2.1	7.6
D2.GD	K ^d	5.7	D ^b	6.3	12.0
CBA/H	K ^k	4.9±0.1(3)	D ^k	2.0±0.05(4)	6.9
B10.BR	K ^k	4.4	D ^k	1.5±0.3(3)	5.9
B10.A	K ^k	4.4	D ^d	3.8±0.1(4)	8.2
B10.A(2R)	K ^k	4.3±0.2(5)	D ^b	4.8±0.2(3)	9.1
C57BL/10 (B10)	K ^b	4.2±0.1(7)	D ^b	4.9±0.2(3)	9.1
C57BL/6	K ^b	4.3,4.0	D ^b	5.0	9.2
B10.A(5R)	K ^b	4.2±0.1(3)	D ^d	3.9±0.1(5)	8.1
DBA/1j	K ^q	4.1±0.1(4)	D ^q	4.1±0.1(3)	8.2
B10.G	K ^q	3.2±0.1(4)	D ^q	4.2±0.1(4)	7.4
B10.T(6R)	K ^q	3.1±0.1(3)	D ^d	3.8	6.9
B10.AQR	K ^q	3.3±0.05(4)	D ^d	3.8	7.1

a, b See Table 3.

TABLE 5
 ESTIMATES OF H-2 ANTIGEN EXPRESSION
 IN H-2 CONGENIC C57BL/10 STRAINS

Relative H-2 Antigen Expression ^a			
	K antigen		D antigen
K ^d	4.50 ± 0.51 (3) ^{b,c}	D ^d	3.86 ± 0.04 (14)
K ^k	4.36 ± 0.13 (7) ^f	D ^k	1.52 ± 0.28 (3) ^d
K ^b	4.18 ± 0.06 (12) ^{e,f}	D ^b	4.79 ± 0.11 (8)
K ^q	3.20 ± 0.06 (11)	D ^q	4.23 ± 0.10 (4) ^e

a, b See Table 3.

c Average estimate over all strains tested = 5.72 ± 1.18 (14)

d Average estimate over all strains tested = 1.83 ± 0.10 (9)

e Not significantly different at the 5% level.

f See e. All other pairs of estimates are significantly different. (This comparison does not include K^d.)

INTRODUCTION

The K and D regions of the H-2 gene complex, the major histocompatibility complex in mice, contain the structural genes for I-E antigens, which interact with apparently similar antigens derived by mice (1974). H-2 molecules play a critical role in the stimulation of H-2 restricted T cell (thymus-derived lymphocyte) immune responses (reviewed by Klein, 1976). It is clear that H-2 molecules control the presentation of foreign antigens (e.g., virus or tumor antigens) to T lymphocytes only in association with self-derived H-2 molecules.

The quantitative control of H-2 antigen expression is partly understood, but it is clear that H-2 genes express all 4 polymorphic H-2 antigens (K, D, E, and I-E) in a single individual that also

CHAPTER 5

H-2 antigens are expressed in a single individual as H-2K, H-2D, H-2E, and H-2I-E. While H-2K and H-2D are expressed in a single individual (Chapter 2 and 3), H-2E and H-2I-E are expressed in a single individual (Chapter 2 and 3).

QUANTITATIVE VARIATION IN H-2 ANTIGEN EXPRESSION

II. EVIDENCE FOR A DOMINANCE PATTERN

IN H-2K AND H-2D EXPRESSION IN F_1 HYBRID MICE

Antigenic variation in H-2K and H-2D expression in F_1 hybrid mice is very different from that observed in H-2K and H-2D expression in H-2K and H-2D mice. The results show that the H-2K and H-2D antigens are expressed in a single individual as H-2K and H-2D. Furthermore, expression of H-2K and H-2D antigens is controlled by the H-2K and H-2D alleles themselves (see Table 1).

INTRODUCTION

The *K* and *D* regions of the *H-2* gene complex, the major histocompatibility complex in mice, contain the structural genes for 2 different molecules each with apparently similar antigenic function (reviewed by Klein 1975). *H-2* molecules play a crucial role in the stimulation of *H-2* restricted T cell (thymus-derived lymphocyte) immune responses (reviewed by Snell 1978), such that cytotoxic T cells (Tc cells) can recognise foreign antigens (e.g. viral or minor H (histocompatibility) antigens) only in association with self *H-2K* or *H-2D* molecules.

Thus far, the genetic control of *H-2* antigen expression is poorly understood, but it is known that F_1 hybrids express all 4 parental *K* and *D* antigens (Cullen *et al.* 1972). Earlier observations that some *H-2* antigens are expressed in similar amounts on F_1 hybrid and parental cells, while others are expressed in much less amount (Chapters 2 and 3), argue against a simple gene dosage effect for the regulation of *H-2* antigen expression. Since cell surface *H-2* concentration is now known to be a variable determining the effectiveness of particular *H-2* antigens in their immune response role (Chapters 2 and 3), *K* and *D* antigen expression in many different F_1 hybrids was therefore investigated. The results show that the phenomenon is common among F_1 hybrids and, furthermore, expression of an antigen appears to be dependent on the *K* and *D* allelic composition of the F_1 hybrid.

MATERIALS AND METHODS

Mice

Mice were bred in the John Curtin School of Medical Research. Mice of the same age and sex were used in each experiment. An F₁ hybrid breeding program was established and mice from several breedings were used in this study. The H-2 haplotypes of mice used are indicated in Table 1.

Antisera

Anti-H-2 sera were generously provided by Dr Ian McKenzie (University of Melbourne, Australia). They were produced by repetitive immunization with lymphoid cells, in strain combinations which differed where possible by just an H-2K or H-2D private specificity (McKenzie and Snell 1973). Antisera were tested prior to use by complement-mediated lysis and in the ¹²⁵I-protein A radioimmunoassay (RIA) for crossreactivity on relevant strains. The sera, and their strains of origin, are listed in Table 2. Only slight crossreactivity was detectable for some of the antisera (i.e. <50% lysis by a 1/4 dilution of antiserum). This activity was absorbed out on spleen cells (100 μl antiserum per 10⁸ spleen cells, 60 minutes, 4°C), until cytotoxic activity was removed.

Two-Stage Microcytotoxicity Assay

This method for measuring complement-mediated lysis has already been described in Chapter 2.

Quantitative Absorption Analysis

The method used to compare parental and F₁ hybrid cells for their ability to absorb activity from anti-H-2 sera has been previously described (McKenzie *et al.* 1976; Chapter 2). Briefly, a two-stage

microcytotoxicity assay was carried out in U-bottomed Linbro 6mm microtitre trays. 50 μ l of Isopaque/Ficoll purified spleen cells in doubling dilution (<4% dead cells), was added to 50 μ l of antisera (used at a dilution which gave 80% lysis of control cells in a routine titration) for 30 minutes at room temperature. After centrifugation, each sample of the absorbed antiserum was then titrated by complement-mediated lysis on parental strain cells. Unabsorbed antisera and rabbit complement alone were tested as controls. Results were plotted as percent dead cells versus number of absorbing cells (log 2 scale), from which 50% end points were calculated. Data sets were compared by the likelihood ratio test (LRT) for significance in each experiment.

Radioimmunoassay Using 125 I-Protein A

Methods for cell preparation, radioiodination of protein A, and the RIA have been outlined previously (Chapters 3 and 4).

Briefly, the binding of 125 I-protein A is used as an approximation of antibody binding. The relative number of antibody binding sites or H-2 antigens on F_1 hybrid and parental strain spleen cells, and the relative dissociation constants for antibody binding were estimated by plotting the reciprocal of bound 125 I-protein A against the reciprocal of antibody dilution. The intercept on the Y-axis represents the reciprocal of the number of H-2 molecules to which antibody would bind at infinite antibody concentration, while the intercept on the X-axis is an estimate of the dissociation constant for the antibody-antigen interaction. Estimates of H-2 antigen expression are given only in terms of the number of counts per minute (cpm) incorporated and in any one experiment estimates were made on both F_1 hybrid and parental cells. Estimates made on different strains were compared for significance by the Wilcoxon Rank Sum Test.

RESULTS

Not all F_1 hybrids show the same selective expression of one set of parentally-derived H-2 antigens as does the [B10.A(5R) x B10.A(2R)] F_1 hybrid (Chapter 2). This was found when several other F_1 hybrids were screened for expression of H-2 antigens relative to parental strains firstly by quantitative absorption analysis and secondly in an ^{125}I -protein A RIA involving estimation of H-2 concentration on the cell. The results were reproducible both in male and female adult mice and in many repetitive experiments on mice from several breedings. At least two and as many as four quantitative absorption experiments were conducted for each H-2 antigen in 6 C57BL/10 H-2 congenic F_1 hybrids. A representative result using the (B10 x B10.BR) F_1 hybrid is shown in Figure 1, and the results from absorptions using other F_1 hybrids are presented in summary form in Tables 3 and 4. Between 3 and 5 ^{125}I -protein A RIA estimates were made for each H-2 antigen in 4 different F_1 hybrids. The pooled results are presented in Table 5. Differences in the binding of anti- K^b and anti- K^d to (B10 x B10.D2) F_1 and parental cells are shown in Figure 2A. Use of the reciprocal plot to estimate H-2 antigen expression is shown in Figure 2B. Results obtained by this method complemented the quantitative absorption results in showing that some H-2 antigens in F_1 hybrids are expressed in 2 fold less amount than in parental cells, while others are expressed more closely to parental amounts (see Table 5). Despite differences in the sensitivity of the 2 assays (see Discussion), the same general pattern of variation in antigen expression was seen. A summary of H-2 antigen expression in all the F_1 hybrids studied is presented in Table 6.

Equal Expression of H-2 Antigens in Different F_1 Hybrids Carrying the Same H-2K and H-2D Antigens

Irrespective of different *I*-region coded alleles, and the chromosomal location of the *K* and *D* alleles, both the [B10.A(5R) x B10.A(4R)] F_1 and (B10 x B10.A) F_1 mice show the same pattern of antigen expression as do [B10.A(5R) x B10.A(2R)] F_1 mice (Chapter 2), i.e. D^b and K^k are expressed approximately equally in both F_1 hybrid and parent and K^b and D^d in approximately 2-3 fold less amount (Tables 3 and 5). Furthermore, when these three F_1 hybrids were compared with each other for antigen expression (see Table 3), K^b and D^d were expressed equally in [B10.A(5R) x B10.A(2R)] F_1 , [B10.A(5R) x B10.A(4R)] F_1 and (B10 x B10.A) F_1 mice, which excludes an *I*-region or maternal influence on H-2 antigen expression, and implicates an effect due to the combination of the 4 K^k , K^b , D^b and D^d alleles in the one cell, regardless of the chromosomal location of each allele.

Further Evidence for Weak K^b Expression in F_1 Hybrids Carrying K^k

When (B10 x B10.BR) F_1 mice were compared with the parental strains B10 and B10.BR, they showed equal expression of K^k , but two-fold reduced expression of K^b (see Table 4, and Figure 1). There was also a two-fold reduced expression of both D^b and D^k .

The Reduced Expression of D^d in the Presence of D^b Maps to the *D*-Region

Weak D^d expression in F_1 hybrids expressing D^b is also seen in both (B10 x B10.D2) F_1 and [B10.A x B10.A(2R)] F_1 mice (Tables 4 and 5). By quantitative absorption analysis, the relative amount of D^d is slightly greater than in [B10.A(5R) x B10.A(4R)] F_1 and (B10 x B10.A) F_1 mice, but still excludes a major influence by a different combination of *K* alleles (K^b and K^d) on the expression of D^b and D^d . The results

in [B10.A x B10.A(2R)] F_1 mice map the regulation of H-2 antigen expression to the vicinity of the *D*-locus, and suggests partial dominance of D^b over D^d .

Dominance Relationship Between K^d , K^k and K^b Alleles

There is also differential expression of K^d and K^b in the (B10 x B10.D2) F_1 (see Tables 4 and 5). K^d is expressed equally in the F_1 hybrid and in the B10.D2 parent, while K^b is in much less amount than in B10. When compared with the relative expression of K^d and K^k in (B10.BR x B10.D2) F_1 mice, where K^d is again equally expressed in the F_1 and B10.D2 parent, but K^k is in two-fold less amount, a pattern of partial dominance in expression emerges. Both K^b and K^k alleles are weakly expressed in the presence of K^d , K^b being weakly expressed in the presence of K^k .

Dominance Relationship Amongst *D* Alleles

Although both combinations of D^d and D^k and of D^b and D^k alleles in (B10.BR x B10.D2) F_1 and (B10 x B10.BR) F_1 give slightly weaker expression of all *D* antigens relative to parental strains, the only significant difference is seen for D^b expression in the latter F_1 hybrid (Table 4). This suggests partial dominance of D^k over D^b , just as was seen for D^b over D^d (Table 5). Since the combination D^d and D^k does not appear to lead to preferential reduction in the expression of either *D*-coded antigen, the dominance pattern amongst *D* alleles is not as clear-cut as with *K* alleles. Other factors may influence allelic preference when the D^k allele is present.

Background Genotype Does Not Influence Differences in Relative H-2
Expression Between F₁ Hybrid and Parental Strain Mice

The RIA results presented in Table 5 show that the same variation in H-2 antigen expression is seen in the (C57BL/6 x BALB/c)F₁ as in the (B10 x B10.D2)F₁. The (BALB/c x CBA/H)F₁ also shows the same pattern of antigen expression compared with parental cells as does the (B10.BR x B10.D2)F₁, i.e. K^d is the only antigen expressed equally in parental and F₁ cells, while K^k, D^k and D^d are weakly expressed to some extent despite slight differences in the results obtained by the two different methods.

DISCUSSION

The results presented here support earlier results in showing that quantitative differences in H-2 antigen expression are a common feature of F₁ hybrids (Chapters 2 and 3). The effect is not due to maternal influence, nor does *K* or *D* gene expression seem to be influenced by other *I*-region genes. H-2 expression in F₁ hybrids is dependent on the *K* and *D* allelic composition of the F₁ hybrid in that some alleles seem to 'compete' with and partially dominate others. Background genes do not appear to affect relative H-2 antigen expression between F₁ hybrid and parental cells, despite minor differences in antigen expression amongst homozygotes with different background genotypes (see Chapter 4).

The most interesting issue raised by these results is the nature of the mechanism leading to 'strong' and 'weak' antigen expression in F₁ hybrids. Regulation of expression appears to be confined to just the *K* or *D* regions, firstly because of the consistency in expression of K^k/K^b and D^b/D^d in several F₁ hybrids studied here, and secondly, since the results presented in the preceding chapter indicate that

H-2K and H-2D antigen expression is not influenced by the expression of other *H-2* gene products on the same cell. H-2 expression in F_1 hybrids appears to involve some competition or 'dominance' between allelic products, which could act at the level of regulator genes and the initiation of transcription of the *K* and *D* genes.

Although small, differences of 2-3 fold in H-2 antigen expression between F_1 hybrid and parental cells were consistent for any pair of *K* or *D* alleles and by the 2 different assay systems. Despite differences in the sensitivity of the 2 methods, the same pattern of antigen expression was found for 3 different F_1 hybrids analysed by both methods. Since quantitative absorption analysis is an indirect assay, preabsorption of antisera may selectively remove high affinity antibodies so that differences in antibody binding may be expanded by this method. This could explain why up to 3 fold differences in antigen expression were detected by this method but no greater than 2 fold differences were detectable in the ^{125}I -protein A RIA. Furthermore, this could also account for slight differences in the expression of H-2 antigens in $H-2^k/H-2^d$ F_1 hybrids detectable by the two methods despite general similarity in the pattern of antigen variation. The RIA results indicate that while most antigens are weakly expressed to some extent in F_1 hybrids, particular antigens are expressed predominantly more weakly.

F_1 hybrids appear to be characterised by greater variability in the expression of their *K* and *D* antigens than do homozygotes (see Chapter 4), and greater total numbers of H-2 antigens on their cell surface (see Table 5). This could give F_1 hybrids a selective advantage over homozygotes in their T cell response capacity, and may help to explain the enhanced responsiveness of F_1 hybrid mice over

parental strains seen by Doherty and Zinkernagel (1975) following inoculation with lymphocytic choriomeningitis virus. The dominance relationship between K and D antigens indicates that the expression of an antigen can be varied by placing that antigen into different F_1 hybrids. The K and D allelic composition of the F_1 hybrid, and the parental origin of the cell, will determine its expression, and could ultimately influence the stimulating ability of the antigen. However, as pointed out in the previous chapter, the role of quantitative differences in H-2 expression in T cell responsiveness may be clouded by other helper and suppressor T cell effects, immune response gene effects, the nature and expression of the viral or minor histocompatibility antigens and the size of the T cell pool.

In line with the results of Shearer *et al.* (1979) where H-2-linked differences in responsiveness to trinitrophenyl-modified cells were only detectable when stimulator cells were modified with low concentrations of the conjugating reagent, it is expected that the small, 2-3 fold differences in H-2 expression seen here may have most importance for T cell recognition when cells carry limited amounts of the foreign antigen.

In this regard, it is very interesting to examine the present results in the context of the pattern of responsiveness to the male-specific HY antigen in F_1 hybrid female mice (von Boehmer *et al.* 1977, Simpson and Gordon 1977, Hurme *et al.* 1977). If an inoculum of male spleen cells is a relatively inefficient priming stimulus, it follows that the antigen threshold needed for detectable priming might be most often achieved with the more strongly expressed K and D antigens, so that a secondary *in vitro* response will be detected only in association with these 'strong' alleles. This could explain why F_1

hybrids tend to show preference for one of the two parental strain male targets when primed with F_1 male cells (Gordon *et al.* 1977).

The pattern of antigen expression in the (B10 x B10.BR) F_1 hybrid is particularly relevant to anti-HY responsiveness. Of all the F_1 hybrids tested, this is the only one which expresses D^b more weakly than the B10 parental strain. With respect to anti-HY responsiveness, D^b is known to be a permissive responder allele in both homozygous and F_1 hybrid strains (Simpson and Gordon 1977). However, the (CBA/H x B10) F_1 hybrid ($H-2$ identical to the (B10 x B10.BR) F_1) differs from all other D^b -carrying strains in that priming with F_1 male cells produces a detectable response against $H-2^k$ and not $H-2^b$ targets (Gordon *et al.* 1977). Since parental B10 male cells can adequately stimulate an anti- D^b response, these results are consistent with a role for antigen concentration on the stimulator cells in determining anti-HY Tc cell responsiveness.

In line with observations made in the previous paper, the RIA results are consistent with a quantitative reduction in H-2 antigen expression in F_1 hybrid mice, but the possibility that some qualitative change in the F_1 hybrid antigens is affecting their antibody binding capacity can not be eliminated until appropriate monoclonal antibodies are found. The existence of either qualitative or quantitative differences in H-2 expression in F_1 hybrids is pertinent to the recent results of Warner and Cudkowicz (1979) and of Ishikawa and Dutton (1979), which suggest that qualitatively different H-2 antigens may be the targets for F_1 anti-parent responses.

With the results presented here, and evidence for F_1 -specific I-region determinants (Fathman and Nabholz 1977), for multigenic control of Ia antigens (Jones *et al.* 1978), and for at least 2 H-2-

like molecules which code in the *D* region (Démant *et al.* 1977, McKenzie *et al.* 1977), regulation of expression of H-2 antigens portends to be much more complicated than previously assumed.

SUMMARY

Further evidence for quantitative variation in H-2 antigen expression in F_1 hybrids is described. Spleen cells from 9 different F_1 hybrids were compared with parental strain cells for differences in H-2K and H-2D antigen expression by quantitative absorption analysis and in an ^{125}I -radioimmunoassay using anti-H-2 sera. Some K and D antigens are expressed in 2 to 3 fold less amount in F_1 hybrids while others are approximately equally expressed. There was no evidence for either *I*-region control or for parental influence in the genetic control of H-2 antigen expression. Expression of K and D antigens in an F_1 hybrid (compared with parental cells) is dependent on the K and D allelic composition of the hybrid, in that some alleles tend to dominate others. In 4 different F_1 hybrids K^k was dominant over K^b , while in other F_1 hybrids, K^d predominated over both K^k and K^b . Similarly, D^b dominated D^d in 5 different F_1 hybrids, while no obvious dominance pattern was found in F_1 hybrids carrying D^k .

R E F E R E N C E S

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Fig. 1 Quantitative absorption of anti-H-2 serum by spleen cells of the F_1 hybrid (B10 x B10.BR) in comparison with B10 and B10.BR cells. The F_1 hybrid was compared with B10 for absorption of anti- K^b and anti- D^b serum and with B10.BR for anti- K^k and anti- D^k serum. F_1 (\blacktriangle); B10 (\blacksquare); B10.BR (\bullet). Remaining antibody activity was titred on appropriate parental spleen cells. Lysis by unabsorbed antiserum plus rabbit complement (AS) and by rabbit complement alone (RC) are shown as controls. Each experiment was repeated at least twice, but only one representative result is shown. The number of cells absorbing was plotted on a log 2 scale.

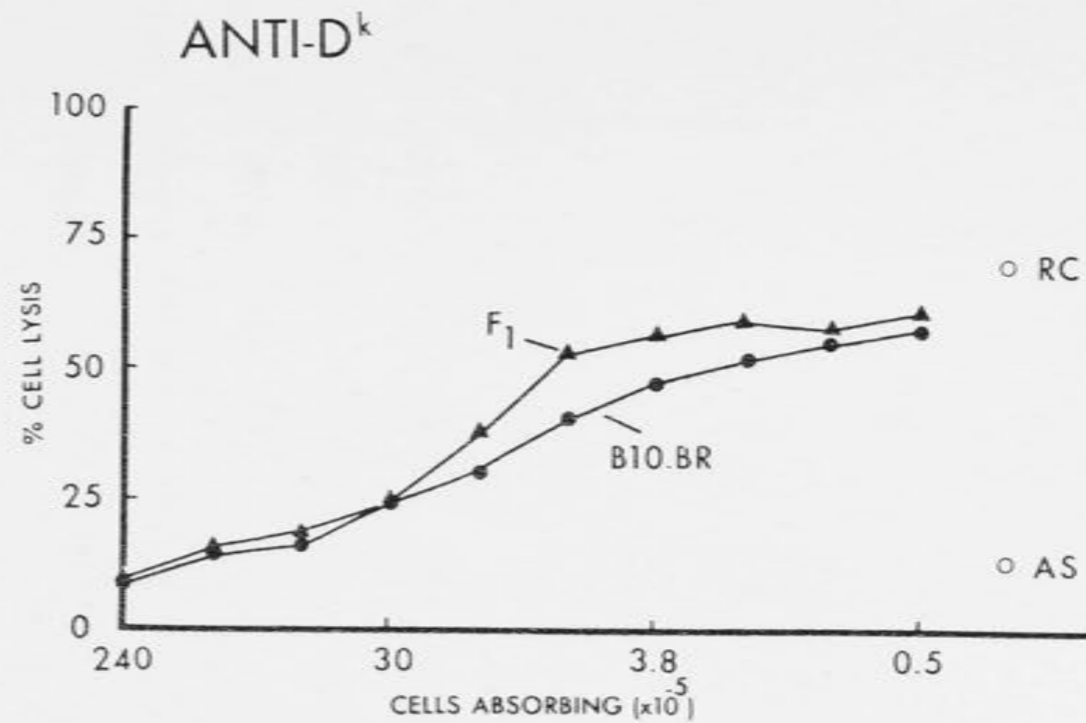
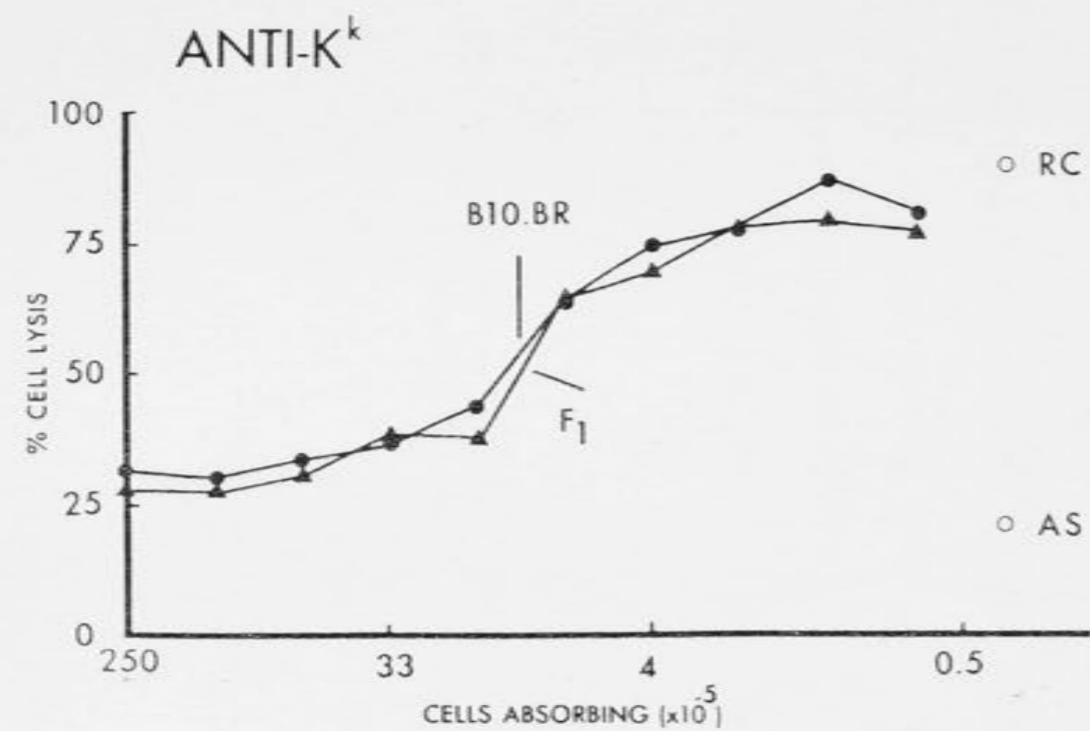
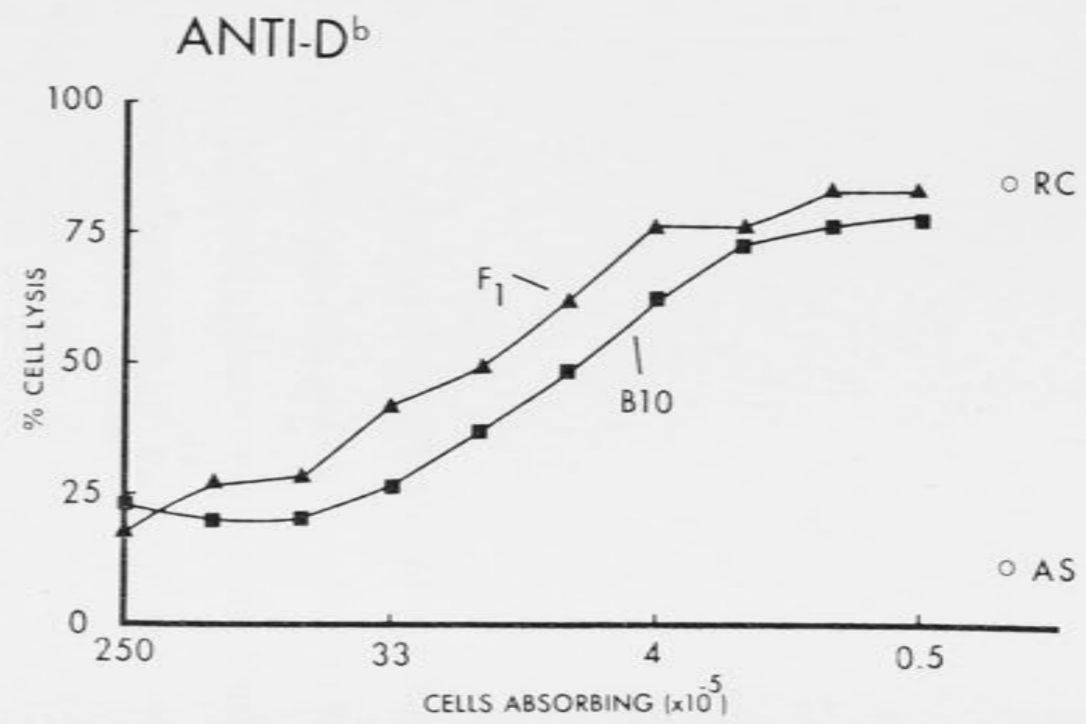
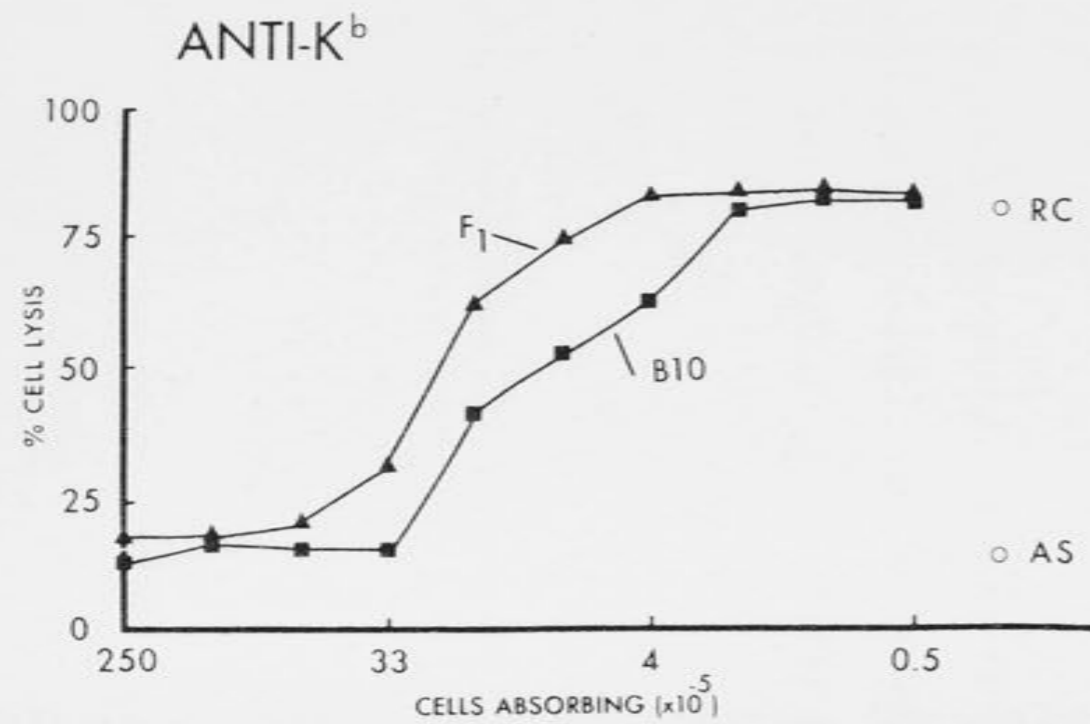


Fig. 2A Spleen cells of the (B10 x B10.D2) F_1 hybrid (■), B10 (●) and B10.D2 (▲) mice were compared for their uptake of 125 I-protein A following exposure to serial dilutions of either anti-H-2K^b serum (AS924A) or anti-H-2K^d serum (AS43). Binding of 125 I-protein A to cells preabsorbed with normal mouse serum were included as controls (open symbols).

Fig. 2B Reciprocal plot of 125 I-protein A binding versus antiserum dilution. The line of best-fit was plotted through points representing the linear region of the binding curve in Figure 2A.

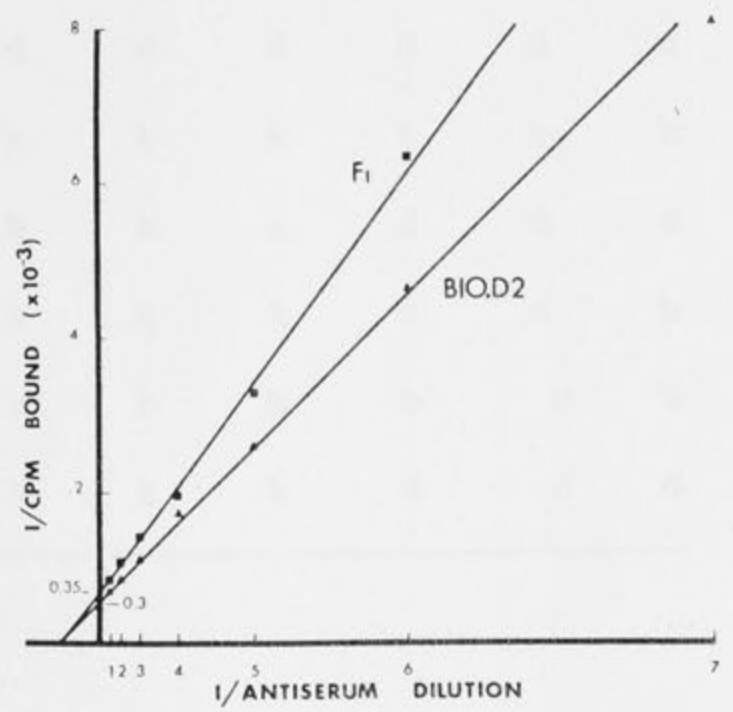
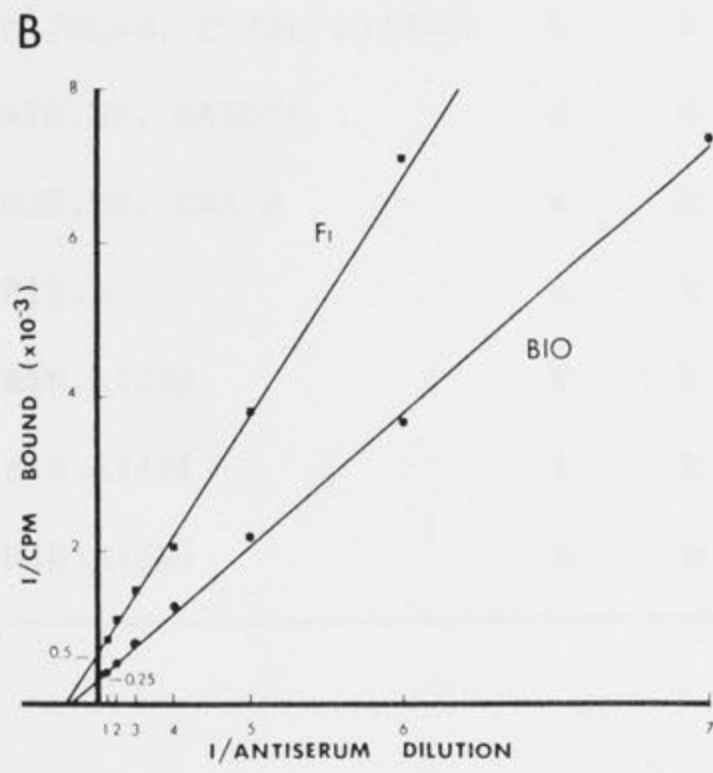
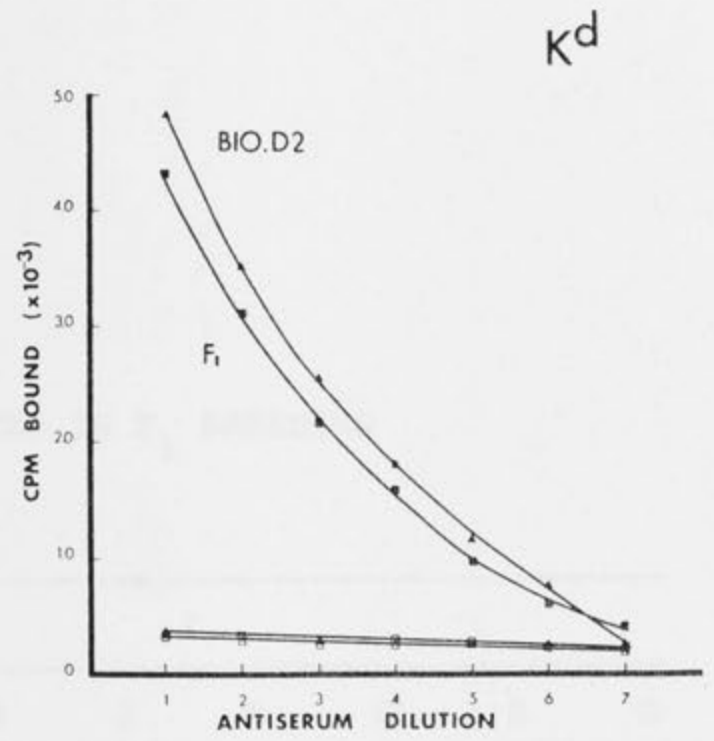
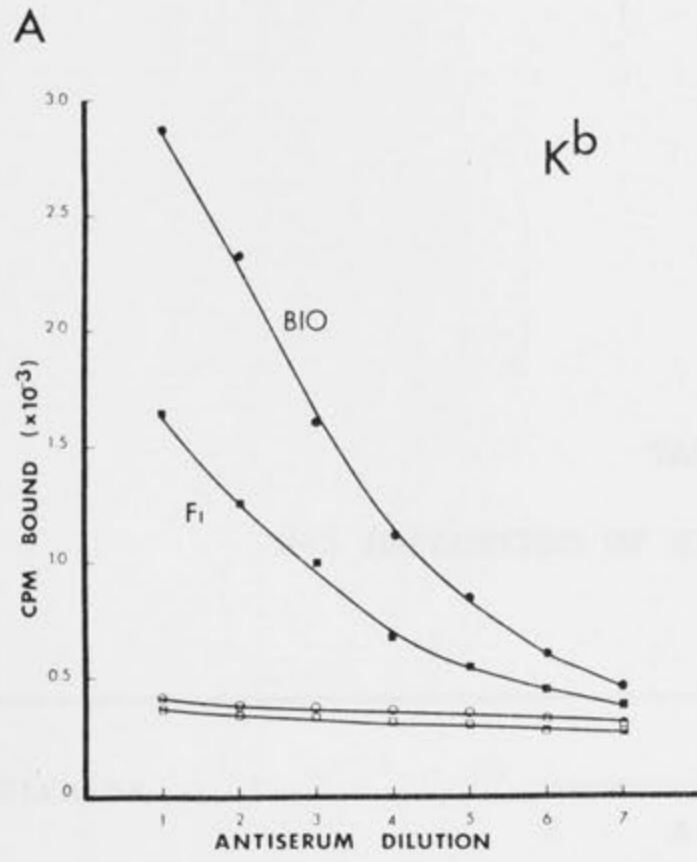


TABLE 1
H-2 HAPLOTYPES OF MICE USED IN F₁ BREEDING

Strains	I							
	K	A	B	J	E	C	S	D
C57BL/6, C57BL/10 (B10)	b	b	b	b	b	b	b	b
B10.D2, BALB/c	d	d	d	d	d	d	d	d
B10.BR, CBA/H	k	k	k	k	k	k	k	k
B10.A	k	k	k	k	k	d	d	d
B10.A(2R)	k	k	k	k	k	d	d	b
B10.A(4R)	k	k	b	b	b	b	b	b
B10.A(5R)	b	b	b	k	k	d	d	d

TABLE 2
ANTISERA USED IN THIS STUDY

Catalogue Number	Specificity	Recipient	Donor	Titre ^a	Crossreactivity ^b
AS924A	H-2.33(K ^b)	(B10.D2 x A)F ₁	B10.A(5R)	$\frac{1}{800}$	No crossreactivity on B10.A(2R), B10.A, B10.D2, B10.BR.
AS946	H-2.2(D ^b)	(B10.D2 x A)F ₁	B10.A(2R)	$\frac{1}{128}$	No crossreactivity on B10.A(5R), B10.A, B10.BR, B10.D2.
AS43	H-2.31(K ^d)	(B6 x A)F ₁	B10.D2	$\frac{1}{4096}$	No crossreactivity on B10, B10.BR, B10.A.
AS285	H-2.4(D ^d)	(B10.AKM x 129)F ₁	B10.A	$\frac{1}{320}$	No crossreactivity in B10.A(2R), B10. Slight crossreactivity on B10.BR was absorbed out on spleen cells.
AS508	H-2.23(K ^k)	A.TL	A.AL	$\frac{1}{1024}$	No crossreactivity on B10, B10.A(5R). Slight crossreactivity on B10.D2 was absorbed out on spleen cells.
AS292	H-2.23(D ^k)	(C3H.SW x B10.A(2R))F ₁	C3H	$\frac{1}{2560}$	Absorbed on A.TL spleen prior to use. No crossreactivity on B10. Slight crossreactivity on B10.D2 was absorbed out on spleen cells.

a Titres determined by complement-mediated lysis of spleen cells.

b Antisera were tested by complement-mediated lysis for crossreactive binding to other K and D antigens carried by F₁ hybrid and parental strains under test.

TABLE 3

QUANTITATIVE ABSORPTION ANALYSIS OF DIFFERENT F₁ HYBRIDS CARRYING THE SAME K AND D ALLELES

F ₁ Hybrid	Antiserum Specificity	Dilution Used for Absorption	Cell Number Required to Reduce Cytotoxicity of Antiserum by 50% (x 10 ⁻⁵) ^a			LRT ^b χ ² (d.f.) ^c	H-2 Antigens Expressed Equally in F ₁ and Parental Cells ^e	H-2 Antigens Expressed More Weakly in F ₁ than Parental Cells ^e
			F ₁	B10	B10.A			
(B10xB10.A)F ₁	K ^k (H-2.23)	1/100	9.2		8.5	7.82 (10)		
	D ^b (H-2.2)	1/25	8.6	6.9		5.23 (10)	K ^k , D ^b	K ^b , D ^d
	K ^b (H-2.33)	1/300	18.0	6.8		37.58 (10) ^d		
	D ^d (H-2.4)	1/120	14.0		5.3	20.03 (10) ^d		
[B10.A(5R)xB10.A(4R)]F ₁	K ^k (H-2.23)	1/100	9.8		7.6	8.23 (8)		
	D ^b (H-2.2)	1/25	5.5		4.8	9.44 (10)	K ^k , D ^b	K ^b , D ^d
	K ^b (H-2.33)	1/300	18.0	5.3		36.56 (10) ^d		
	D ^d (H-2.4)	1/120	15.0	3.8		48.33 (10) ^d		
Comparison Between F ₁ Hybrids			(B10xB10.A)F ₁ [B10.A(5R)xB10.A(2R)]F ₁					
	K ^b (H-2.33)	1/300	4.2		3.2	2.76 (8)	-	-
	D ^d (H-2.4)	1/120	11.0		13.0	11.15 (9)		
			[B10.A(5R) x B10.A(4R)]F ₁ [B10.A(5R) x B10.A(2R)]F ₁					
	K ^b (H-2.33)	1/300	18.0		21.0	6.48 (10)	-	-
	D ^d (H-2.4)	1/120	15.0		15.0	2.64 (10)		

a F₁ hybrid spleen cells were compared with parental cells (or other F₁ cells) for ability to absorb out activity from anti-H-2 serum. The 50% end-point of titration is given as the number of cells required to absorb out 50% of antiserum activity as titred on parental strain cells by complement-mediated lysis.

b Likelihood Ratio Test Results.

c d.f.; degrees of freedom.

d Significantly different at the 5% level.

e Classification into 2 groups is determined by LRT results.

TABLE 4

QUANTITATIVE ABSORPTION ANALYSIS OF UNRELATED F₁ HYBRIDS

F ₁ Hybrid	Antiserum Specificity	Dilution Used for Absorption	Cell Number Required to Reduce Cytotoxicity of Antiserum by 50% (x10 ⁻⁵) ^a			LRT ^b χ ² (d.f.) ^c	H-2 Antigens Expressed Equally in F ₁ and Parental Cells ^e	H-2 Antigens Expressed More Weakly in F ₁ than Parental Cells ^e
			F ₁	B10	B10.BR			
(B10xB10.BR) F ₁	K ^b (H-2.33)	1/300	26.0	11.0		39.12 (10) ^d		
	D ^b (H-2.2)	1/25	8.5	4.0		23.90 (10) ^d	K ^k , D ^k	K ^b , D ^b
	K ^k (H-2.23)	1/120	12.0		11.0	7.97 (10)		
	D ^k (H-2.32)	1/200	6.1		3.8	7.38 (10)		
(B10xB10.D2) F ₁	K ^b (H-2.33)	1/300	50.0	14.0		91.91 (9) ^d		
	D ^b (H-2.2)	1/25	7.5	8.0		4.54 (10)	K ^d , D ^b	K ^b , D ^d
	K ^d (H-2.31)	1/250	12.0		8.6	10.25 (10)		
	D ^d (H-2.4)	1/120	27.0		14.0	36.52 (8) ^d		
(B10.BRxB10.D2) F ₁	K ^k (H-2.23)	1/100	16.0	5.9		46.31 (7) ^d		
	D ^k (H-2.32)	1/200	7.0	5.0		6.36 (10)	K ^d , D ^k , D ^d	K ^k
	K ^d (H-2.31)	1/250	4.7		4.7	2.39 (10)		
	D ^d (H-2.4)	1/120	6.5		4.2	2.06 (9)		
[B10.AxB10.A(2R)] F ₁	D ^d (H-2.4)	1/120	11.0	5.6		21.22 (8) ^d	D ^b	D ^d
	D ^b (H-2.2)	1/25	75.0		57.0	4.26 (10)		

a, b, c, d, e See Table 3.

TABLE 5

^{125}I -PROTEIN A BINDING ESTIMATES OF RELATIVE H-2
EXPRESSION ON F_1 HYBRID AND PARENTAL SPLEEN CELLS

F_1 Hybrid	Specificity	Estimate of relative H-2 antigen concentration on 2×10^6 spleen cells ($\times 10^{-3}$) ^a		
		F_1	B10.A(5R)	B10.A(2R)
<u>[B10.A(5R) x B10.A(2R)]F_1</u>	K^k (H-2.23)	3.3 ^b		4.2
	D^b (H-2.2)	3.6		4.3
	K^b (H-2.33)	2.2	4.2	
	D^d (H-2.4)	2.2	4.0	
<u>(B10 x B10.D2)F_1</u>	K^b (H-2.33)	2.3	4.0	
	D^b (H-2.2)	4.0	5.0	
	K^d (H-2.31)	4.5 ^c		4.5
	D^d (H-2.4)	2.2		4.0
<u>(C57BL/6 x BALB/c)F_1</u>	K^b (H-2.33)	1.9	4.0	
	D^b (H-2.2)	4.0	5.0	
	K^d (H-2.31)	6.2 ^c		6.2
	D^d (H-2.4)	2.2		5.0
<u>(BALB/c x CBA/H)F_1</u>	K^d (H-2.31)	5.2 ^c	5.8	
	D^d (H-2.4)	3.1	5.0	
	K^k (H-2.23)	3.3		5.0
	D^k (H-2.32)	1.3		2.0

a Estimates are taken from the Y-axis intercept of the reciprocal binding plot. They represent cpm ^{125}I -protein A which can bind to 2×10^6 spleen cells absorbed with an infinite amount of anti-H-2 serum.

b All values are means of between 3 and 5 estimates. Estimates made for any one antigen varied by less than 2.0×10^2 cpm.

c F_1 hybrid estimate not significantly different from parental estimate by the Wilcoxon Rank Sum Test ($p = 0.05$).

TABLE 6

SUMMARY OF EVIDENCE FOR A DOMINANCE PATTERN
IN H-2 ANTIGEN EXPRESSION IN F₁ HYBRID MICE

F ₁ Hybrid	H-2 Haplotype							Dominance Pattern
	K	IA, IB, IJ, IE, IC	D					
<u>B10.A(5R)</u> ^a	(b) ^b	b	b	k	k	d	(d)	K ^k > K ^b ; D ^b > D ^d
B10.A(2R)	k	k	k	k	k	d	b	
<u>B10.A(5R)</u>	(b)	b	b	k	k	d	(d)	K ^k > K ^b ; D ^b > D ^d
B10.A(4R)	k	k	b	b	b	b	b	
<u>B10</u>	(b)	b	b	b	b	b	b	K ^k > K ^b ; D ^b > D ^d
B10.A	k	k	k	k	k	d	(d)	
<u>B10.A</u>	k	k	k	k	k	d	(d)	D ^b > D ^d
B10.A(2R)	k	k	k	k	k	d	b	
<u>B10</u>	(b)	b	b	b	b	b	(b)	K ^k > K ^b ; D ^k > D ^b
B10.BR	k	k	k	k	k	k	k	
<u>B10</u> ; <u>C57BL/6</u>	(b)	b	b	b	b	b	b	K ^d > K ^b ; D ^b > D ^d
B10.D2 ; <u>BALB/c</u>	d	d	d	d	d	d	(d)	
<u>B10.BR</u> ; <u>BALB/c</u>	(k)	k	k	k	k	k	k	K ^d > K ^k ; D ^k = D ^d
B10.D2 ; <u>CBA/H</u>	d	d	d	d	d	d	d	

a From Chapter 2.

b Circled H-2 antigens are expressed significantly more weakly by F₁ hybrid than parental cells. Classification is defined by LRT results which determine significance of differences between F₁ and parental quantitative absorption results (p = 0.05).

INTRODUCTION

The D and E regions of the major histocompatibility complex each code for 4,700 polypeptide chains... The D region... The E region... The D region... The E region...

CHAPTER 6

MONOCLONAL ANTIBODY DETECTION OF TWO CLASSES OF H-2K^k MOLECULES

Monoclonal antibodies... The H-2K^k molecule... The H-2K^k molecule... The H-2K^k molecule...

Small size... The D region... The E region... The D region... The E region...

INTRODUCTION

The *K* and *D* regions of the murine major histocompatibility complex each code for 45,000 molecular weight glycoprotein molecules with similar immunological functions (Klein 1975). Extensive serological analysis has revealed many crossreactive (public) determinants as well as unique (private) determinants specific for each of the different allelic molecules. Until recently, private and public specificities were thought to be carried by the same molecule, but there are now exceptions which suggest that this is not true for some public specificities. By the differential redistribution method, Lemmonier and others (1975) originally showed that some of the H-2D^d public specificities (H-2.27,28,29) were detectable on H-2 molecules which lacked the H-2D^d private specificity (H-2.4). This result has since been confirmed in different ways by other workers (Morello *et al.* 1977, Hansen *et al.* 1977, McKenzie *et al.* 1977, Hansen and Sachs 1978, Néauport-Sautés *et al.* 1978), and has led to the definition of the 'H-2L' molecule (Démant and Néauport-Sautés 1978) which carries H-2D public antigens but lacks H-2D private specificities. Such a molecule has now been identified in several haplotypes (Demant *et al.* 1979) though an *H-2L* locus has never been separated from the *H-2D* region by recombination.

Until now there has been no evidence for similar complexity in the *K* region (Néauport-Sautés *et al.* 1978), but with the use of two different anti-H-2K^k monoclonal antibodies (Lemke *et al.* 1978, 1979, Hämmerling *et al.* 1979) it has been possible to detect H-2K^k molecules which carry different antigenic determinants. This chapter contains the experimental evidence which supports this conclusion.

MATERIALS AND METHODS

Animals

Mice were bred in the John Curtin School of Medical Research and were used as spleen cell donors when 6-12 weeks old. Male and female mice were used interchangeably in all experiments. The *H-2* haplotypes of all strains used are listed in Table 1.

Antisera

Monoclonal antibodies 27R9 and 30R3 have been described in detail by Lemke *et al.* (1978, 1979) and Hämmerling *et al.* (1979). They were obtained from Dr G.J. Hämmerling (Institute for Genetics, University of Cologne, Cologne, FRG) as freeze dried ascitic fluid produced in mice by hybridomas derived from fusion of the myeloma P3-X63-Ag8 and spleen cells obtained from BALB/c mice immunized against CBA/H spleen cells. 27R9 appears to detect H-2.25, and 30R3, H-2.5. Since 27R9 is IgG_{2a} and 30R3 is IgG_{2b}, both of them bind protein A (Goding 1978). Two different preparations of antisera were used and gave similar results in all experiments described herein, when tested on Bl0.A targets. By a rosetting assay, the two preparations of 27R9 had titres of 1/16,000 and 1/2,000,000, and the two batches of 30R3 had titres of 1/8,000 and 1/50,000.

Goat anti-mouse Ig conjugated with fluorescein isothiocyanate (FITC-GAMIG) was purchased from Hyland Laboratories (Los Angeles, Ca.), and goat anti-mouse Ig conjugated with tetramethylrhodamine isothiocyanate (TRITC-GAMIG) from Nordic Immunology (London, England). Each conjugate was preabsorbed on a 1:1 mixture of Bl0.A thymus and Bl0.A Ig-capped spleen cells prior to use.

Highly specific anti-H-2 sera were provided by Dr Ian McKenzie (University of Melbourne, Australia). The sera were produced by

repetitive inoculation with lymphoid cells in strain combinations which differed where possible by just the H-2K or H-2D private specificity (McKenzie and Snell 1973). Anti-D^d serum (AS954) was raised in (Bl0.AKM x 129)F₁ mice by priming with Bl0.A cells. It has a titre by rosetting of 1/600 on Bl0.A spleen cells. Two different anti-H-2K^k sera were used: AS116E was produced by priming (C57BL/6J x LP.RIII)F₁ mice with Bl0.A(2R) lymphoid cells, and AS508, by priming A.TL mice with A.AL cells. Antiserum 508 was used routinely in all rosetting assays and has a titre of 1/960 on Bl0.A spleen cells. It is highly specific for H-2.23, the private H-2K^k specificity, but has very weak activity for the public specificities H-2.3 and I1 (1/40). Antiserum 116E is specific only for the H-2.23 private specificity, and has a rosetting titre of 1/200 on Bl0.A spleen cells. When it was necessary to preabsorb antisera, 100 µl of undiluted serum was absorbed for 30 minutes at 4°C with 10⁸ spleen or thymus cells.

Preparation of Spleen Cell Suspensions

Spleen cell suspensions of high viability (85-95%) were prepared as described by Parish and McKenzie in 1978 in Eagle's minimal essential medium (F15, Grand Island Biological Co., Grand Island, N.Y.) containing 5% fetal calf serum (FCS). The cell suspensions were depleted of red cells and dead cells by centrifugation on a cushion of Isopaque/Ficoll (Davidson and Parish 1975). For alloantisera studies spleen cells were cleared of surface Ig by a capping procedure (Parish and McKenzie 1978). This procedure consists of incubating the cells (10⁷/ml) for 75 minutes at 37°C in F15/5% FCS containing 1 mg/ml of sheep IgG specific for mouse Ig. During incubation the mixture was gassed with 10% CO₂, 7% O₂ in N₂. Following Ig-capping the cells were washed twice with medium prior to use.

Rosetting Assay for Alloantibodies

The binding of alloantibodies to mouse spleen cells was detected by a rosetting procedure which was developed by Parish and McKenzie (1978). However, for this study, the procedure was adapted to microtitre plates in the following manner: 10 μ l of ice-cold, Ig-capped spleen cells (4×10^6 /ml in F15/5% FCS) were mixed with 10 μ l dilutions of antiserum in each well of a 96 well U-bottomed microtitre plate (Linbro Chemical Co., New Haven, Conn.) and incubated on ice for 30 minutes. Each well of the microtitre plate was then filled with 200 μ l of ice-cold medium, the cells sedimented by centrifugation for 1 minute at 4°C and the supernatant discarded by flicking the plate. The cells in each well were washed twice more with 200 μ l of ice-cold medium, 10 μ l of medium then added to each well and the cell pellet resuspended by vortexing the plate. 10 μ l of a 2% suspension of sheep erythrocytes coated, via CrCl_3 , with sheep anti-mouse Ig (Parish and McKenzie 1978) was added to each well and the red cell-lymphocyte mixture pelleted by centrifugation. The plates were then stored on ice for at least 15 minutes and, just prior to reading, each pellet was gently resuspended in its supernatant with a short pasteur pipette. Methyl violet staining solution (20 μ l) (Parish and McKenzie 1978) was then added to the wells, each sample transferred to a hemocytometer chamber and the percentage of rosette forming cells assessed.

Solubilisation of Spleen Cells

Spleen cells at a concentration of 2×10^8 cells/ml were solubilised in 0.5% (v/v) Nonidet P-40 (NP-40) in phosphate-buffered saline (PBS) containing 1.5 mM MgCl_2 and 10^{-3} M phenylmethylsulphonyl fluoride. This method, together with the procedure for removing NP-40 from a cell lysate is discussed in detail in the Appendix.

Immunoprecipitation of Antigens from Cell Lysates

The method for immunoprecipitating molecules from cell lysates using protein A-bearing *Staphylococcus aureus* (Cowan I strain) bacteria is fully outlined in the Appendix. Preparations were tested for complete removal of alloantibody used for immunoprecipitation by the rosetting assay, and subsequently for alloantigen content by the rosette inhibition assay.

Rosette Inhibition Assay

Serial dilutions of various NP-40 lysates of spleen cells were tested for their ability to inhibit the binding of a constant amount of antibody to spleen cells in the rosette inhibition assay which is discussed in detail in the Appendix. Briefly, 10 μ l serial dilutions of extract were preincubated with 10 μ l of a given amount of antiserum and the remaining activity was measured in the rosetting assay after the addition of 10 μ l of Ig capped B10.A spleen cells. In each experiment a control (unabsorbed) treatment was included, which consisted of serial dilutions of NP-40 lysate absorbed with medium rather than antiserum.

Antibody Blocking Assays

B10.A spleen cells (4×10^7) were incubated with 250 μ l of a saturating concentration of monoclonal antibody for 60 minutes at 4°C. The concentration of blocking antibody used was routinely ten-fold greater than the highest dilution of antiserum that gave maximum rosetting. After washing with medium, cells were resuspended in 40 μ l of medium, 20 μ l doubling dilutions were plated out in the microtitre plates, and 20 μ l of the appropriate antibody at the highest dilution which gave 80% rosette forming cells added to each well. After incubation for 30 minutes at 4°C the absorbing cells were pelleted by centrifugation

and 20 μ l of supernatant harvested from each well. The reactivity of the absorbed antiserum was then tested on B10.A spleen cells by the rosetting assay. In all experiments a control treatment was included that consisted of doubling dilutions of B10.A spleen cells that had not been preincubated (i.e. blocked) with monoclonal antibodies.

Immunofluorescence Procedures

Cocapping experiments involved the use of the double-labelled fluorescence method for detecting the differential redistribution of antigenic specificities. This method was originally developed by Lemonnier *et al.* (1975).

Ig-capped B10.A spleen cells were prepared as previously described, but were maintained in medium supplemented with cyclohexamide (100 μ g/ml). For absorption of the first antiserum 20 μ l of a saturating concentration of anti-H-2 serum was absorbed twice to 20 μ l of cells (10^5 cells/ml) in the wells of a microtitre plate. After washing thrice with medium, the cells were then absorbed twice for 30 minutes with 40 μ l TRITC-GAMIC (1/10) (10 mg/ml). The first absorption was done at 4°C, the second at 37°C to facilitate capping. After washing, the cells were capped in the presence of medium for a further 30 minutes at 37°C. Sodium Azide (0.1% w/v final concentration) was then added to the medium to prevent further capping of cells. The cells were absorbed twice at 4°C for 30 minutes with the second anti-H-2 serum and after washing, were absorbed with FITC-GAMIG (1/10) (10 mg/ml) for a further 30 minutes at 4°C. The cells were fixed with 100 μ l 1% paraformaldehyde in PBS and a drop of the cell suspension was air-dried on a slide and mounted in glycerol. The stained cells were scored using a Leitz Orthoplan microscope equipped with filters for fluorescein and rhodamine stains. Double-blind readings were made on

at least 300 cells. In all experiments involving capping with a first antiserum, 100% of the cells showed rhodamine stained caps. Following absorption of the second antiserum, the green staining FITC-GAMIG also entered these caps. Cells with bright granular staining were scored as 'intense', and those with diffuse green staining, as 'weak'.

Radioimmunoassay Using ^{125}I -Protein A

The methods for radioiodination of protein A, cell preparation and use of the reciprocal plot estimation method for estimating relative numbers of H-2 antigens expressed on spleen cells have been previously described in Chapters 3 and 4.

Briefly, the assay involves absorption of serial dilutions of antiserum to spleen cells, followed by washing and absorption of saturating amounts of ^{125}I -protein A to cell-bound antibody (Goding 1978, Chapter 3). Normal mouse serum was used as a control for non-specific binding. The relative amounts of H-2 antigen on different cell populations and the relative dissociation constants are estimated by plotting the reciprocal of bound ^{125}I -protein A (an approximation of the amount of antibody bound) against the reciprocal of antibody dilution (Chapters 3 and 4). The intercept on the Y-axis represents the reciprocal of the number of H-2 molecules to which antibody would bind at infinite antibody concentration, while the intercept on the X-axis is an estimate of the dissociation constant for the antibody-antigen interaction. The X-axis intercepts are known to be the same for cells of all strains tested with any of the antisera used herein (Chapter 4). Hence no X-axis values are given, and estimates of relative H-2 expression are presented only in terms of the number of counts per minute (cpm) bound.

RESULTS

Capping Studies with Monoclonal Antibodies

In order to determine the relationship between the H-2K^k molecules recognised by the two anti-H-2K^k monoclonal antibodies, 27R9 and 30R3, differential redistribution or cocapping studies were performed. The two monoclonal antibodies and an anti-H-2K^k serum (either 508 or 116E), which was highly specific for the H-2K^k molecule (see Materials and Methods), were each tested for their ability to cap off molecules specific for each of the other two antibodies. The results of one of three such experiments using antiserum 508 as the anti-H-2K^k serum are given in Table 2, but similar results were obtained in experiments using antiserum 116E.

Each of the anti-H-2K^k sera bound to all B10.A spleen cells since 100% of uncapped cells showed diffuse green fluorescence. When initially capped with antiserum 508, very few cells showed subsequent reactivity with either antiserum 508, or the two monoclonal antibodies, 27R9 and 30R3. This indicated that all molecules which react with 508 also carry 27R9 and 30R3 binding sites. However, following capping with either 27R9 or 30R3, molecules reactive with antiserum 508 still remained uncapped on 70-80% of cells. The uncapped molecules were not antigens controlled by some other region of the H-2 complex of B10.A mice as similar results were obtained following prior absorption of antiserum 508 with B10.AQR spleen cells (H-2 identical with B10.A except in the K region). Further evidence that 27R9 and 30R3 did not cap off all H-2K^k molecules was provided by the finding that after capping with one monoclonal antibody, approximately 70% of cells were diffusely stained with the other monoclonal antibody. In contrast, a combination of the two monoclonal antibodies, each added at the same concentration

as used individually, completely capped off all of the H-2K^k molecules recognised by conventional anti-H-2K^k alloantisera.

In all experiments there was approximately 20-30% cocapping of the antigens recognised by the two monoclonal antibodies which was more noticeable when 27R9 was the capping antibody. Similar results were also obtained in separate cocapping experiments using a rosetting method to measure antibody binding (results not shown). Anti-H-2D^d (AS954) serum was used as a control in all experiments. Capping with any of the H-2K^k sera did not cap off any H-2D^d molecules, while no H-2K^k molecules cocapped with H-2D^d determinants (results not shown).

Collectively, the capping studies described above indicate that monoclonal antibodies 27R9 and 30R3 predominantly bind to different H-2K^k molecules on the lymphocyte membrane.

Immunoprecipitation Studies with Solubilised H-2

Antigens

An immunoprecipitation assay was used to confirm that the mutual exclusiveness of the 27R9 and 30R3 binding sites seen in the cocapping experiments was a real phenomenon and not a unique feature of the capping assay. The procedure entailed immunoprecipitating the H-2K^k antigens recognised by one antiserum from an NP-40 lysate of B10.A spleen cells and then measuring in a rosette-inhibition assay the content of H-2K^k antigens remaining in the lysate which react with another antibody. A more detailed analysis of this method is presented in the Appendix.

A control (unabsorbed) B10.A lysate which was preincubated with medium rather than antibody, was highly effective at inhibiting the binding of antiserum 508, and monoclonal antibodies 27R9 and 30R3 to B10.A spleen cells (see Figure 1). When the lysate was preabsorbed with either antiserum 508 or a combination of the two monoclonal

antibodies 27R9 and 30R3, virtually all inhibitory activity for antiserum 508 was removed. Similarly, antiserum 508 very effectively cleared the lysate of the H-2K^k antigens recognised by monoclonal antibodies 27R9 and 30R3. In contrast, each monoclonal antibody could only partially remove the H-2K^k molecules in the NP-40 lysates recognised by antiserum 508, antibody 27R9 appearing to be slightly less effective than antibody 30R3 at absorbing these antigens. On the other hand, the monoclonal antibodies clearly reacted with different molecules in the NP-40 lysate, antibody 27R9 absorbing none of the H-2K^k antigens recognised by 30R3, and 30R3 removing none of the H-2K^k molecules detected by 27R9: a result which confirms the capping studies.

Blocking Studies with Monoclonal Antibodies

The spatial relationship between the 27R9 and 30R3 binding sites on the cell membrane was determined by measuring the ability of one monoclonal antibody to inhibit the subsequent absorption of the other antibody by B10.A spleen cells. The results of these blocking experiments are depicted in Figure 2.

Over the range of cell numbers used for absorption, cells which had been saturated with either 27R9 or 30R3, were unable to absorb any additional antibody of the same specificity. In contrast, cells saturated with antibody 27R9 were just as effective as unabsorbed cells at removing activity from a 30R3 antibody preparation. This result indicates that binding of antibody 27R9 to cells has no effect on the subsequent binding of 30R3. On the other hand, cells preabsorbed with 30R3 were less effective than unabsorbed cells at binding 27R9, suggesting that the binding of 30R3 masks to some extent, the 27R9 binding site. This 'one-way' blocking effect suggests a close association between the two H-2K^k molecules in the cell membrane.

Estimation of the Number of H-2K^k Molecules Detected by Monoclonal Antibodies 27R9 and 30R3

Since the data so far presented implies that monoclonal antibodies 27R9 and 30R3 recognise different H-2K^k molecules on the cell surface, it was important to determine the relative number of these molecules on the plasma membrane.

This information was obtained by using the ¹²⁵I-protein A radioimmunoassay (RIA) combined with a reciprocal plot estimation method which were previously described in Chapters 3 and 4, to make relative estimates of H-2 concentration on cells. Binding of ¹²⁵I-protein A was used as an indicator of the amount of antibody bound to cells. Figure 3A demonstrates the use of this method to show a difference in the binding of ¹²⁵I-protein A to B10.A(2R) spleen cells, preabsorbed with different anti-H-2K^k sera. Use of the reciprocal plot to demonstrate differences in the maximum number of H-2K^k molecules bound by each of the antisera is shown graphically in Figure 3B. This gives a two-fold difference in the Y-axis intercept of the reciprocal plot for the anti-H-2K^k serum, 116E, and each of the monoclonal antibodies, 27R9 and 30R3, indicating a two-fold difference in the estimated number of antibody binding sites, or H-2K^k molecules present on B10.A(2R) spleen cells.

Estimates of absolute numbers of H-2K^k molecules obtained by this method are 10^6 molecules using anti-H-2K^k sera and 5×10^5 using the monoclonal antibodies. This calculation assumes that one molecule of protein A binds to one IgG molecule. Although these values could be overestimates, they are in general agreement with other estimates for H-2 antigen expression on lymphoid cells (discussed in Chapters 3 and 4). Another feature of this assay is that both monoclonal antibodies

and the conventional alloantiserum had comparable binding constants as estimated from the X-axis intercept.

Over many experiments on four different homozygous strains of mice, both 27R9 and 30R3 appeared to bind to only half as many H-2K^k molecules as alloantisera 116E or 508. All results are summarised in Table 3. Although 27R9 and 30R3 are known to bind weakly to H-2D^k (Lemke *et al.* 1978), crossreactive binding on H-2^k strains did not appear to contribute to estimates for H-2K^k expression in this assay.

The results obtained in the two F₁ hybrids were particularly interesting. Firstly, a 3-fold rather than a 2-fold difference in H-2K^k expression was detected by the monoclonal antibodies compared with anti-H-2K^k sera, and secondly, there was also a discrepancy in the relative expression of H-2K^k in F₁ hybrids compared with parental cells, as detected by the different antisera. (The reduced expression of H-2 antigens in F₁ hybrids has already been discussed in Chapter 5.)

DISCUSSION

A new era in the analysis of antigen-antibody interactions was begun with the development of techniques to produce monoclonal antibodies by Köhler and Milstein in 1975. Since then, the value of monoclonal antibodies has become very apparent in many areas of immunology, and is reinforced by the results in this report in which monoclonal antibodies against H-2 antigens have revealed new complexity in an antigenic system already thought to be rigorously defined by heterogeneous anti-H-2 sera.

This work is the first demonstration of the existence of two classes of H-2K^k molecules encoded by the K region and the results were confirmed by three different and complementary experimental approaches. Cocapping experiments revealed the existence of at least

two H-2K^k variant molecules, defined both by anti-H-2K^k serum specific for the H-2.23 private specificity and either of the monoclonal antibodies 27R9 (H-2.25) and 30R3 (H-2.5). Mutual exclusiveness between these molecules was evident both by their inability to cocap and because they absorbed out different H-2K^k molecules from an NP-40 solubilised extract of cells. Since a combination of the two monoclonal antibodies either completely capped off, or absorbed out, all H-2K^k molecules detectable with the anti-H-2K^k sera, this suggested that all H-2K^k molecules (H-2.23) carried at least one of the 27R9 (H-2.25) or 30R3 (H-2.5) binding sites. The H-2 estimation results were consistent with this finding in that the sum of 27R9- and 30R3-defined molecules was equal to the total number of H-2K^k molecules detectable with an anti-H-2K^k (H-2.23) serum. In this respect, F₁ hybrids were an exception, in that the number of molecules detectable with the conventional anti-H-2K^k serum was greater than the sum of those detectable with each of the monoclonal antibodies. This is suggestive of a larger number of H-2K^k variant molecules in F₁ hybrids, and perhaps also the existence of F₁ hybrid specific H-2K and H-2D antigens, a phenomenon already considered by several workers (Ishikawa and Dutton 1979, Chapter 3). The possibility that further subpopulations of H-2K^k molecules exist in homozygotes within the 27R9- and 30R3-defined classes cannot yet be excluded until studies are completed using other monoclonal antibodies.

Results published recently by Lemke *et al.* (1979) conform with our findings. In binding inhibition studies on CBA/H spleen cells using these same two monoclonal antibodies, they showed that the binding of one monoclonal antibody did not interfere with subsequent binding of the second. They interpreted their result to mean that the two antigenic

determinants recognised by these antibodies were far apart, but on the same molecule, whereas evidence here demonstrates that they are, in fact, on separate molecules. While their results agree with the general finding, they did not detect the one-way blocking effect of 30R3 for 27R9 binding, but this could be due to differences in the two assays. Such results suggest a close spatial relationship between the two different molecules. Furthermore, evidence from two different sources supports the notion that there may be some specific association between these two molecules. Firstly, such an association could explain the small degree of cocapping seen in Table 1, where 30R3 molecules tend to cocap weakly with molecules bound by 27R9 and vice versa. Secondly, in the initial attempts to define experimental conditions required for preabsorption of H-2K^k molecules from NP-40 lysates of cells, there also appeared to be a degree of specific binding between the two different molecules. In order to show complete identity between the H-2K^k molecules which bound to each monoclonal antibody, it was necessary to preabsorb lysates immediately after cell dissociation, and in the presence of 0.5% NP-40. The two different molecules tended to aggregate with time and when NP-40 was removed from the preparation.

The existence of two classes of K region coded H-2 molecules complements the molecular complexity already defined at the D region. Heterogeneity amongst D-region molecules carrying certain public specificities has already been well documented with the definition of H-2D and H-2L molecules in many different haplotypes (Démant and Néauport-Sautés 1978, Demant *et al.* 1979). Recent preliminary results published by Iványi and Démant (1979) would seem to suggest that the existence of different H-2K^k molecules bearing the private specificity

might also have a counterpart at the *D* region. They claim to have found heterogeneity amongst H-2D^d molecules carrying the H-2.4 private specificity, and as a result have defined the 'H-2M' molecule which lacks the H-2.28 public specificity carried by the H-2D and H-2L molecules.

Currently, three possible explanations are under consideration for the existence of two classes of H-2K^k molecules. 1. The two molecules could be products of different H-2K^k structural genes as has been postulated for H-2D and H-2L with the definition of the *L* locus distal to *D* (Démant and Néauport-Sautés 1978). Only recombination data will verify this proposition. 2. Variant forms of H-2 molecules may arise by posttranslational modification of a primary gene product. For example, posttranslational modification or glycosylation could lead to minor differences in the tertiary structure of the H-2 glycoprotein. 3. The two classes of H-2K^k molecules could be chemically very different. The existence of both glycolipid and glycoprotein Ia molecules both of which map to the *I*-region, has been shown by Parish, McKenzie and coworkers (see review by Parish and McKenzie 1980), and has recently been demonstrated very conclusively with the use of monoclonal antibodies (Higgins *et al.* 1980), but so far, there is neither evidence nor precedence for the existence of a glycolipid H-2K or H-2D molecule. The latter two possibilities are currently under investigation.

At a late stage in the preparation of this chapter, the existence of more than one class of H-2K^k molecule was confirmed in preliminary experiments using a third H-2K^k monoclonal antibody. Whatever the explanation for heterogeneity amongst H-2K^k antigens, the role of these antigens in T cell recognition, and the mechanism of

thymic processing (Zinkernagel 1978) in establishing self tolerance portends to be much more complicated as a result of these findings.

SUMMARY

Studies described in this paper indicate that two anti-H-2K^k monoclonal antibodies, namely 27R9 (H-2.25) and 30R3 (H-2.5) recognise different H-2K^k molecules on the surface of lymphocytes. Initial experiments in support of this conclusion were cocapping experiments which showed mutual exclusiveness between H-2K^k molecules which bind either of the two monoclonal antibodies 27R9 (H-2.25) or 30R3 (H-2.5) whereas conventional anti-H-2K^k (H-2.23) alloantiserum binds to both types of H-2 molecules. This result was confirmed by experiments using solubilised H-2 antigen preparations to inhibit antibody binding to spleen cells. Preabsorption of the preparation with one monoclonal antibody did not remove its inhibitory activity for the other monoclonal antibody, and only partially removed its inhibitory activity for the conventional anti-H-2K^k serum. These results suggest that at least two antigenically distinct H-2K^k molecules are controlled by the H-2K region. Subsequent blocking studies have indicated that the two different molecules are associated, to some extent, in the cell membrane. Furthermore, in an ¹²⁵I-protein A radioimmunoassay, each monoclonal antibody was found to bind to only half of the estimated total number of H-2K^k molecules recognised by conventional anti-H-2K^k sera. Several interpretations for the existence of the two classes of H-2K^k molecules are discussed.

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Fig. 1 Inhibition of binding of different anti-K^k antibodies to B10.A spleen cells by NP-40 lysates of B10.A splenocytes. Each graph is headed with the anti-K^k antibody being inhibited. The lysate used for inhibition was either unabsorbed (closed symbols) or immunoprecipitated with 27R9 (○), 30R3 (□), 27R9 + 30R3 (■) or 508 (△) antibodies prior to addition to the assay. The dotted line represents antibody activity in the absence of inhibition, antibody binding being measured by a rosetting assay.

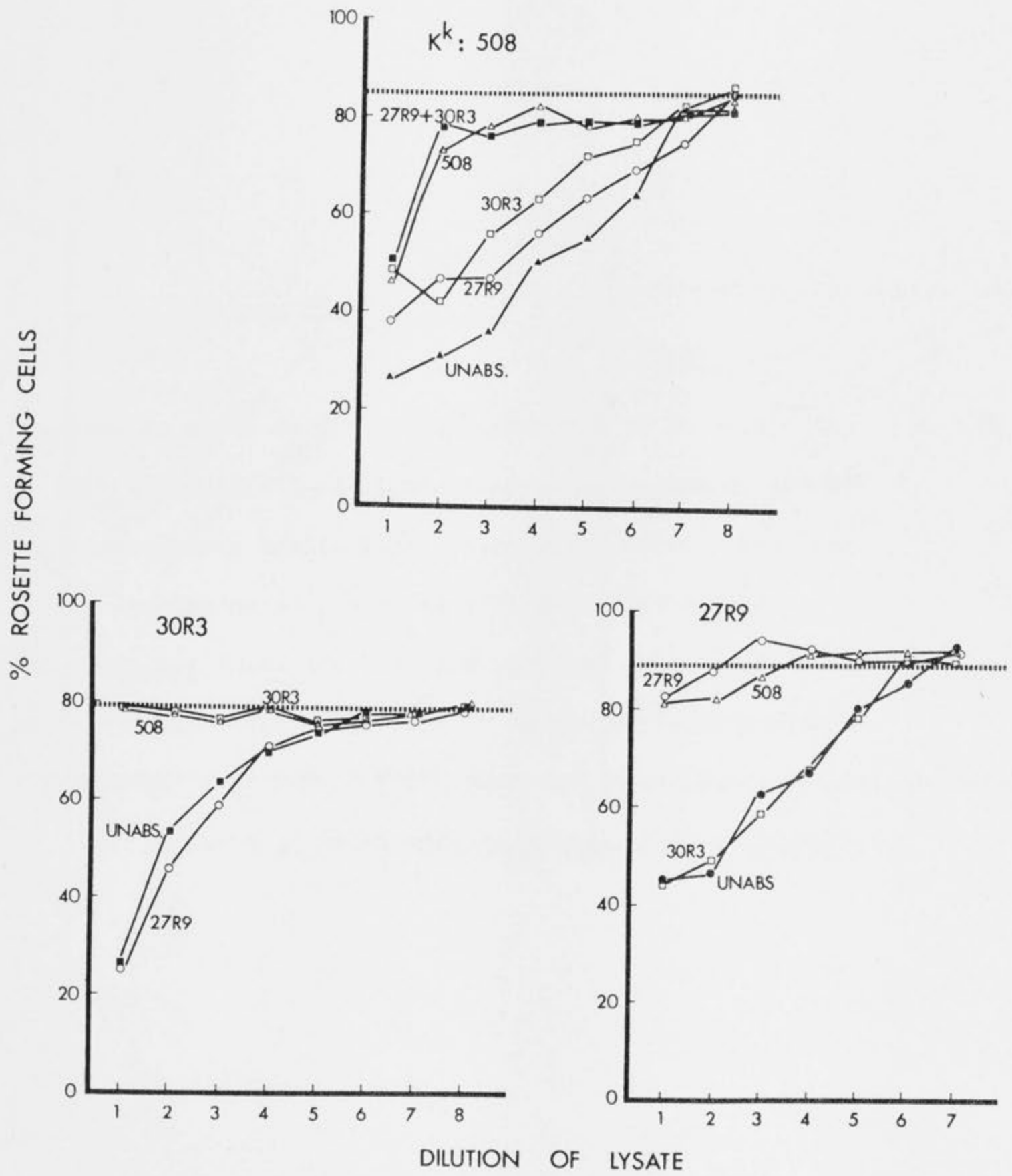


Fig. 2 Inhibition of antibody binding to spleen cells as measured by the rosetting assay using monoclonal antibodies 27R9 and 30R3. B10.A spleen cells were either untreated (●) or saturated with 27R9 (○) or 30R3 (Δ) monoclonal antibodies prior to being measured for their ability to absorb a constant amount of 27R9 (left hand graph) or 30R3 (right hand graph) antibody. The dotted line represents antibody activity prior to absorption with cells.

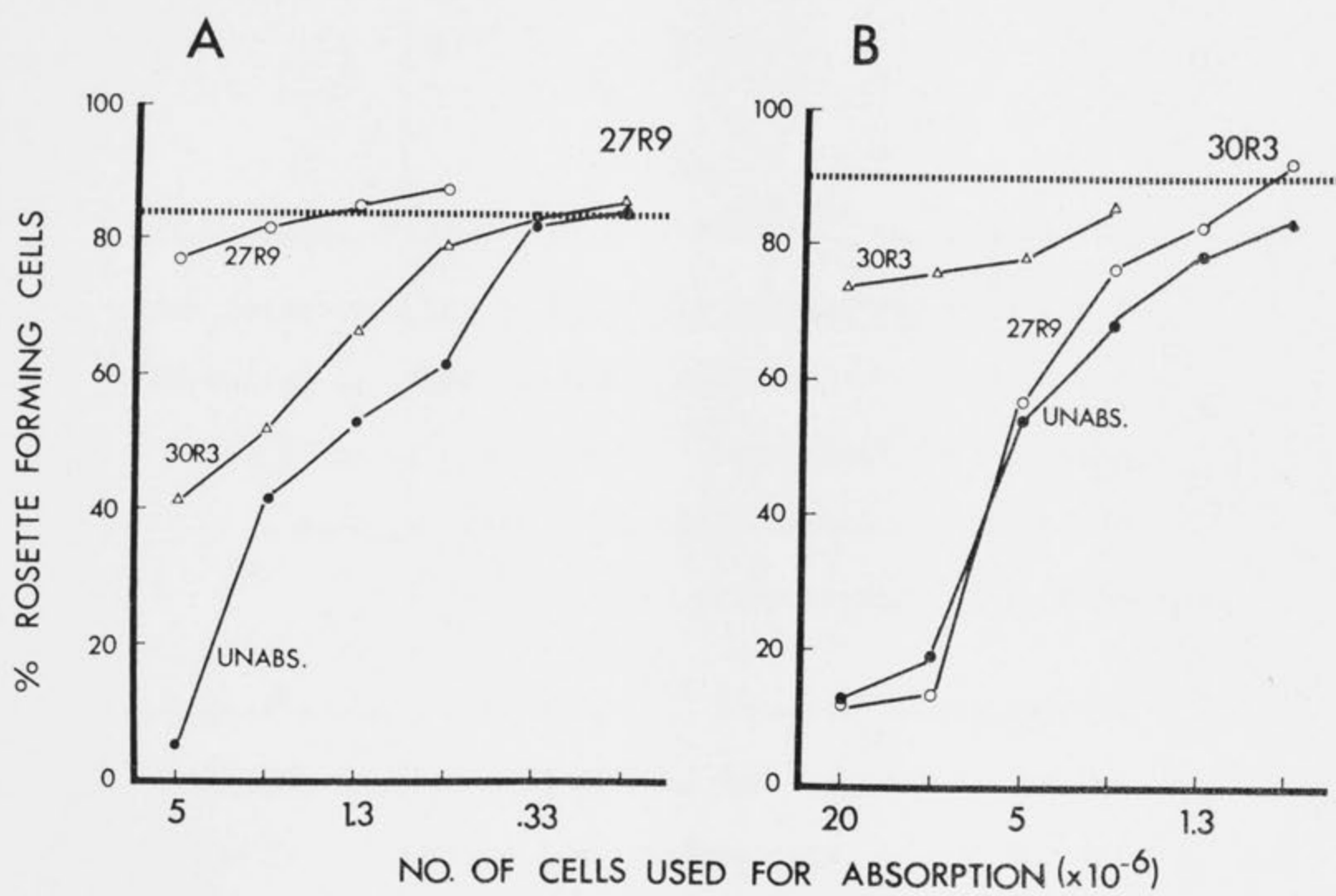
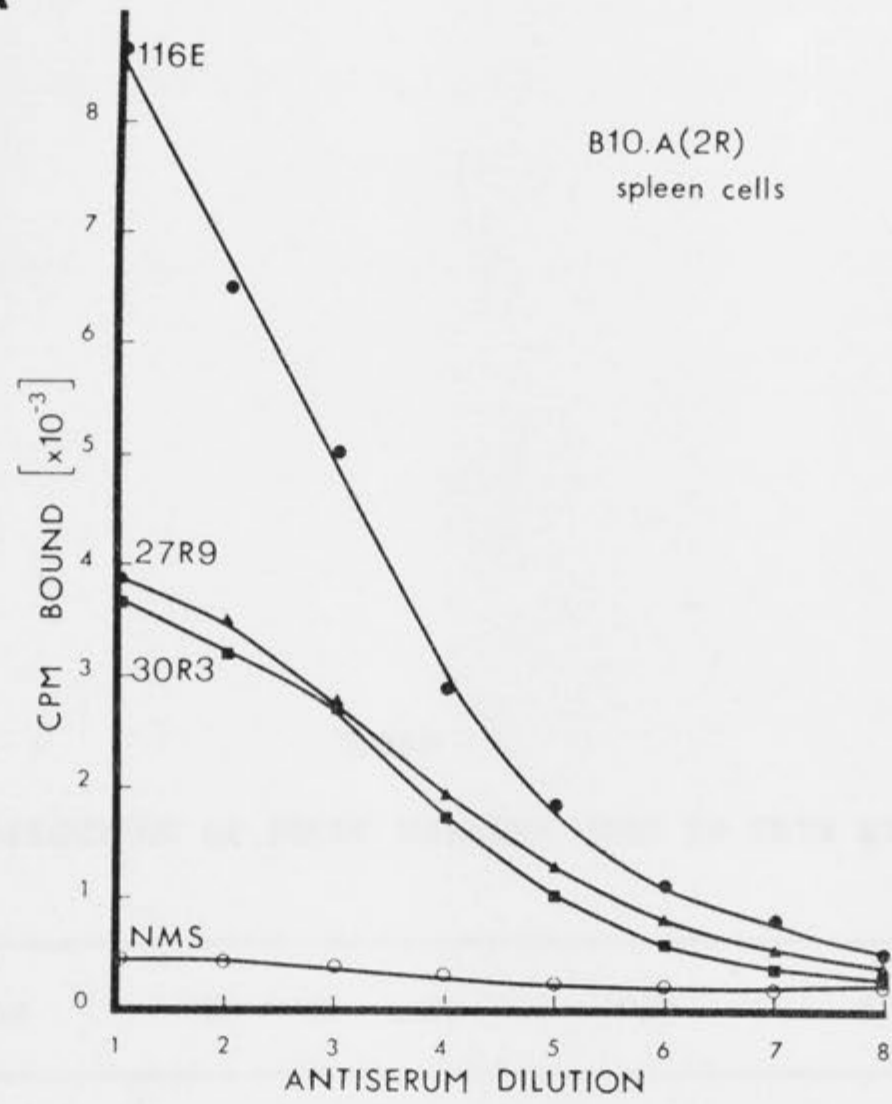


Fig. 3 A: Binding of ^{125}I -protein A to B10.A(2R) spleen cells pretreated with serial dilutions of the monoclonal antibodies 27R9 (\blacktriangle) and 30R3 (\blacksquare) and 116E, an anti-H-2K^k serum (\bullet). Binding of ^{125}I -protein A to cells preabsorbed with normal mouse serum (\circ) was used as a control for non-specific binding.

B: Reciprocal plots of ^{125}I -protein A binding versus antibody dilution. The line of best fit was plotted through points representing the linear region of the binding curves in Figure 3A.

A



B

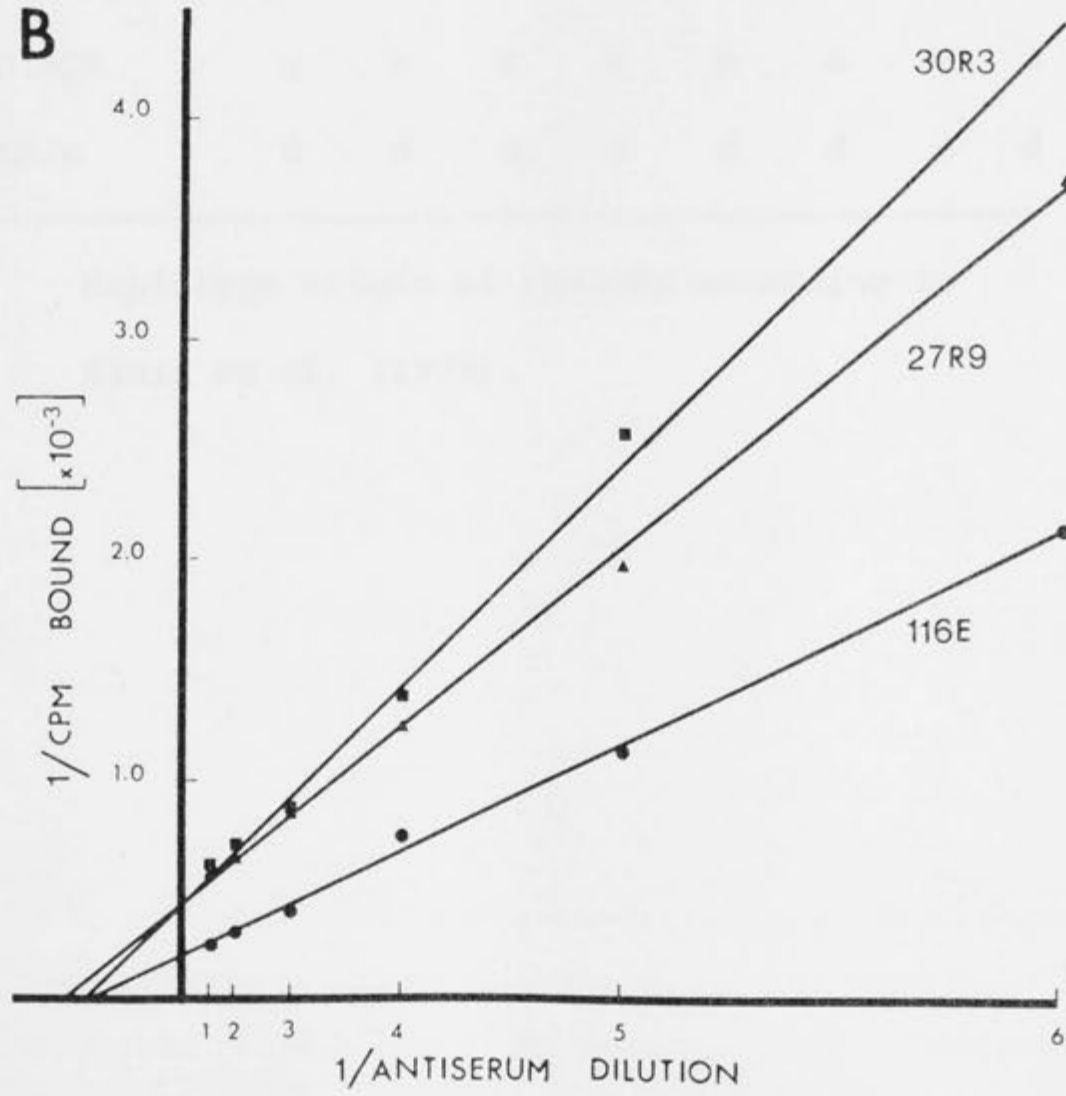


TABLE 1

H-2 HAPLOTYPES OF MOUSE STRAINS USED IN THIS STUDY

Strains	K	A	B	J	E	C	S	D
B10.BR, CBA/H	k ^a	k	k	k	k	k	k	k
B10.A	k	k	k	k	k	d	d	d
B10.A(2R)	k	k	k	k	k	d	d	b
B10.A(5R)	b	b	b	k	k	d	d	d
B10.AQR	q	k	k	k	k	d	d	d
BALB/c	d	d	d	d	d	d	d	d

a Haplotype origin of regions according to Klein *et al.* (1978).

DISTRIBUTION OF H-2K^k MOLECULES ON B10.A SPLEEN CELLS
AFTER CAPPING WITH CONVENTIONAL AND MONOCLONAL ANTI-K^k
ANTIBODIES

First treatment followed by TRITC-GAMIG	Second treatment followed by FITC-GAMIG	% cells with FITC labeling outside TRITC-labeled caps ^a		
		Null	Weak	Intense ^b
Medium	Medium	0	0	0
	27R9	0	70	30
	30R3	0	81	19
	508	0	70	30
	954	0	68	32
	508 Absorbed ^c	0	46	54
508(H-2.23)	Medium	100	0	0
	27R9	83	14	3
	30R3	86	12	2
	508	93	6	1
	954	12	77	11
27R9(H-2.25)	Medium	100	0	0
	27R9	87	13	0
	30R3	34	59	7
	508	17	47	36
	954	11	38	51
	508 Absorbed ^c	0	49	51
30R3(H-2.5)	Medium	100	0	0
	27R9	23	67	10
	30R3	88	12	0
	508	20	61	19
	954	6	47	47
	508 Absorbed ^c	18	80	2
27R9 + 30R3	Medium	100	0	0
	27R9	92	7	1
	30R3	84	14	2
	508	82	17	1
	954	10	75	15

a Values are taken from counts made on up to 500 individual cells.

b Fluorescence was scored as either 'null', 'weak' (faint, diffuse staining), or 'intense' (bright, granular staining).

c Anti-H-2K^k (AS508) was absorbed with B10.AQR spleen cells.

TABLE 3

ESTIMATES OF H-2K^k EXPRESSION ON SPLEEN CELLS USING DIFFERENT ANTI-H-2K^k SERA

Strain	Antiserum:	Relative Number of H-2K ^k Molecules ^a		
		30R3	27R9	508/116E ^c
CBA/H		2.4 ± 0.1(5) ^b	2.6 ± 0.3(3)	4.9 ± 0.1(3)
B10.BR		2.2(1)	2.3(1)	4.4(1)
B10.A		2.2 ± 0.2(2)	2.3 ± 0(2)	4.4(1)
B10.A(2R)		2.2 ± 0.1(4)	2.1 ± 0.1(7)	4.3 ± 0.2(7)
(CBA/H x BALB/c)F ₁		1.0 ± 0(3)	1.1 ± 0.1(2)	3.3 ± 0(2)
[B10.A(5R) x B10.A(2R)]F ₁		0.9 ± 0(3)	0.9 ± 0(5)	3.3 ± 0(2)

a Values in the table are estimates of maximum ¹²⁵I-protein A (cpm x 10⁻³) bound by 2 x 10⁶ spleen cells following absorption with an infinite amount of antiserum.

b Estimates are means ± S.E., sample size bracketed.

c Estimates obtained with the two different antisera were not significantly different and were therefore pooled.

CHAPTER 7

MONOCLONAL ANTIBODY DETECTION OF
CARBOHYDRATE-DEFINED AND PROTEIN-DEFINED H-2K^k ANTIGENS

In the previous chapter, evidence was presented for the existence of two antigenically distinct H-2K^k antigens defined by monoclonal antibodies. The studies reported here have shown that H-2K^k molecules expressed on the cell surface are the following: those possibly representing the antigen.

(1) The two antigens are products of two different structural genes in the D region of the H-2 complex: the H-2K and H-2L with the assistance of the D region (Gallatly and Macgregor, 1970).

INTRODUCTION

The major histocompatibility complex (*MHC*) in the mouse, the *H-2* complex, contains genes controlling the expression of many cell surface antigens involved in cellular interactions (reviewed by Klein 1975). The most widely studied are the *K*, *D* and *Ia* antigens, the gene products of the *K*, *D* and *I* regions of the *MHC*. Genetic studies using recombinant inbred strains of mice have shown that these antigens play a unique role in self-recognition processes by lymphocytes (reviewed by Snell 1978, Benacerraf and Germain 1978), but despite intensive speculation, their exact functional role is unknown. Glycoprotein *H-2* and *Ia* molecules have been well characterized by immunoprecipitation studies using highly specific antisera (Cullen *et al.* 1976, Vitetta and Capra 1978). However, recent evidence for the existence of glycolipid molecules carrying carbohydrate-defined *Ia* antigens has been viewed with scepticism despite the potential functional implications inherent in a system of biochemically different molecules carrying antigenic determinants controlled by the same gene (Parish *et al.* 1978, Parish and McKenzie 1980, Higgins *et al.* 1980).

In the previous chapter, evidence was presented for the existence of two antigenically distinct $H-2K^k$ molecules defined by monoclonal antibodies. This finding suggested heterogeneity amongst $H-2K^k$ molecules expressed on the cell surface and the following three possible interpretations were proposed.

(i) The two molecules are products of two different structural genes in the *K* region as has been postulated for *H-2D* and *H-2L* with the definition of the *L* locus (Démant and Néauport-Sautés 1978).

(ii) Heterogeneity of $H-2K^k$ gene products arises by some process of posttranslational modification or, finally,

(iii) the two molecules are biochemically very different. To investigate the latter two possibilities, the biochemical nature of the $H-2K^k$ molecules detected by three different monoclonal antibodies have been examined, and evidence is presented for the existence of carbohydrate-defined $H-2K^k$ antigens. The evidence favours the existence of two biochemically different $H-2K^k$ molecules which map to the K region; one appears to be a glycoprotein and the other a glycolipid, analogous to the system already described for Ia antigens (Parish *et al.* 1978, Parish and McKenzie 1980, Higgins *et al.* 1980).

MATERIALS AND METHODS

Animals

Mice were bred in the John Curtin School of Medical Research and were used as spleen cell donors when 6-12 weeks old. Male and female mice were used interchangeably in all experiments.

Antisera

Monoclonal antibodies 27R9 and 30R3 have been described in detail by Lemke *et al.* (1978, 1979) and Hämmerling *et al.* (1979). They were obtained from Dr G.J. Hämmerling (Institute for Genetics, University of Cologne, Cologne, FRG), as freeze dried ascitic fluid, produced in mice by hybridomas derived from fusion of the myeloma P3-X63-Ag8 and spleen cells obtained from BALB/c ($H-2^d$) mice immunised against CBA/H ($H-2^k$) spleen cells. 27R9 appears to detect H-2.25, and is largely specific for $H-2K^k$. 30R3 reacts strongly with $H-2K^k$, but cross-reacts on cells of other haplotypes. It appears to detect the H-2.5 public specificity. Two different preparations of antisera were

used and gave similar results in all experiments described herein, when tested on B10.A (K^k, D^d) targets. By a rosetting assay, the two preparations of 27R9 had titres of 1/16,000 and 1/2,000,000, and the two batches of 30R3 had titres of 1/8,000 and 1/50,000.

Monoclonal antibody 11-4 was purchased from Becton Dickson (Mountain View, Ca.) as mouse ascitic fluid. It was derived from a hybridisation of mouse NS-1 myeloma cells with spleen cells from BALB/c ($H-2^d$) mice immunised with CKB ($H-2^k$) spleen cells (Oi *et al.* 1978). Although it reacts strongly with $H-2K^k$, and like 30R3, crossreacts on cells of several other haplotypes, its reactivity maps to no known alloantibody defined-H-2 specificity. Two preparations were used in these experiments that had titres of 1/5,000 and 1/12,000 measured in the rosetting assay on B10.A (K^k, D^d) targets.

In all experiments involving monoclonal antibodies, B10.A (K^k, D^d) spleen cells were used to avoid crossreactive binding to any other cell surface antigen besides the $H-2K^k$ molecule. Since 11-4 and 27R9 are IgG_{2a} and 30R3 is IgG_{2b} , all bind protein A (Goding 1978).

Preparation of Spleen Cell Suspensions

Spleen cell suspensions of high viability (85-95%) were prepared as previously described (Chapter 6) in Eagle's minimal essential medium F15 (Grand Island Biological Co., Grand Island, N.Y.) containing 5% fetal calf serum (FCS). The cell suspensions were depleted of red cells and dead cells by centrifugation on a cushion of Isopaque/Ficoll (Davidson and Parish 1975). For alloantisera studies spleen cells were cleared of surface Ig by a capping procedure (Parish and McKenzie 1978). Briefly, this procedure consists of incubating the cells (10^7 /ml) for 75 minutes at 37°C in F15/5% FCS containing 1 mg/ml of sheep IgG specific for mouse Ig, followed by two washes with medium.

Rosetting Assay for Alloantibodies

The binding of alloantibodies to mouse spleen cells was detected by a rosetting procedure which has been described in detail in Chapter 6 (Parish and McKenzie 1978). Briefly, 10 μ l of ice cold Ig-capped spleen cells (4×10^6 /ml in F15/5% FCS) were absorbed for 30 minutes with 10 μ l doubling dilutions of antiserum in microtitre plates (Linbro Chemical Co., New Haven, Conn.) After washing with medium 10 μ l of a 2% suspension of sheep erythrocytes coated, via CrCl_3 , with sheep anti-mouse Ig was added and the mixture centrifuged gently to form rosettes. Methyl violet staining solution was used to determine the percentage of rosette forming cells (RFC).

Solubilisation of Spleen Cells

Spleen cells at a concentration of 2×10^8 cells/ml were solubilised in either 0.5% (v/v) Nonidet P-40 (NP-40) or 25mM sodium cholate (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) containing 1.5 mM MgCl_2 and 10^{-3} M phenylmethylsulphonyl fluoride. This method, together with the procedure for removing NP-40 from cell lysates is outlined in the Appendix. Sodium cholate was removed from cell lysates by extensive dialysis according to the method outlined by Higgins *et al.* (1980).

Enzyme Treatments

Both intact lymphoid cells and solubilised cell preparations were treated with various enzymes. Cells were depleted of red and dead cells and were cleared of endogenous surface Ig by capping (see above) prior to treatment. They were resuspended in F15/0.1% (w/v) sodium azide at a concentration of 4×10^6 /ml, gassed to the appropriate pH using either 10% CO_2 /7% O_2 in N_2 or 95% CO_2 in air and were incubated at 37°C for 30 minutes in the presence of enzyme. They were washed twice in ice cold

medium before use in the rosetting assay. Cell lysates (2×10^8 cell equivalents/ml) were dialysed for 2 hours against PBS before treatment with enzyme. The lysate-enzyme mixture was gassed to the appropriate pH and incubated for 24 hours at 37°C in the presence of 0.1% (w/v) sodium azide. Enzyme activity was stopped by the addition of medium containing FCS to cells and by the maintenance of the lysate at 4°C when it was used in the rosette-inhibition assay.

The following conditions were employed for the various enzyme treatments. Cell lysates were treated with either pronase (Calbiochem, B Grade, San Diego, Ca.), at a concentration of 1 mg/ml and a pH of 7.3, or with mixed glycosidases (Mills Laboratories Inc., Kankakee, Il.) at a concentration of 200 µg /ml and a pH of 6.5 supplemented with 10 units/ml of neuraminidase (NA) (*Vibrio cholera*, B grade, Calbiochem., San Diego, Ca.). For treatment of cells, NA was used at a concentration of 10 units/ml and cells were maintained at pH 6.0 throughout the incubation. When cells were treated with α-mannosidase (Sigma Chemical Co., St. Louis, Mo.) they were gassed to pH 7.0 in the presence of 0.8 units of enzyme per ml. For α-galactosidase (Sigma Chemical Co., St. Louis, Mo.) treatment, cells were incubated at pH 7.0 in the presence of 0.3 units/ml of enzyme while β-galactosidase (Sigma Chemical Co., St. Louis, Mo.) treatment involved 10 units/ml, again at pH 7.0. When cells were treated with mixed glycosidases, a concentration of 100 µg/ml was used, supplemented with 10 units/ml NA and cells were maintained at pH 6.5. For all treatments, control (untreated) preparations were always included.

Immunoprecipitation of Antigens from Cell Lysates

The method of immunoprecipitating molecules from cell lysates has been described in detail in the Appendix. Briefly, lysates were absorbed

with antiserum, and then antibody and antigen-antibody complexes were cleared from the lysates by protein A-bearing *Staphylococcus aureus* (Cowan I strain) bacteria. Prior to use, lysates were depleted of NP-40 using XAD-8 resin as described in the Appendix, and tested for complete removal of alloantibody by the rosetting assay. Subsequently, alloantigen content was measured in the rosette-inhibition assay.

Preparation of Glycolipid Extracts

The method for extraction of the glycolipids from spleen cells and serum was developed by Higgins and Parish (1980). Briefly, the glycolipid components of serum or erythrocyte-free spleen cells were obtained by extracting vigorously twice with buffered pyridine and removing insoluble material by centrifugation. The extracted material was dried and then partitioned into aqueous and organic solvent soluble components using the Folch procedure (Folch *et al.* 1957). The aqueous phase which contains glycolipids was taken to dryness then solubilised in F15 containing 5% FCS at a concentration of 4×10^8 cells/ml for cell extracts or 75% of the starting volume for serum extracts. The extracts have been shown to have high levels of both carbohydrate and lipid components (i.e. sphingosine, neutral hexose and sialic acid), yet contain no detectable protein (Higgins and Parish 1980).

Rosette Inhibition Assays

Serial dilutions of various extracts of spleen cells and serum were tested for their ability to inhibit the binding of a constant amount of alloantibody to spleen cells in the rosette inhibition assay which is also described in the Appendix. Briefly, 10 μ l serial dilutions of extract were preincubated with 10 μ l of a given amount of antiserum and the remaining activity was measured in the rosetting assay after the addition of 10 μ l of Ig capped B10.A spleen cells.

Extracts preincubated with medium rather than antiserum were included as controls.

Sugar Inhibition Studies

Antibodies were tested for sugar inhibition using greater than 30 mono-, di-, and oligosaccharides as previously described (Higgins *et al.* 1980, McKenzie *et al.* 1977a). For these experiments 10 μ l of each sugar (20 mg/ml in PBS) was added to 10 μ l of antiserum in microtitre plates and the mixtures incubated on ice for 60 minutes. 10 μ l of Ig-capped spleen cells were then added to each well and the rosetting assay performed as described above.

RESULTS

Susceptibility of Solubilised H-2 Antigens to Pronase and Mixed Glycosidase

Initially the three monoclonal anti-H-2K^k antibodies 27R9, 30R3 and 11-4 were tested for their ability to interact with protease-susceptible or glycosidase-susceptible antigenic determinants. For these experiments, solubilised lysates of B10.A spleen cells which had been pretreated with either pronase or mixed glycosidases were compared with untreated lysates in a rosette inhibition assay for their ability to inhibit the binding of a given amount of each monoclonal antibody to B10.A target cells. Pronase treatment should destroy any protein-defined H-2 antigens in lysates while mixed glycosidases should selectively degrade carbohydrate moieties. Both NP-40 and sodium cholate solubilised preparations of spleen cells gave similar results in these experiments, despite the fact that low molecular weight material generated by enzyme treatment is removed only from the sodium cholate preparation by the dialysis step.

The results in Figure 1 show a clear difference in the pronase sensitivity of the antigenic determinants recognised by the monoclonal antibodies. While pronase treatment of a sodium cholate lysate of B10.A spleen cells completely eliminated the 27R9 inhibitory activity, it did not affect those determinants to which 11-4 and 30R3 bound, since the treated lysate was just as inhibitory as the untreated one for these two antibodies. In contrast, the results in Figure 2 show that mixed glycosidase treatment of an NP-40 lysate of B10.A spleen cells removed approximately 97% of the inhibitory activity for the 30R3 and 11-4 antibodies, but had no effect on the inhibition of 27R9. Taken together, these results suggest that the 27R9 antibody recognises a protein determinant, while both the 30R3 and 11-4 antibodies bind to carbohydrate determinants.

Susceptibility of Cell Bound H-2 Antigens to Specific Glycosidase

Treatments

Since glycosidases cleave terminal sugar residues from carbohydrates, while having no direct effect on proteins, treatment of cells with these enzymes was used to confirm the protein or carbohydrate nature of the antigenic determinants recognised by each of the monoclonal antibodies. The effect of each enzyme was assessed by measuring the reduction in antibody titre resulting from treatment of B10.A spleen cells. While the binding of the 27R9 antibody to cells was unaffected by any of the enzyme treatments, the antigens recognised by the 30R3 and 11-4 antibodies were susceptible to certain glycosidases, a result consistent with these monoclonal antibodies distinguishing between protein and carbohydrate H-2K^k antigens. Titration curves showing the extent of these effects are depicted in Figures 3, 4 and 5, and a complete summary of the results is given in Table 1.

Only antigens recognised by the 30R3 and 11-4 antibodies on B10.A spleen cells were susceptible to treatment with mixed glycosidases (see Figure 3) as were the same antigens in a solubilised preparation of cells (see previous section). There was approximately an 8-fold reduction in the titre of both 30R3 and 11-4 antibodies on the treated cells. In similar experiments using NA to treat target cells (see Figure 4) only the binding of the 11-4 antibody was affected by enzyme treatment, while the binding of 27R9 and 30R3 was unaffected. Since NA is known to specifically cleave the terminal sialic acid residues of oligosaccharide chains, this implies that a sialic acid residue contributes to the determinant recognised by 11-4. Several other glycosidases were found to influence, in a selective manner, the antigens recognised by the 30R3 and 11-4 antibodies. Firstly, α -mannosidase treatment drastically reduced the binding of both 30R3 and 11-4 antibodies to target cells (see Figure 5), suggesting involvement of an α -linked D-mannose residue in the determinants recognised by both antibodies. Both α -galactosidase and β -galactosidase treatments reduced the titres of 11-4 (see Figure 5) suggesting that terminal D-galactose units in both α - and β -linkage participate in this antigen. The specificity of these enzymes was confirmed by blocking their effects with appropriate sugar substrates (data not shown). Thus the effect of α -mannosidase on B10.A spleen cells was completely blocked by the addition of D-mannose or mannan (a polymer of D-mannose) to the incubation mixture (20 mg/ml). Similarly, the effect of α -galactosidase was completely removed by the addition of lactose (20 mg/ml) and the effect of α -galactosidase was largely overcome by the addition of melibiose in high concentrations (80 mg/ml). Furthermore, the effect of a particular enzyme treatment

was not due to contaminating glycosidases since the effects observed were only abrogated by the appropriate specific sugar.

It should be noted that those glycosidases which had an effect removed 80-90% of the antigens detected by the 30R3 and 11-4 antibodies. No greater loss of 11-4 antigens was obtained by treating cells with various mixtures of NA and α - and β -galactosidases.

Sugar Inhibition Studies

Hapten inhibition studies using mono-, di-, and oligosaccharides have been successively used to partially characterise carbohydrate Ia antigens (Higgins *et al.* 1980, McKenzie *et al.* 1977a). Using this same approach, with the same thirty haptens as previously used by McKenzie *et al.* (1977), the binding of two of the three antibodies was found to be inhibited by certain simple sugars. The positive inhibition results are shown in Figure 6. 27R9 was not inhibited by any of the sugars tested, a result consistent with the protein nature of the antigen recognised by 27R9. On the other hand, the binding of 30R3 and 11-4 to B10.A spleen cells was inhibited by at least one hapten, providing further evidence for the involvement of sugar units in the antigenic determinants which these antibodies recognise. In these experiments, D-glucose was used as a control for osmotic effects, but the same antibody titres were obtained in its absence (results not shown).

Since the binding of 30R3 to B10.A spleen cells was inhibited by D-mannose and no other hapten, this result, in combination with the sensitivity of the determinant to α -mannosidase, suggests that the immunodominant sugar of this antigen is D-mannose. In contrast, the binding of 11-4 was inhibited by both lactose [D-galactose (β 1 \rightarrow 4)-D-glucose] and N-acetyl-neuraminic acid. Coupled with the lack of inhibition by D-galactose, this result suggests that the determinant

recognised by 11-4 is comprised of at least a β -linked D-galactose and a sialic acid unit. This result is also consistent with the effects of β -galactosidase and neuraminidase on the 11-4-defined antigen. Although α -mannosidase and α -galactosidase are known to substantially alter this antigen, neither D-mannose nor melibiose [D-galactose (α 1 \rightarrow 6)-D-glucose] were effective inhibitors of 11-4 binding. This discrepancy, however, merely implies that free D-mannose and melibiose do not closely mimic the orientation of α -linked D-mannose and α -linked D-galactose in the 11-4 determinant.

Inhibition Studies with Glycolipid Extracts

The data in the preceding sections suggest that monoclonal antibodies 30R3 and 11-4 recognise carbohydrate H-2K^k antigens. Since a system of carbohydrate-defined Ia antigens has already been described that are expressed as glycolipids on cells and in serum (Parish *et al.* 1978, Parish and McKenzie 1980) it was important to determine whether the H-2K^k antigens were also glycolipid in nature.

Preparations of glycolipids were obtained from cells and serum of various strains of mice using organic solvent extraction procedures (Higgins and Parish 1980). Such preparations would be expected to inhibit antibodies reacting against glycolipid H-2 determinants but not protein H-2 antigens. Extracts of spleen cells from several strains of mice and of serum from CBA/H (H-2^k) mice were tested for their ability to inhibit the binding of the three different monoclonal antibodies 27R9, 30R3 and 11-4 to B10.A spleen cells in the rosette inhibition assay. The results of these inhibition assays are presented in Figure 7 and Table 2.

The binding of 27R9 was not inhibited by any of the glycolipid extracts used (see Figure 7), whereas the binding of both 30R3 and 11-4

was inhibited by several spleen cell extracts. This result is consistent with other evidence presented in this paper for the protein nature of the antigen recognised by 27R9 and suggests that the carbohydrate structures recognised by 30R3 and 11-4 are present on glycolipid molecules.

The CBA/H ($H-2^k$) spleen cell extract inhibited the binding of both 30R3 and 11-4 antibodies to B10.A spleen cell targets more efficiently than did the extracts from other strains (see Table 2). Inhibition was never greater than 50% (Figure 7), but it has since been found that this can be improved by incorporating the glycolipids into liposomes, thus giving rise to multipoint binding structures. However, strong crossreactive inhibition by B10 ($H-2^b$) was evident for both antibodies, and weaker inhibition of 30R3 was detectable with an SJL ($H-2^s$) extract, and of 11-4 with a B10.RIII ($H-2^r$) extract. These results correlate, to some extent, with the published crossreactivity of these two antibodies on strains carrying other H-2 antigens (Lemke *et al.* 1978, Hämmerling *et al.* 1979). Although other workers have reported weak crossreactive binding of both 30R3 and 11-4 antibodies to $H-2^b$ targets, strong crossreactions have been found (see Table 2), a result which can only be attributed to a difference in the sensitivities of the antibody assay systems used. In contrast to the cell extracts, the CBA/H ($H-2^k$) serum extracts did not inhibit the binding of either 30R3 or 11-4 to spleen cells (see Figure 7). This same extract is known to effectively inhibit the binding of monoclonal antibodies against carbohydrate-defined Ia^k antigens to spleen cells (Higgins *et al.* 1980).

Molecular Relationship Between Antigenic Determinants Recognised by 30R3 and 11-4 antibodies

In the previous chapter, an immunoprecipitation assay was used to demonstrate the mutual exclusiveness between the 27R9- and 30R3-defined antigenic sites. This same method was used in this report to analyse the molecular relationship between these two sites and the antigenic determinant recognised by 11-4. The procedure entailed immunoprecipitating the H-2K^k antigens recognised by one antibody from an NP-40 lysate of B10.A spleen cells, and then measuring, by a rosette-inhibition assay, the content of H-2K^k antigens remaining in the lysate that reacted with another antibody.

A control (unabsorbed) B10.A lysate which was preincubated with medium rather than antibody, was highly effective at inhibiting the binding of each of the monoclonal antibodies to spleen cell targets (see Figure 8). However, when the lysate was preabsorbed with the 27R9 antibody, only the inhibitory activity for 27R9 was removed. On the other hand, the monoclonal antibodies 11-4 and 30R3 clearly reacted with the same molecule in the NP-40 lysate, since each antibody, when used alone, effectively cleared the lysate of H-2K^k antigens recognised by both monoclonal antibodies. These results are consistent with just two H-2K^k molecules, one carrying the protein-defined 27R9 antigenic determinant, the other carrying carbohydrate-defined antigens recognised by the 30R3 and 11-4 monoclonal antibodies.

DISCUSSION

The results of experiments described in this chapter are summarised in Table 3 and clearly show that two of the three monoclonal anti-H-2K^k antibodies examined recognise carbohydrate antigenic determinants.

Sugar inhibition studies and glycosidase treatments indicate that α -linked D-mannose is the immunodominant sugar in the determinant recognised by the 30R3 (H-2.25) monoclonal antibody. The 11-4 antibody binds to a more complicated branched chain structure containing terminal sialic acid, D-mannose, and α - and β -linked D-galactose units (see Table 3). This was evident from the observation that four different exoglycosidases acted on the same structure, suggesting the existence of at least 4 different terminal sugars in the antigenic site. The accommodation of such a large number of carbohydrate units in the binding site of an antibody is not unusual and has already been described for the O antigens of *Salmonella* (Lüderitz *et al.* 1966), for the *Pneumococcal* polysaccharides (Mage and Kabat 1963) and by Kabat (1960) using human anti-dextran antibodies.

Immunoprecipitation-inhibition studies revealed that the protein-defined antigen recognised by the 27R9 monoclonal antibody is carried by one H-2K^k molecule whereas the carbohydrate-defined antigens detected by the 30R3 and 11-4 antibodies are carried on another molecule (Figure 8). Furthermore, the carbohydrate H-2K^k antigens appear to be expressed on cell surface glycolipids (Figure 3 and Table 2). Thus the K region of the murine MHC appears to control two separate families of alloantigens, one family consisting of cell surface glycolipids, and the other being comprised of cell surface polypeptides. An analogous system of glycoprotein - glycolipid antigens has been proposed previously for the murine I-region (reviewed by Parish and McKenzie 1980).

Unless the carbohydrate-defined H-2 molecules exist as aggregates of several smaller molecules, they would appear to be much larger than most glycolipid Ia molecules which have molecular weights of about

5,000 daltons and are readily dialysable (Parish *et al.* 1978, Parish and McKenzie 1980, Higgins *et al.* 1980). One exception, however, is the glycolipid molecule defined by an Ia.17 monoclonal antibody, which is also non-dialysable (Higgins *et al.* 1980). Glycolipid H-2 molecules also appear to differ from Ia glycolipid molecules in that they are not readily detectable in serum, a phenomenon well-characterised for the Ia molecules, and which may reflect some inherent functional difference (Parish *et al.* 1978, Parish and McKenzie 1980, Parish *et al.* 1976a,b,c).

At this point, it should be emphasised that a great diversity of structures can be generated by joining a limited number of different monosaccharides in an oligosaccharide chain. For example, Sharon (1975) has calculated that as many as 1,056 isomeric structures can be generated by joining just three different monosaccharides. At the serological level, more than 60 different specificities have been identified on the carbohydrate-defined O antigens of *Salmonella* bacteria (Lüderitz *et al.* 1966). Thus, carbohydrate structures could readily accommodate the serological diversity of H-2 antigens.

Although there have been several earlier reports which suggest that carbohydrate-defined H-2 antigens may exist, most evidence has been indirect (reviewed by Klein 1975). However, the results of Sanderson *et al.* (1971) on human histocompatibility antigens are probably the most definitive. These workers were able to detect HLA antigenic determinants in both the carbohydrate and protein fractions of purified cell extracts. There is also early evidence from Davies (1962) for lipid carriers of H-2 antigenicity which has never been satisfactorily refuted. Subsequent gel filtration experiments which demonstrated that H-2 antigenic activity resided in a glycoprotein molecule (Shimada and Nathenson 1969), and in its polypeptide component (Muramatsu and Nathenson 1970), directed

interest away from the former results, but did not disprove them. With the discovery of the phenomenon of 'H-2 restriction' in 1974 (Zinkernagel and Doherty 1974, Shearer 1974), very little attention has been paid to the important results of Sanderson *et al.* (1971) and, instead, most interest in this field has been directed at demonstrating a requirement for H-2 antigens in T cell recognition. The general failure of earlier workers to demonstrate glycolipid H-2 molecules could stem from the use of inappropriate organic solvent extraction methods (Higgins and Parish 1980), or column purification procedures e.g. lentil lectin coupled Sepharose beads could specifically absorb these molecules by virtue of their chemical nature. The availability of appropriate alloantiserum preparations could also impair detection of glycolipid molecules; e.g. if anti-protein antibodies predominated, the detection of both types of molecules in inhibition assays would be difficult.

On the other hand, glycoprotein H-2 molecules can be easily characterised by gel filtration and electrophoresis methods (Vitetta and Capra 1978), but probably the most conclusive evidence that a 45,000 molecular weight molecule carries H-2 antigenic activity is the recent amino acid sequencing data which indicates significant changes in amino acid sequences between H-2K and H-2D molecules from strains of mice carrying different H-2 haplotypes (Vitetta and Capra 1978, Silver and Hood 1976, Cook *et al.* 1978, Coligan *et al.* 1978). Furthermore, changes in the amino acid sequence of H-2 molecules from mutant mouse strains correlates with changes in H-2K and H-2D antigens defined by functional tests such as skin graft rejection and H-2 restricted cytotoxic T cell responsiveness (Brown and Nathenson 1977, Brown *et al.* 1978, McKenzie *et al.* 1977b).

Attempts to immunoprecipitate H-2 molecules with monoclonal antibodies and to characterise them on SDS-polyacrylamide gel electrophoresis, in general, have proved to be difficult, and the results have been variable. This difficulty has also been reported for monoclonal antibodies by other workers (Robinson and Schirrmacher 1979). However, Oi *et al.* (1978) have shown that the 11-4 monoclonal antibody can precipitate a protein H-2 molecule. Since this finding is inconsistent with the prediction that this antibody recognises a glycolipid molecule, an attempt has been made to find an explanation for their results. In the previous chapter, it was shown that the molecules defined by the 27R9 and 30R3 monoclonal antibodies tended to aggregate in a specific manner both on the cell surface, and in solubilised cell preparations, which were stored for some time. One interpretation is that because of this specific aggregation, a glycolipid-specific antibody could spuriously immunoprecipitate a protein H-2 molecule. At present, this can only be offered as an explanation for the results of Oi *et al.* (1978). However, using a modified gel electrophoresis procedure, preliminary experiments suggest that the carbohydrate-defined and protein-defined antigens described here are present on different molecules with very different molecular weights.

The demonstration of both protein- and carbohydrate-defined H-2K^k antigens on different molecules has important theoretical implications. The most obvious implication is that the K-region of the murine MHC must, in some way, control the action of glycosyltransferase enzymes. The simplest interpretation is that the K region directly codes for glycosyltransferases. Another, more complex possibility, is that the K region produces regulators of glycosyltransferases that are coded

for by genes located outside the *MHC*. Another intriguing question is the genetic relationship between the protein and carbohydrate $H-2K^k$ antigens. It is conceivable that the protein and carbohydrate antigens are controlled by separate genes that map to the *K* region. A more provocative hypothesis, which has already been proposed for the protein and carbohydrate families of Ia antigens (Parish *et al.* 1977, Parish and McKenzie 1980), is that the $H-2K^k$ protein antigen is a glycosyltransferase (or glycosyltransferase regulator) and the $H-2K^k$ carbohydrate antigen, a product of the transferase. A definitive answer to these questions awaits the isolation and characterization of the proposed glycosyltransferases.

Whatever the outcome of the above proposals, the demonstration of both protein and glycolipid molecules mapping to the *K* and *I* regions (Parish and McKenzie 1980) of the *H-2* complex, as well as evidence for the carbohydrate nature of the *H-2* linked T/t antigens (Cheng and Bennett 1980), suggests a common functional basis for the different *H-2* gene products. An attractive hypothesis is that the *H-2* complex controls several families of glycosyltransferase enzymes that mediate communication between lymphoid cells in a manner originally proposed by Roseman (1980). In this model glycosyltransferases on the surface of one cell interact with their carbohydrate substrate (an incompletely glycosylated structure) on the other cell. Thus, the 'anti-self receptors' proposed to explain *H-2* restricted phenomena (Doherty *et al.* 1976, Shearer *et al.* 1976) may well be glycosyltransferases and the structures they recognise on target cells, incompletely glycosylated glycolipids.

In this context, carbohydrate structures have already been shown to be involved in a variety of recognition processes (reviewed by McKenzie *et al.* 1977a) and recent work by Sia and Parish (personal communication), has shown that carbohydrate structures on red blood cells are the combining sites for autorosetting thymocytes. The thymocyte receptor is a protein determinant, and since the interaction leading to autorosetting is known to involve *H-2* restricted anti-self receptors (Sia and Parish 1980a,b), this work could be the first demonstration of an *H-2*-linked protein-carbohydrate interaction. Work is currently in progress to determine the nature of *H-2* antigens involved in allogeneic and *H-2* restricted T cell recognition.

Finally, it should be noted that several workers have used the 27R9 and 30R3 monoclonal antibodies to block *H-2K^k* recognition by alloreactive and *H-2* restricted cytotoxic T cells (Lindahl and Lemke 1979, Blanden *et al.* 1979). Although both monoclonal antibodies blocked anti-*H-2K^k* recognition, such an experimental approach does not appear to be definitive enough to characterise the target antigen involved. Problems such as the existence of glycolipid-glycoprotein complexes on cells (see Chapter 6), as well as the need to restrict antibody binding to either the target or the effector cells, makes these methods too indirect. Other approaches are needed to answer these questions.

SUMMARY

Three different monoclonal anti-*H-2K^k* antibodies, 27R9, 30R3 and 11-4 were examined for the biochemical nature of the antigenic determinants they recognise. When these were compared on the basis of their sensitivity to pronase and various glycosidases, 27R9 was shown to bind to protein-defined *H-2K^k* antigens, while 30R3 and 11-4 bound to

H-2 antigens defined by carbohydrate. From sugar inhibition studies, and treatments with specific glycosidases, D-mannose appears to be the immunodominant sugar involved in the antigenic site recognised by 30R3, while several sugars, namely sialic acid, D-mannose and α - and β -linked D-galactose appear to be components of the antigenic site bound by 11-4. The carbohydrate determinants were shown to be present on glycolipid molecules, since both the 30R3 and 11-4 antibodies could be inhibited by glycolipid extracts from spleen cells of the appropriate H-2 haplotype, as well as from several other strains of mice previously shown to be crossreactive targets for these antibodies. This finding is supported by evidence that the molecule carrying the protein-defined antigen is distinct from that carrying the carbohydrate-defined antigens. The results are discussed in the light of current information on the nature of glycolipid Ia antigens, as well as the role of H-2 antigens in T cell interactions.

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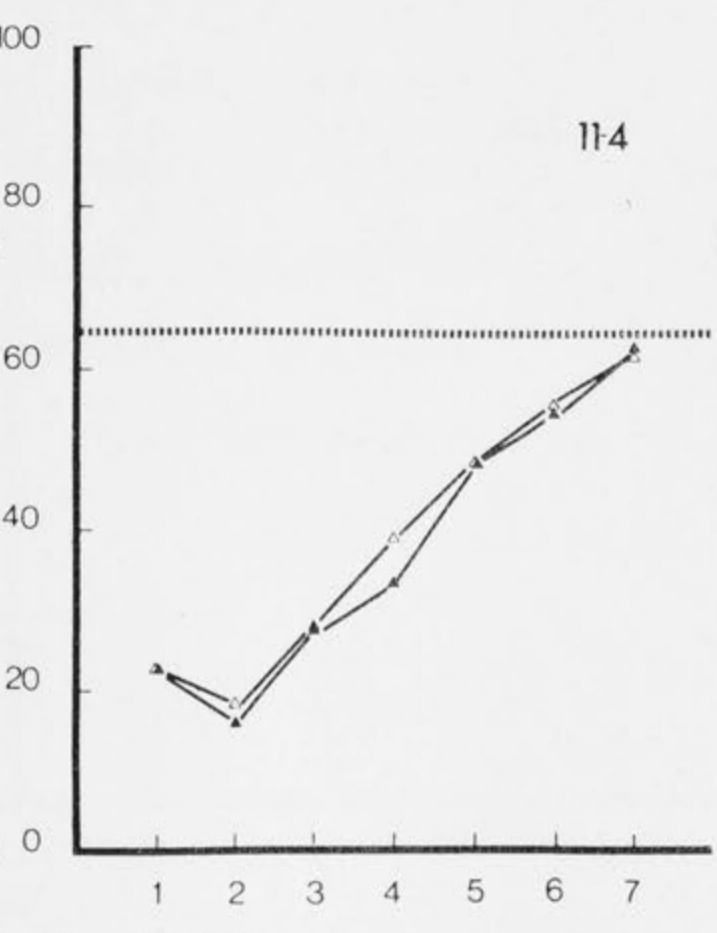
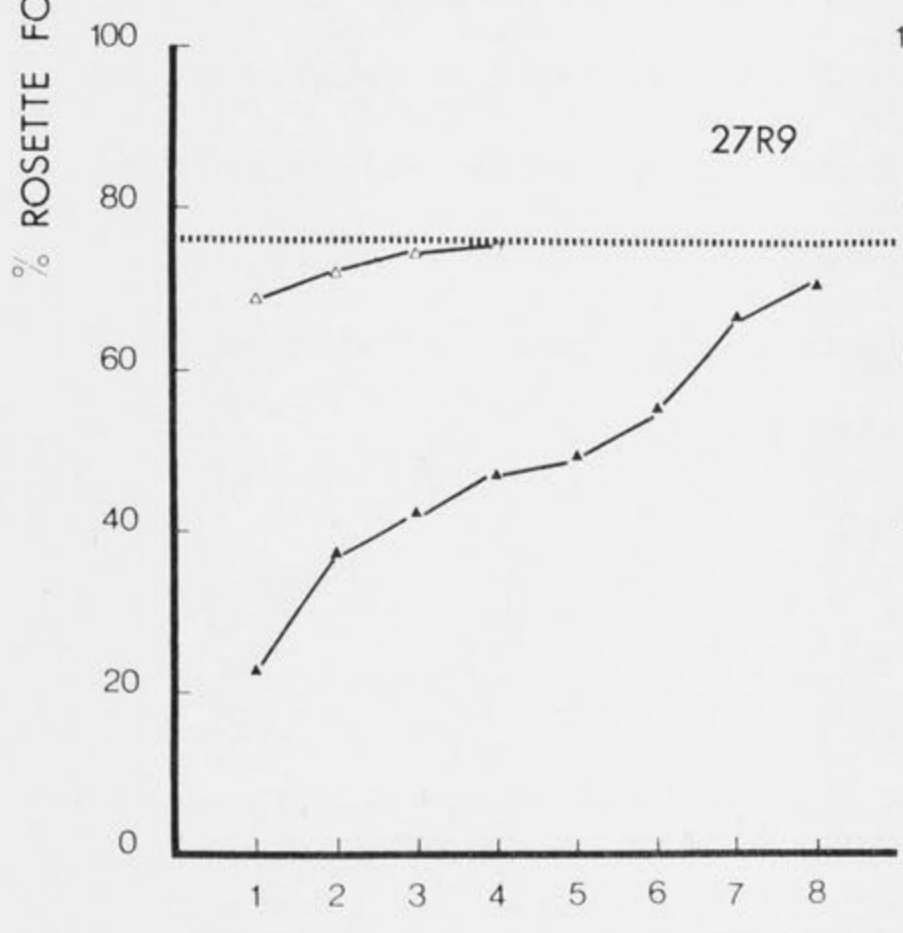
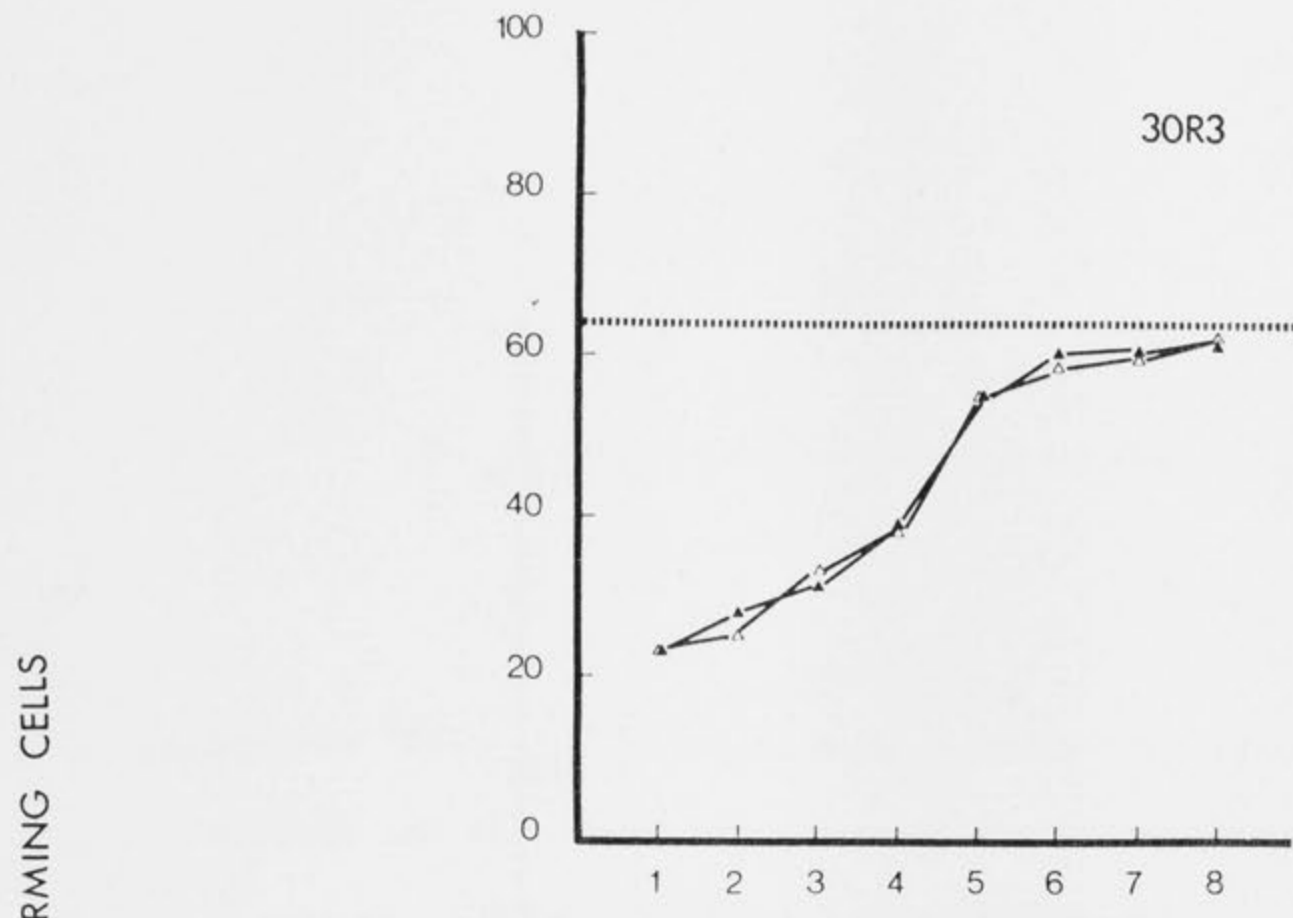
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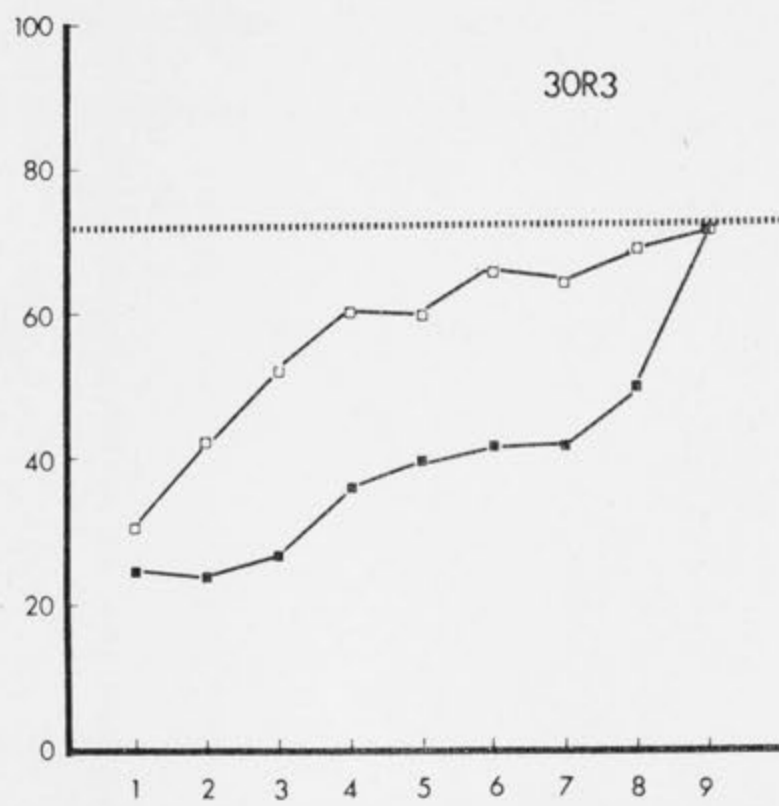
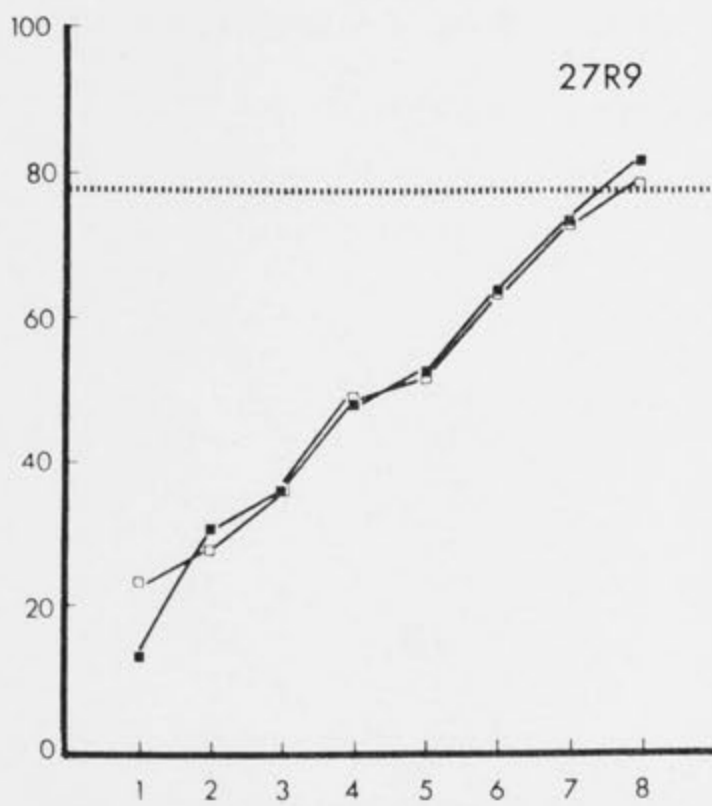
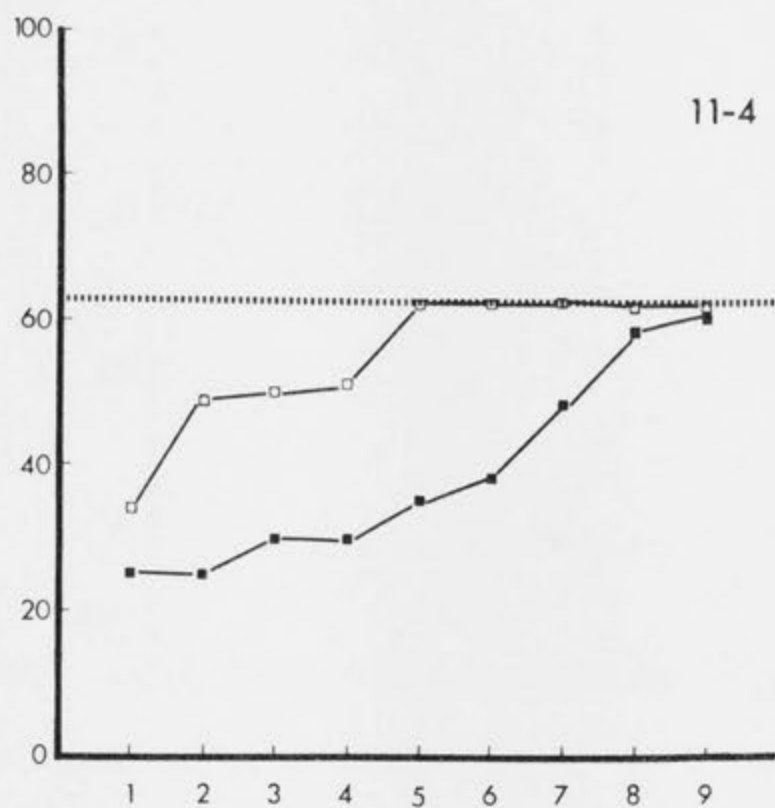
Fig. 1 Inhibition of binding of monoclonal anti-H-2K^k antibodies to B10.A spleen cells by a sodium cholate lysate of the same cells. Serial two-fold dilutions of lysate from 2×10^8 cell equivalents/ml were used to inhibit the binding of a constant amount of monoclonal antibodies 27R9, 30R3 and 11-4 to spleen cells, antibody content being measured by a rosetting assay. Lysates were either untreated (\blacktriangle) or treated with pronase (\triangle) before use in the inhibition assay. The dashed line represents % RFC in the absence of lysate.



DILUTION OF LYSATE

Fig. 2 Inhibition of binding of monoclonal anti-H-2K^k antibodies to B10.A spleen cells by a NP-40 lysate of the same cells. Serial two-fold dilutions of untreated (■), or mixed glycosidase treated (□) lysate were used to inhibit the binding of a constant amount of monoclonal antibodies 27R9, 30R3 and 11-4. The dashed line represents % RFC in the absence of lysate, and tube 1 represents undiluted lysate derived from 2×10^8 cells/ml.

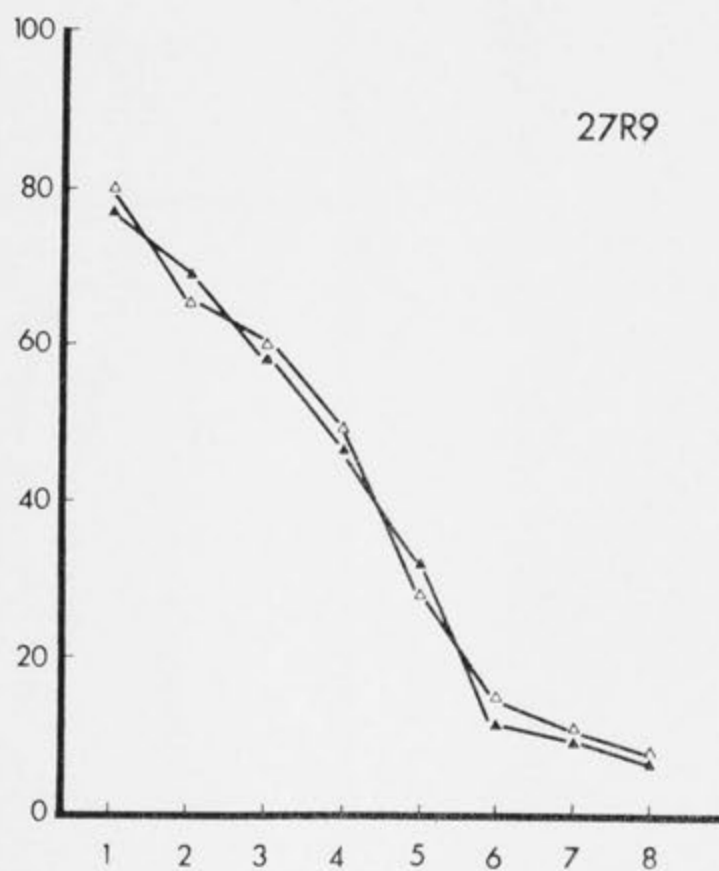
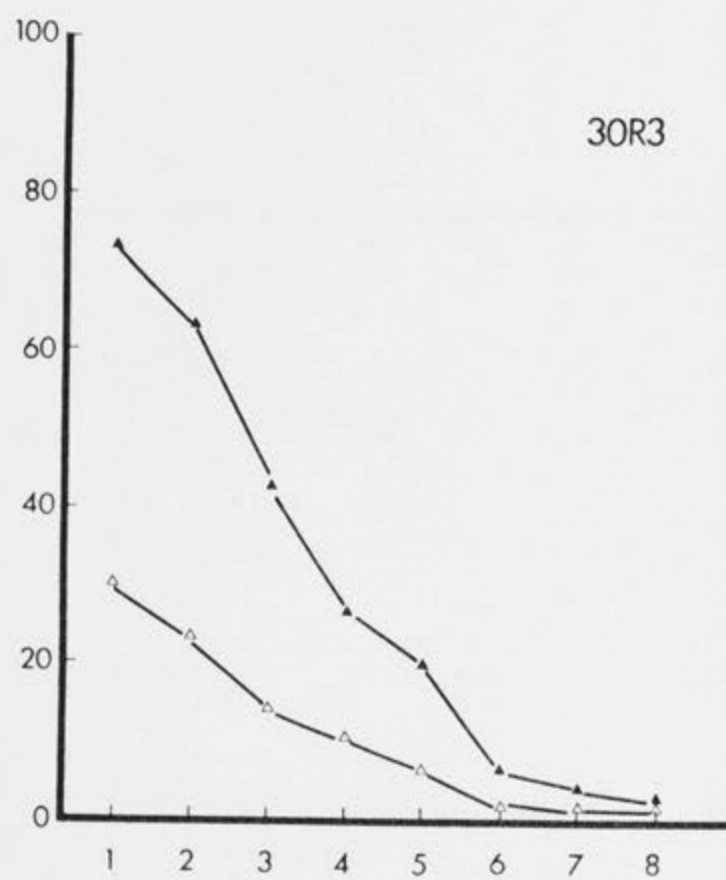
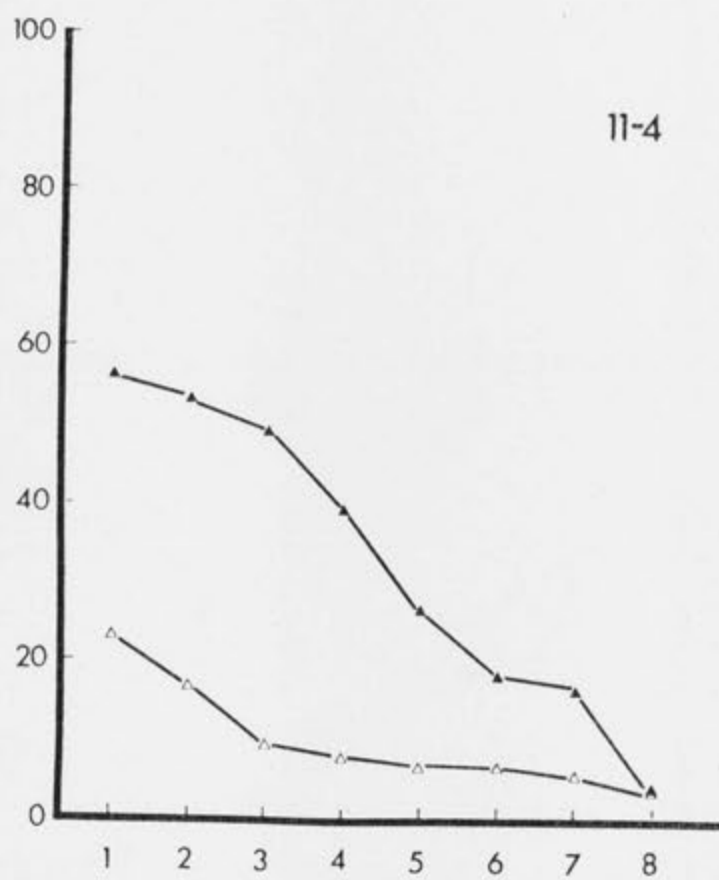
% ROSETTE FORMING CELLS



DILUTION OF LYSATE

Fig. 3 Binding of monoclonal antibodies 27R9, 30R3 and 11-4 to either untreated (▲) or mixed glycosidase treated (△) B10.A spleen cells. Antibody binding was measured by a rosetting assay using serial two-fold dilutions of antibody.

% ROSETTE FORMING CELLS



ANTISERUM DILUTION

Fig. 4 Binding of monoclonal antibodies 27R9, 30R3 and 11-4
to either untreated (●) or NA treated (○) B10.A
spleen cells.

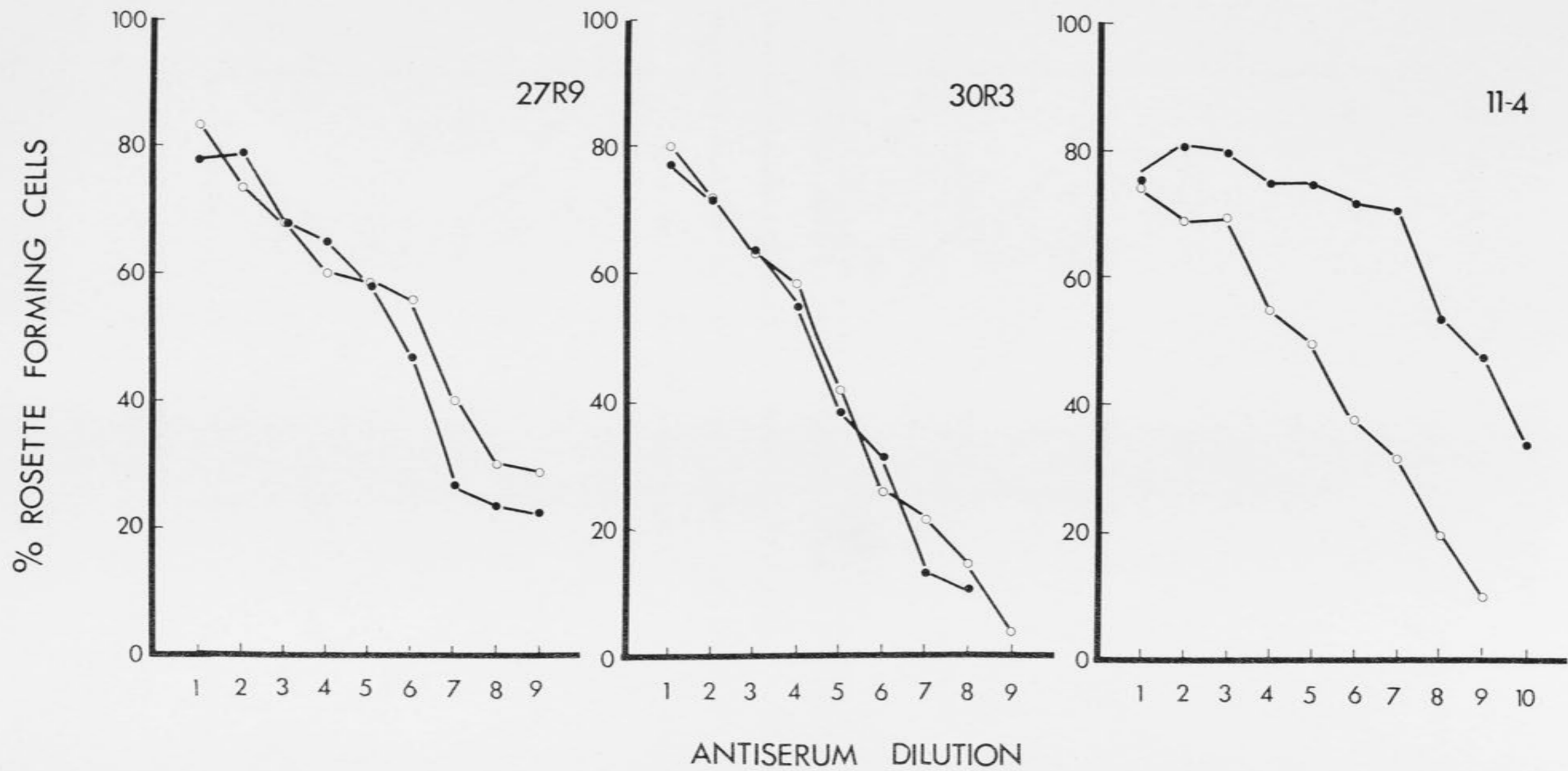
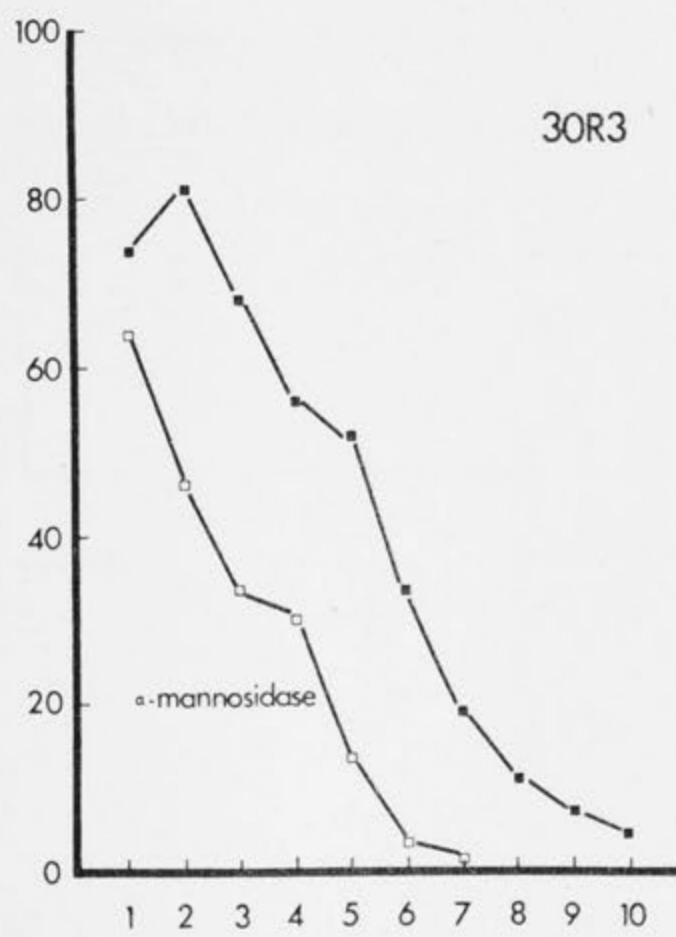
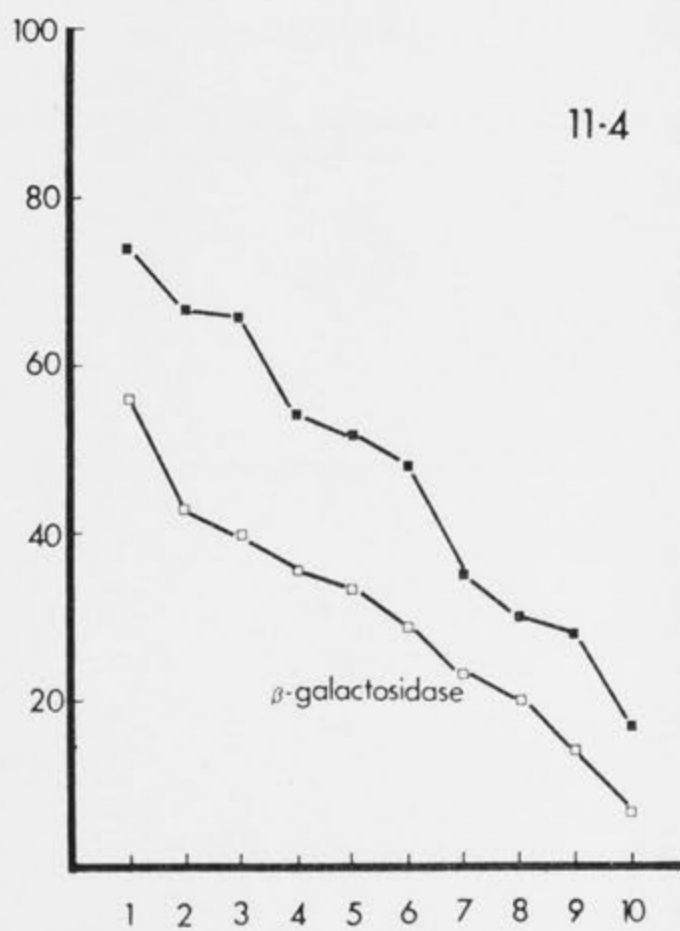
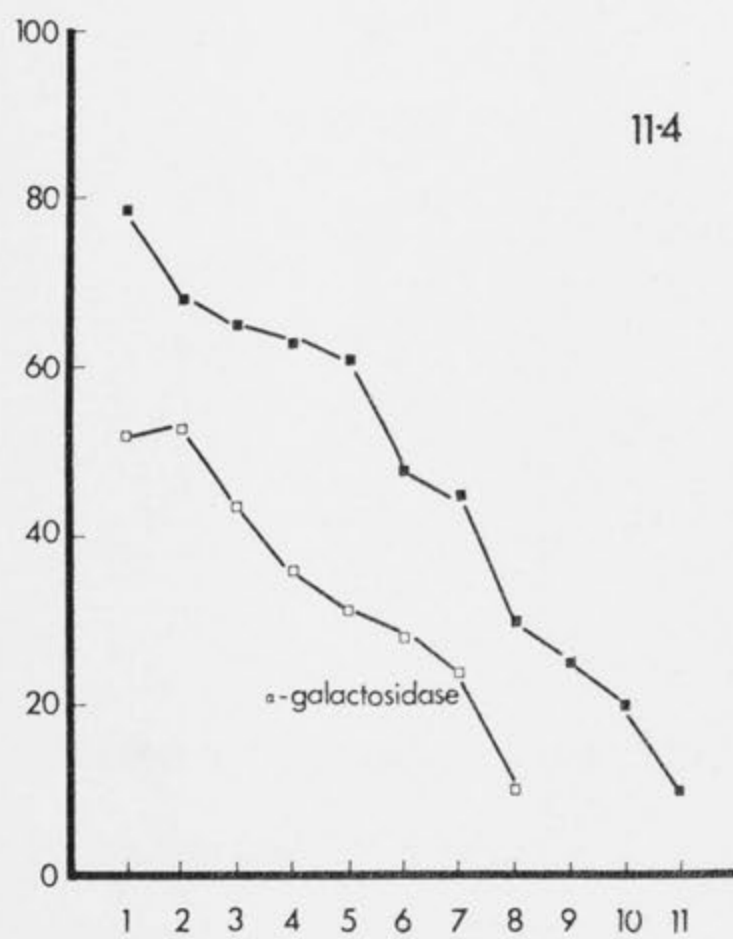
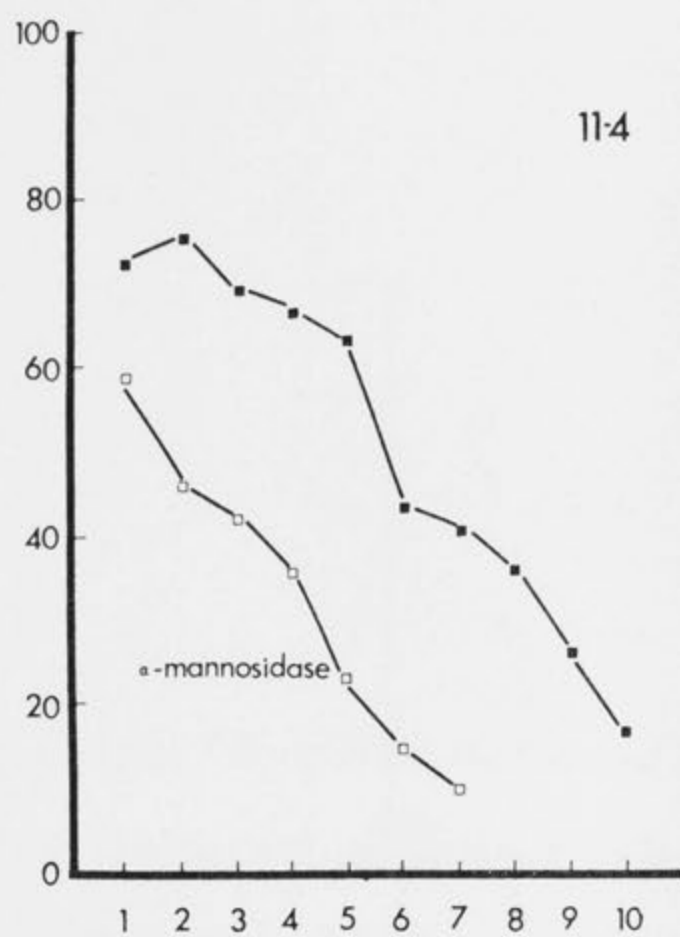


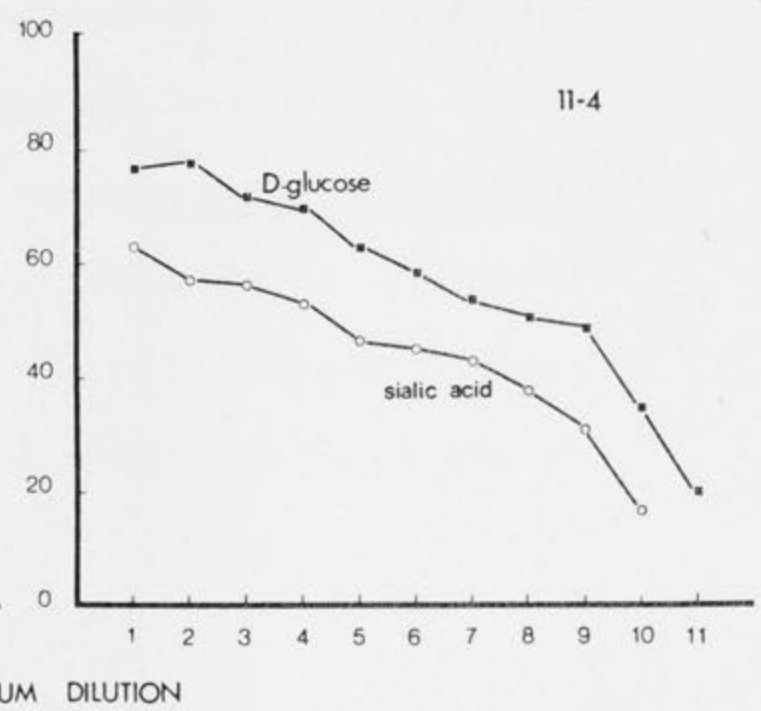
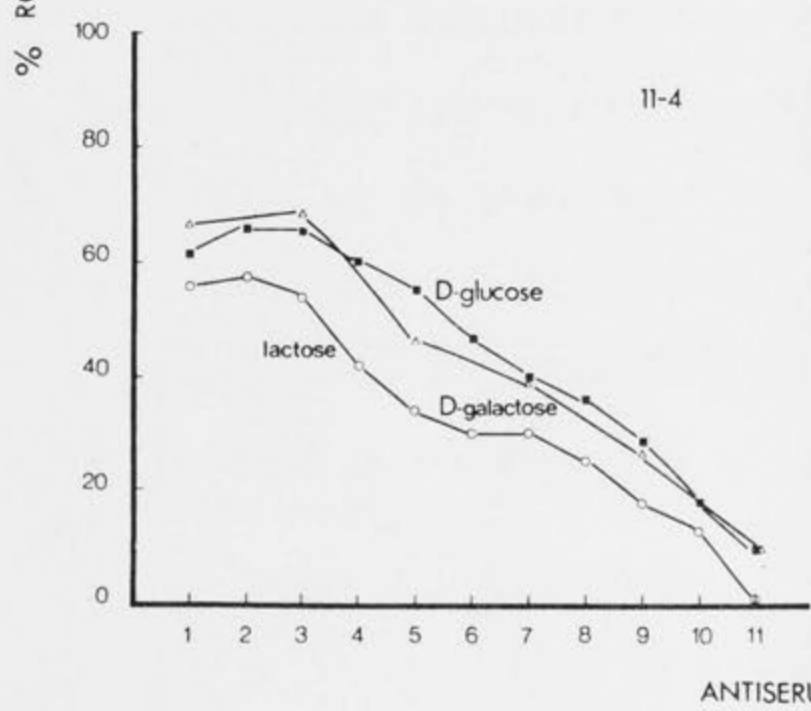
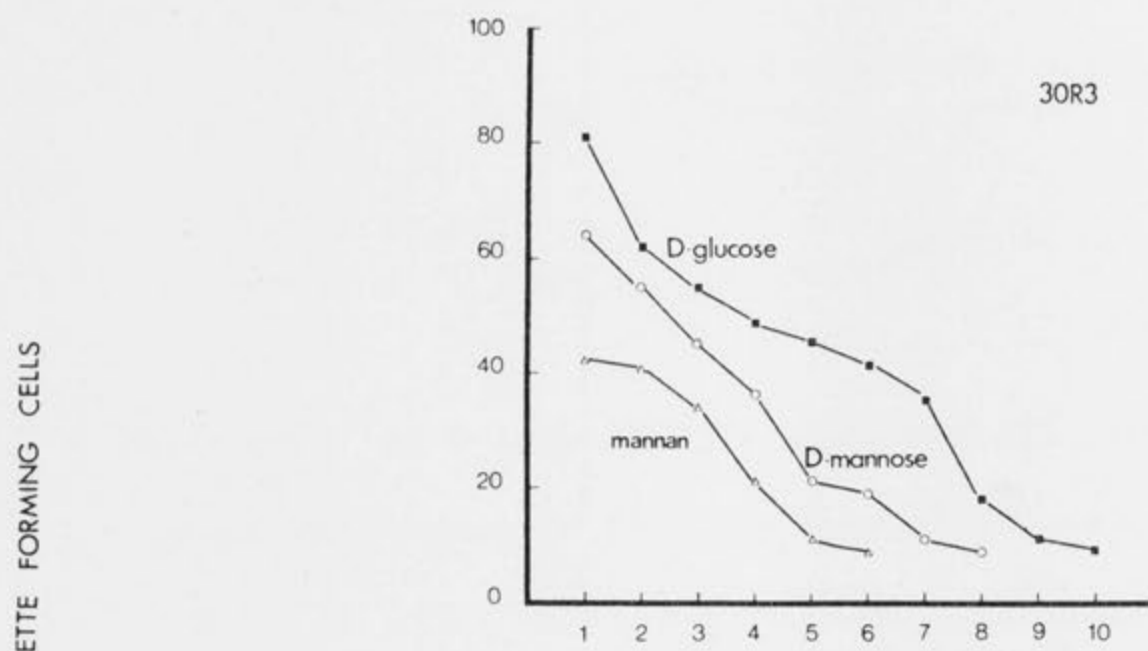
Fig. 5 Effect of specific enzyme treatments on the ability of B10.A spleen cells to bind the monoclonal antibodies 27R9, 30R3 and 11-4. The binding of serial two-fold dilutions of antibody was measured by the rosetting assay.

% ROSETTE FORMING CELLS



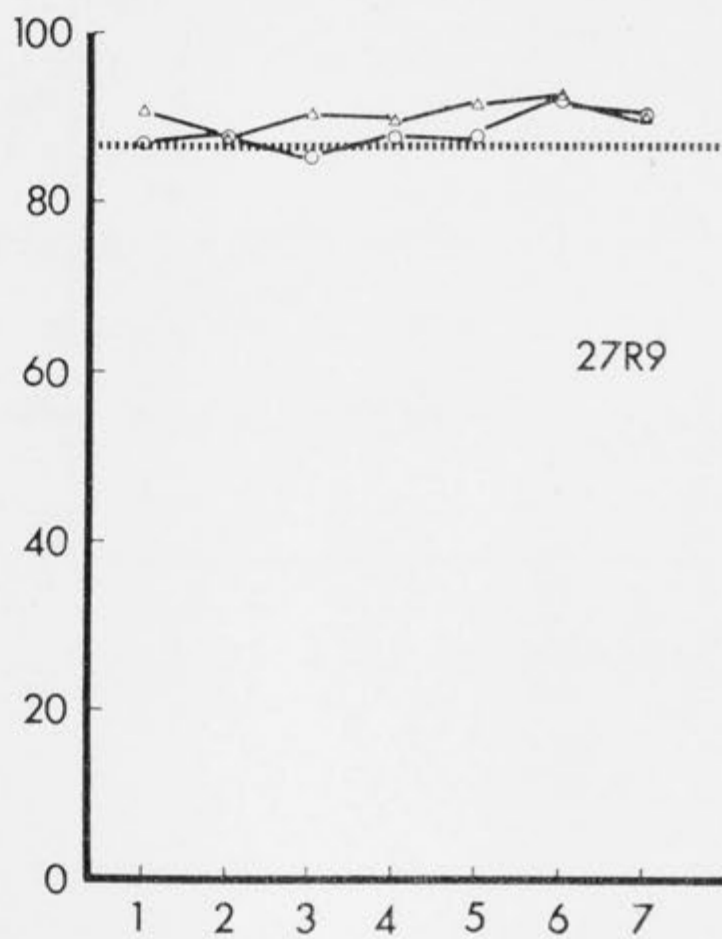
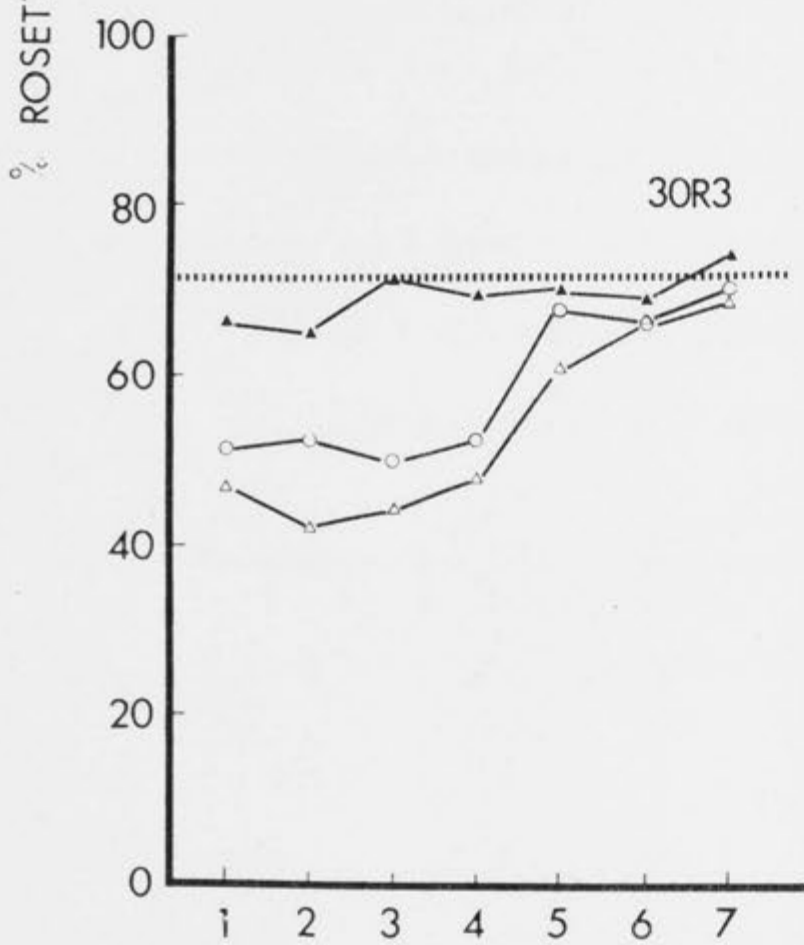
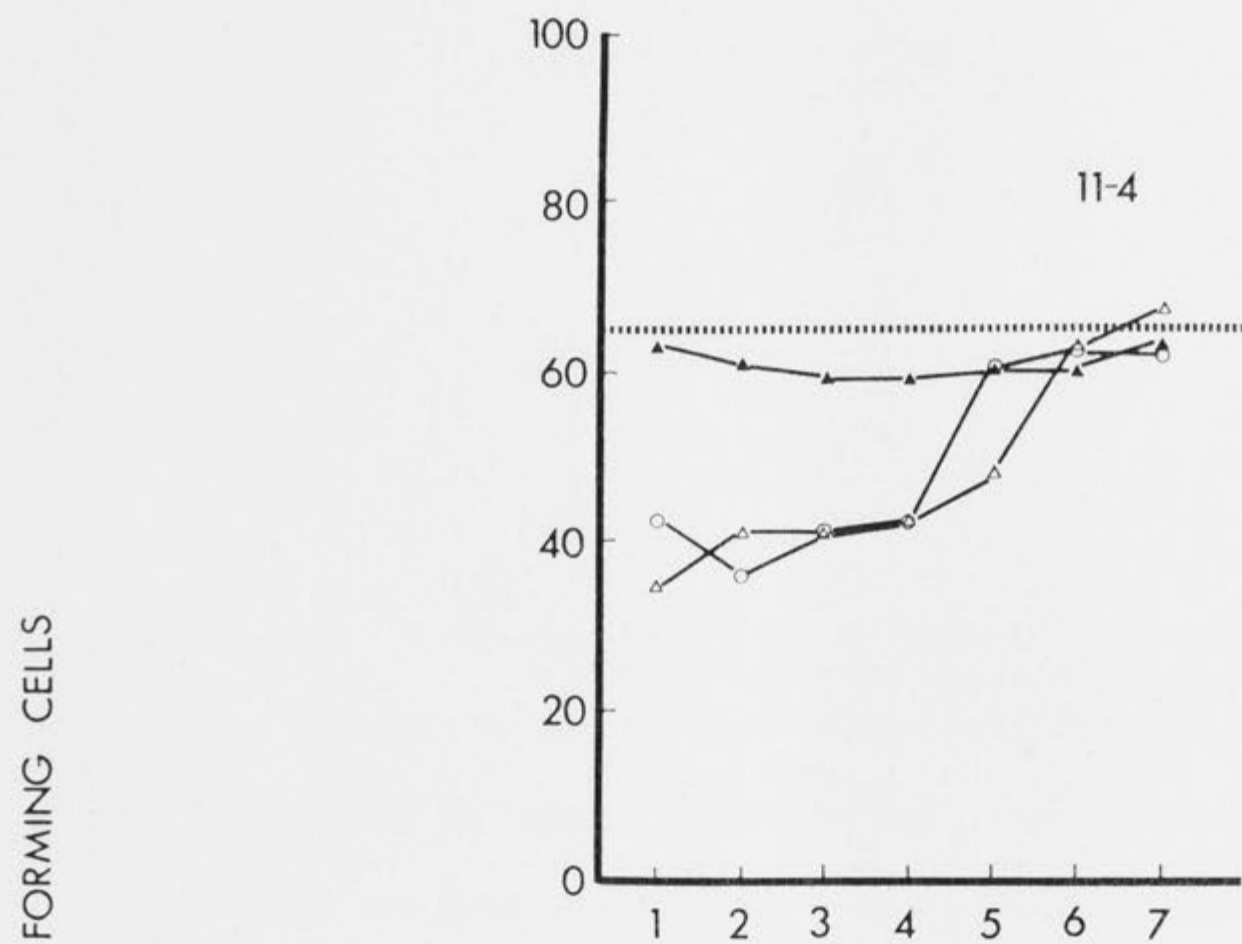
ANTISERUM DILUTION

Fig. 6 Inhibition of binding of monoclonal anti-H-2K^k antibodies to B10.A spleen cells by simple sugars. Serial two-fold dilutions of monoclonal antibodies 30R3 or 11-4 were incubated with B10.A spleen cells in the presence of a constant concentration (10 mg/ml) of different monosaccharides and antibody binding measured by a rosetting assay.



ANTISERUM DILUTION

Fig. 7 Inhibition of binding of monoclonal anti-H-2K^k antibodies to B10.A spleen cells by glycolipid extracts from CBA/H spleen cells (Δ), C57BL/6 spleen cells (O), or CBA/H serum (\blacktriangle). A constant amount of monoclonal antibodies 27R9, 30R3 or 11-4 was incubated with serial two-fold dilutions of the extracts and the binding of the antibodies to target cells was measured by the rosetting assay. The dashed line represents % RFC in the absence of inhibitor and Tube 1 equals undiluted lysate derived from 4×10^8 cells/ml.



DILUTION OF LIPID EXTRACT

Fig. 8 Inhibition of binding of different anti-H-2K^k monoclonal antibodies to B10.A spleen cells by NP-40 lysates of B10.A splenocytes. Each graph is headed by the anti-K^k antibody being inhibited. The lysate used for inhibition was either unabsorbed (closed symbols) or immunoprecipitated with 27R9 (○), 30R3 (□) or 11-4 (△) antibodies prior to addition to the assay. Antibody binding was measured by a rosetting assay. The dotted line represents % RFC in the absence of inhibitor and Tube 1 equals undiluted lysate derived from 2×10^8 cells/ml.

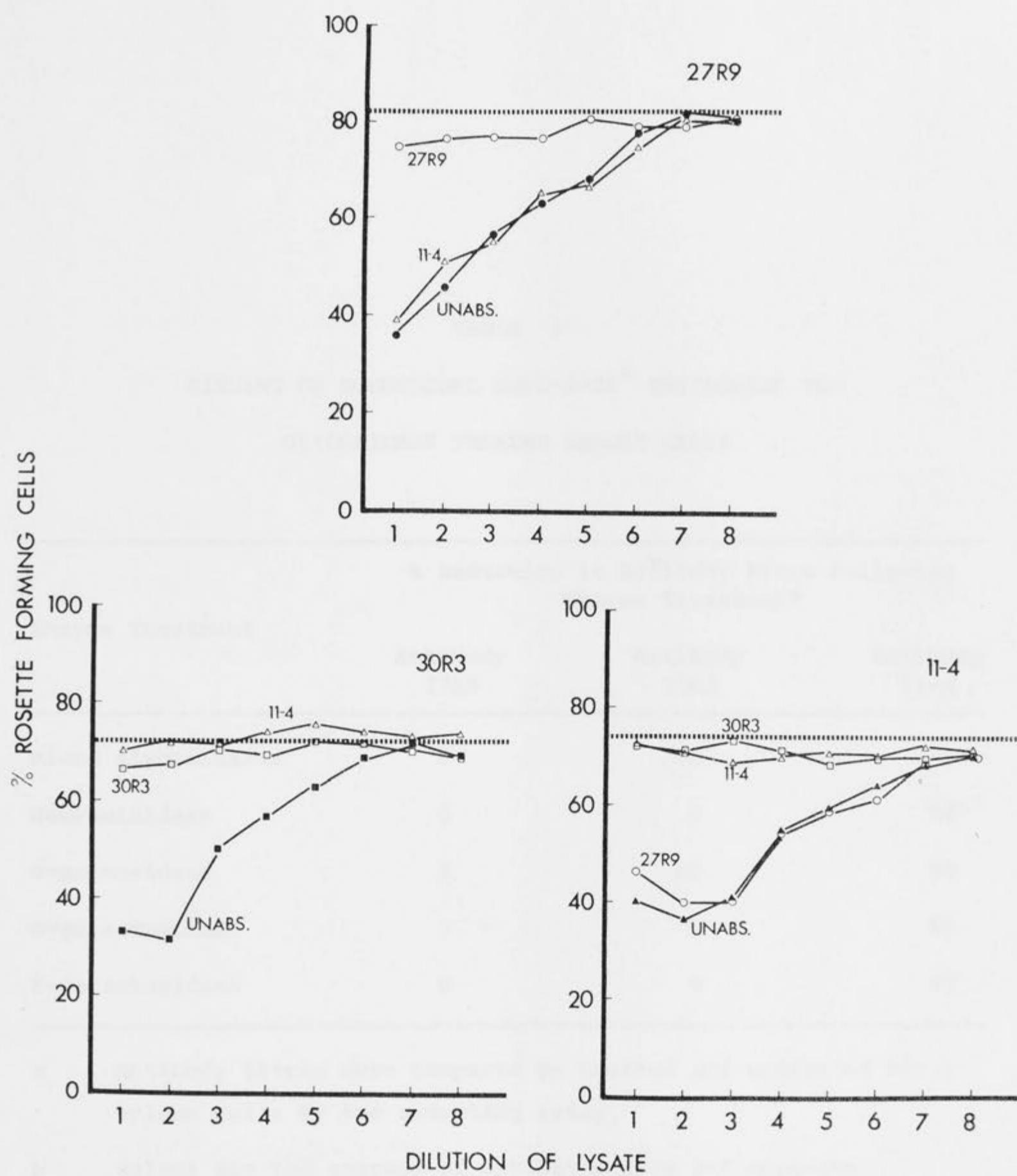


TABLE 1
 BINDING OF MONOCLONAL ANTI-H-2K^k ANTIBODIES TO
 GLYCOSIDASE TREATED TARGET CELLS

Enzyme Treatment	% Reduction in Antibody Titre Following Enzyme Treatment ^a		
	Antibody 27R9	Antibody 30R3	Antibody 11-4
Mixed glycosidases	0	>80 ^b	>80
Neuraminidase	0	0	88
α -mannosidase	0	89	90
α -galactosidase	0	0	89
β -galactosidase	0	0	87

a Antibody titres were compared on treated and untreated B10.A spleen cells by the rosetting assay.

b Values are the average of estimates from 2-4 separate experiments.

TABLE 2
 INHIBITION OF BINDING OF MONOCLONAL ANTI-H-2K^k ANTIBODIES
 BY GLYCOLIPID EXTRACTS FROM DIFFERENT STRAINS OF MICE

Strain origin of glycolipid extract of spleen cells	Minimal concentration of extract (cell equivalents per ml x 10 ⁻⁷) required to inhibit antibody binding ^a			
	Antibody 30R3		Antibody 11-4	
CBA/H (H-2 ^k)	2.7	(32,000) ^b	2.2	(16,000)
C57BL/10 (H-2 ^b)	3.6	(20,000)	3.6	(3,200)
BALB/c (H-2 ^d)	>40	(<<100)	>40	(<<100)
SJL (H-2 ^s)	13.1	(6,400)	>40	(<<100)
B10.RIII (H-2 ^r)	>40	(100)	11.3	(800)
DBA/1j (H-2 ^q)	>40	(100)	>40	(100)

a Antibody binding was measured by the rosetting assay using B10.A spleen cells as targets.

b Titre⁻¹ of antibody measured by the rosetting assay on cells of the strain from which the glycolipid extract was derived.

TABLE 3
SUMMARY OF EVIDENCE FOR PROTEIN AND CARBOHYDRATE H-2K^k ANTIGENS

Monoclonal Antibody	Speci- ficity Detected	Sensitivity of Antigen to Pronase	Sensitivity of Antigen to Mixed Glycosi- dases	Sensitivity of Antigen to Specific Glycosi- dases	Sugar Inhibition	Inhibitable with Glycolipid Extracts	Nature of Antigen
27R9	H-2.25	+	-	-	-	-	Protein
30R3	H-2.5	-	+	α -mannosidase	D-mannose	+	Carbohydrate
11-4	Unknown	-	+	Neuraminidase β -galactosidase α -galactosidase α -mannosidase	N-acetyl-neuraminic acid Lactose	+	Carbohydrate

DISCUSSION

Extensive genetic analysis of the murine major histocompatibility complex (MHC), also known as the H-2 complex, has so far predominantly defined the structure of two distinct regions, namely K, L, E and D (Klein 1970). A fifth region, designated F, has been proposed, but recent evidence has cast doubt on the existence of this region (Klein and Klein 1970). Thus, of the regions K, L and E control cell surface alloantigens whereas the D region controls secreted levels of the lymphokines I-A and I-E (Klein 1970, Schwartz 1970). At present the F region is probably the most complex, being subdivided into the D-F, D-G, D-H, D-I, D-J and D-K subregions (Klein 1970, Schwartz and Klein 1970, Klein and Klein 1970).

CHAPTER 8

A NEW REGION AT THE D-END OF THE MURINE MHC CONTROLLING Ia-LIKE ANTIGENS

It is well known that the murine major histocompatibility complex (MHC) is a highly polymorphic gene complex. The D region of the MHC is particularly polymorphic and has been subdivided into several subregions (D-F, D-G, D-H, D-I, D-J, D-K) (Klein 1970, Schwartz and Klein 1970, Klein and Klein 1970). The D region is known to control the expression of several Ia-like antigens (I-A, I-E, I-G, I-H, I-I, I-J, I-K) (Klein 1970, Schwartz and Klein 1970, Klein and Klein 1970). The D region is also known to control the expression of several other genes (D-F, D-G, D-H, D-I, D-J, D-K) (Klein 1970, Schwartz and Klein 1970, Klein and Klein 1970). The D region is therefore a highly complex and polymorphic region of the MHC.

The D-F and D-G subregions, products of the F and G regions respectively, are expressed on most cells and have been characterized as 25,000 dalton molecular weight glycoprotein molecules (Klein et al. 1970). In contrast, the D-H, D-I, D-J and D-K subregions are expressed only on certain cells and have been characterized as 25,000 dalton molecular weight glycoprotein molecules (Klein et al. 1970). The D-H, D-I, D-J and D-K subregions are therefore highly polymorphic and are known to control the expression of several Ia-like antigens (I-A, I-E, I-G, I-H, I-I, I-J, I-K) (Klein 1970, Schwartz and Klein 1970, Klein and Klein 1970).

INTRODUCTION

Extensive genetic analysis of the murine major histocompatibility complex (*MHC*), also known as the *H-2* complex, has so far conclusively defined the existence of four distinct regions, namely *K*, *I*, *S* and *D* (Klein 1975). A fifth region, designated *G*, has been proposed, but recent evidence has cast doubt on the existence of this region (Huang and Klein 1979). Three of the regions (*K*, *I* and *D*) control cell surface alloantigens whereas the *S* region controls serum levels of the fourth component of complement (Klein 1975, Shreffler 1976). At present the *I* region is genetically the most complex, being subdivided into the *I-N*, *I-A*, *I-B*, *I-J*, *I-E* and *I-C* subregions (David 1976, Shreffler and David 1976, Hayes and Bach 1980). Immune response (*Ir*) gene effects have been mapped to the *I-A*, *I-B* and *I-C* subregions, whereas *I* region-associated (*Ia*) antigens have been mapped to all subregions except *I-B* (David 1976, Shreffler and David 1975, Benacerraf and Germain 1978). Whether the *Ia* antigens are, in fact, *Ir* gene products is not known but the importance of *Ia* antigens in T cell-macrophage interactions (Schwartz *et al.* 1976, 1978) and their association with many immunoregulatory factors (Tada *et al.* 1976, Munro and Taussig 1975, Greene *et al.* 1977, Thèze *et al.* 1977, Delovitch and McDevitt 1977, Rich *et al.* 1977, Moorhead 1977, Howie and Feldman 1977), suggests that they do play a role in *Ir* gene effects.

The H-2K and H-2D antigens, products of the *K* and *D* regions respectively, are expressed on most cells and have been characterised as 45,000 dalton molecular weight glycoprotein molecules (Cullen *et al.* 1976). In contrast, *Ia* antigens have a restricted cellular distribution, being primarily expressed on most B lymphocytes but also

appearing on subpopulations of macrophages, T lymphocytes and epidermal cells (McKenzie and Potter 1979). Furthermore, glycoprotein Ia molecules have been shown, by immunoprecipitation studies, to consist of two noncovalently associated polypeptide subunits: an α -chain of molecular weight 30,000-35,000 daltons and a β -chain of molecular weight 25,000-30,000 daltons (Cullen *et al.* 1976, Cook *et al.* 1978). While the known Ia antigens are the products of genes closely linked to the *K* region, in this chapter evidence is presented for the existence of other Ia-like antigens which map to a new *I*-like region closely associated with the *D* region. Since the antigens involved resemble the known Ia antigens by virtue of their cellular distribution and molecular weight, this new region has been tentatively designated *I'*.

MATERIALS AND METHODS

Animals

Except for B10.AKM mice, which were donated by Dr Ian McKenzie, University of Melbourne, Australia, all mice were bred in the John Curtin School of Medical Research and used when 6 to 12 weeks old. The *H-2* haplotypes of the various strains used in this study are included in the data Tables.

Antisera

The antiserum preparations used in this study are listed in Table 1, and originated from the laboratory of Dr Ian McKenzie, University of Melbourne, Australia. They were produced as anti-*H-2* sera by repetitive immunizations of lymphoid cells in mouse strain combinations which differed, where possible, by just an *H-2* private specificity (McKenzie and Snell 1973). When it was necessary to absorb antisera, 100 μ l of a 1:10 dilution of serum was absorbed for 30 minutes at 4°C with 10^8 spleen cells.

Monoclonal anti-Thy 1.2 antibody (Clone #30-H12) conjugated with biotin was obtained from Becton Dickinson F.A.C.S. Systems (Mountain View, Ca.), together with fluorescein isothiocyanate conjugated avidin (FITC-avidin).

Preparation of Cell Suspensions

Cell suspensions of high viability (85-95%) were prepared as previously described (Chapter 6, Parish and McKenzie 1978) in Eagle's minimal essential medium F15 (Grand Island Biological Co., Grand Island, N.Y.) containing 5% fetal calf serum (FCS). Some spleen cell suspensions were cleared of red and dead cells by centrifugation on a cushion of Isopaque/Ficoll (Davidson and Parish 1975), and for allo-antiseria studies, cells were cleared of surface Ig by a capping procedure (Parish and McKenzie 1978). Briefly, this procedure consists of incubating the cells (10^7 cells/ml) for 75 minutes at 37°C in F15/5% FCS containing 1 mg/ml of sheep IgG specific for mouse Ig, followed by two washes with medium. Pure populations of Ig-negative cells were obtained from spleen by a rosetting procedure which has been previously described by Parish and coworkers (1974).

Lactoperoxidase-Catalysed Iodination of Cells

The procedure used was similar to that described by Parish *et al.* (1978). A spleen cell suspension, depleted of red and dead cells, and whose surface had been cleared of Ig by capping, was prepared. The cells were washed twice in serum-free phosphate-buffered saline (PBS) and then resuspended to a concentration of 2×10^8 cells/ml in PBS containing 400 μ g /ml lactoperoxidase (Calbiochem., San Diego, Ca.). Carrier-free ^{125}I (100 μ Ci/ 10^7 cells) was added, and the reaction then catalysed by two additions of H_2O_2 (1 μ g /ml of reaction mixture) for

2 minutes each at 23°C. The reaction was stopped by the addition of 20 volumes of ice-cold PBS, and the cells were washed twice more with ice-cold PBS, by centrifugation at 4°C, and finally resuspended to a concentration of 10^8 cells/ml in PBS/10% FCS.

Solubilisation of Spleen Cells

B10.A spleen cells, cleared of red cells, were pelleted by centrifugation and resuspended to a concentration of 2×10^8 cells/ml in 0.5% (v/v) Nonidet P-40 (NP-40) in PBS containing 10^{-3} M phenylmethylsulfonyl fluoride (PMSF) and 1.5mM MgCl_2 . Following solubilisation for 30 minutes at 4°C, nuclei and cell debris were removed from the preparation by centrifugation at 20,000 g for 30 minutes at 4°C. Lysates were stored at 4°C in the presence of 0.1% (w/v) sodium azide.

Immunoprecipitation of ^{125}I -labelled Cell Surface Antigens

To 10^7 ^{125}I -labelled B10.A spleen cells was added either 100 μl of a 1:7.5 dilution of AS285 [(B10.AKM x 129) F_1 anti B10.A], or a similar amount of normal B10.A serum, each diluted in PBS/10% FCS. Absorption was carried out for 60 minutes at 4°C, followed by two washes in cold PBS/10% FCS. The cells were then solubilised in 0.5% NP-40 as described above.

To precipitate antibody-antigen complexes, the lysates were incubated for 30 minutes at 4°C with protein A-bearing *Staphylococcus aureus* (Cowan I strain) bacteria (20 μl packed bacteria/0.1 ml of cell lysate). The *S. aureus* bacteria were grown, harvested, and stored as previously described (Kessler 1975). Prior to use, the bacteria were washed twice in PBS containing 0.5% NP-40 and 10^{-3} M PMSF according to the method of Kessler (1976). After absorption of lysate, bacteria

were pelleted, and then washed three times in PBS containing 0.05% NP-40 and 10^{-3} M PMSF in PBS. The bacteria pellet was resuspended in dissolving mix (see below), boiled for 3 minutes to dissociate antigen-antibody complexes, and the bacteria centrifuged out before the precipitated material was electrophoresed.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

NP-40 lysates of Bl0.A spleen cells were analysed by SDS-PAGE, using the SDS-disc polyacrylamide procedure of Maizel (1971), as adapted for use by Parish *et al.* (1978). Cylindrical gels (0.6 cm diameter) comprised a 10 cm resolving gel and a 1 cm stacking gel. Final concentrations in resolving gels were 10% acrylamide (Cyanamid, Australia); 0.266% N, N'-bis-methylene acrylamide (bis-acrylamide; Eastman Chem. Co., Rochester, N.Y.); 0.375M Tris (Sigma Chemical Co., St Louis, Mo.)/HCl, pH 8.9; and 0.1% SDS (Sigma Chemical Co., St Louis, Mo.). Polymerisation was catalysed with N,N,N',N'-tetramethylethylenediamine (TEMED, Eastman Chemical Co., Rochester, N.Y.), final concentration 3.33mM and ammonium persulphate, final concentration 2.2mM. Stacking gels contained 3% acrylamide; 0.08% bis-acrylamide, 0.0625M Tris/HCl, pH 6.7; and 0.1% SDS. Stacking gels were polymerised with 3.33mM TEMED and 4.4mM ammonium persulphate. The electrode buffer contained 0.384mM glycine, 0.0495M Tris, and 0.1% SDS, and was the same for both upper and lower buffer compartments.

Immunoprecipitates, or cell extracts originating from 10^7 cells were dissolved by boiling for 3 minutes in 2% SDS, 10% glycerol, 5% dithiothriitol, 0.002% bromophenol blue and 0.0625M Tris-HCl, and run on 10% polyacrylamide gels. Some cell extracts were run under non-reducing conditions, and were boiled in dissolving mix lacking

dithiothreitol. ^{125}I -labelled bovine serum albumin (BSA), ovalbumin (OV) and sheep anti-mouse Ig (H and L chains) were run as standards at the same time on a separate gel. Electrophoresis was carried out at 1.2 mA per gel, and gels were cut into 1 mm fractions for counting labelled material, and into 4 mm fractions for extraction of antigenic material.

Extraction of Antigens from Polyacrylamide Gels

Gels were cut crosswise into 4 mm segments, and each piece was pulverised with a spatula and then soaked for 36 hours at 4°C in 1 ml of distilled water containing 0.1% (w/v) sodium azide. To remove SDS from the extracts, each sample was added to an equal volume of packed XAD-8 resin beads (Rohm and Haas Co., Philadelphia, Pa.) which had been prewashed with methanol and stored in distilled water as described in the Appendix. Extracts were agitated with the resin at 22°C for 2 hours and then each fraction was dialysed for 36 hours at 22°C against 3 changes of 0.1mM sodium cholate in saline containing 0.1% (w/v) sodium azide. Preabsorption on the beads reduced the SDS concentration to its critical micelle level (see Appendix), and dialysis reduced the concentration to a level that did not lyse lymphocytes. Sodium cholate was then removed by a 2 hour dialysis at 22°C against PBS. The dialysed samples were concentrated by extraction of water using Aquacide 11A (Calbiochem, San Diego, Ca.) and volumes were adjusted to 100 μl using PBS. Samples were stored at 4°C in the presence of 0.1% (w/v) sodium azide and antigenic activity subsequently measured by the rosette inhibition assay.

Rosetting Assay

The binding of alloantibodies to spleen cells was detected by a rosetting procedure which has been described in detail in Chapter 6.

Briefly, 10 μ l of ice-cold Ig-capped spleen cells (4×10^6 /ml in F15/10% FCS) were incubated for 30 minutes at 4°C with 10 μ l doubling dilutions of antiserum in microtitre plates (Linbro Chemical Co., New Haven, Conn.). After washing with medium, 10 μ l of a 2% suspension of sheep erythrocytes coated, via CrCl_3 , with sheep anti-mouse Ig was added and the mixture centrifuged gently to form rosettes. Methyl violet staining solution was used to visualise lymphocytes and the percentage of rosette-forming cells (RFC) then determined.

Rosette Inhibition Assay

Serial dilutions of gel extracts were tested for their ability to inhibit the binding of a constant amount of antiserum to spleen cells in the rosette inhibition assay which has been described in detail in the Appendix. Briefly, 5 μ l serial dilutions of extract were pre-incubated with 5 μ l of a given amount of antiserum, and the remaining activity measured in the rosetting assay after addition of 5 μ l of Ig capped B10.A spleen cells. Extracts preincubated with medium rather than antiserum were included as controls.

RESULTS

Detection of a New Antigen on SDS-PAGE with Anti-D^d Sera

When analysing the H-2D^d molecules immunoprecipitated by anti-D^d sera raised in (B10.AKM x 129)F₁ mice against B10.A lymphoid cells by SDS-PAGE, an unexpected peak of radioactivity was detected which corresponded in molecular weight to the heavy chain of Ia antigens, i.e. 36,000 daltons. The results of one such experiment using anti-D^d serum AS285 to precipitate cell surface antigens of B10.A spleen cells is shown in Figure 1. Only high concentrations of antiserum revealed this peak of radioactivity, and these were usually supraoptimal for

demonstrating the characteristic 45,000 dalton H-2D^d peak. Although this peak could be detected with two other preparations of anti-D^d serum, AS954 and AS45, immunoprecipitation experiments were generally highly variable, and so other serological methods were used to characterise this antigen.

Genetic Mapping Studies Using Anti-D^d Serum

When these same anti-D^d sera were titrated in the rosetting assay on Bl0.A spleen cells, a biphasic titration curve was obtained which suggested that they contained antibodies specific for some determinant besides D^d which appeared to be present on only a subpopulation of cells. Results obtained using anti-D^d serum, AS285, are shown in Figure 2. To analyse this activity further, the anti-D^d sera were absorbed with spleen cells of several recombinant D^d-bearing strains of mice to selectively remove anti-D^d antibodies. While no antibody activity specific for Bl0.A spleen cells or thymocytes remained after absorption of these sera on spleen cells from A.TL and Bl0.T(6R) mice, following absorption on A.TH spleen cells, antibody activity was detectable which reacted with approximately 50% of Bl0.A spleen cells and 30% of thymocytes (see Figure 2). This antibody activity was found to be specific for a new determinant defined by differences in the location of the recombination events in A.TH, A.TL and Bl0.T(6R) mice which gave rise to D^d in these strains.

This was shown when the antisera were preabsorbed on A.TH spleen and tested in the rosetting assay for reactivity with spleen cells from different strains of mice. All antisera reacted similarly and results using AS285 are shown in Table 2. When tested on cells from five independent haplotypes, H-2^b, H-2^d, H-2^s, H-2^q and H-2^k, the preabsorbed antisera reacted only with H-2^d cells. Since four of

the five strains tested were C57BL/10 congenics, the activity appears to be directed against antigens controlled by the *H-2* complex. To map the antigenic specificity more precisely, the antisera were also tested on various recombinant strains derived from *H-2^d* mice. Since the reactions on D2.GD and C3H.OL were negative, but were positive on B10.A, the new antigen must map distal to *I-C*. The new specificity was shown to map between *S* and *D* by the reaction of various recombinant strains containing recombination events between these two loci. Positive reactions were obtained on B10.T(6R) and A.TL, confirming the earlier absorption results, while B10.A(2R) and C3H.OH were negative. A.TFR2, derived from A.TH and A.TFR5, derived from A.TL, reacted as did the parental strains. Two *H-2^d* strains bearing mutations in the *D* region, namely B10.D2-*H-2^{dml}* and BALB/c-*H-2^{dm2}*, also reacted positively with the absorbed antiserum. Since no crossreaction was evident on independent haplotypes, the haplotype origin of possible variants of this new specificity could be assigned.

According to the haplotype designation of this new antigen, it was expected that an A.TH anti-A.TL antiserum, when preabsorbed on B10.G spleen cells should react positively with B10.T(6R) spleen cells. Since no remaining activity was detectable, it was concluded that perhaps a *D*-region difference was needed between donor and recipient strains in order to produce antibodies specific for this new antigen. Furthermore, it should be emphasised that this new antibody activity was not detectable by microcytotoxicity, and could only be revealed by the more sensitive rosetting assay (Parish and McKenzie 1978).

Genetic Mapping Studies Using Anti-D^q Antiserum

Antibodies specific for determinants which map to the same region were also present in an anti-D^q serum, AS30, which was raised in (Bl0.A x LP.RIII)F₁ mice immunized with Bl0.AKM cells. When this antiserum was preabsorbed with Bl0.G spleen cells to remove anti-D^q antibodies, it had residual activity for H-2^k spleen cells, but did not react with cells of any other independent haplotype. These results are shown in Table 3. Since the recombinant strains C3H.OH and C3H.OL both react positively with the absorbed antiserum, the antigenic determinant must map distal to I-C. The fact that the preabsorbed antiserum reacted with cells from the recombinant strain Bl0.AKM but not with A.TL, both of which are recombinants between S and D, maps the new antigenic specificity to the same region as that defined with the anti-D^d serum, i.e. between S and D. Furthermore, since the haplotype origin of antigens found with AS30 correspond to those found with AS285, the two antisera appear to detect allelic variants of the same antigen.

Cellular Distribution of New Antigens

The anti-D^d serum, AS954, was used to determine the cellular distribution of the new antigen. The antiserum was preabsorbed on A.TH spleen cells, and then tested for residual antibody activity on A.TL and A.TH lymphoid cells. These results are presented in Table 4. A.TH cells were included as a control to detect residual anti-D^d activity. The preabsorbed antiserum reacted strongly with varying proportions of A.TL cells, namely 50% of spleen, 35% lymph node, and 30% of thymus. The reaction with bone marrow cells was weak, with only 12% of cells rosetting, and was only slightly higher than the background reaction of the antiserum on A.TH cells. Since the reaction

on A.TL Ig-negative spleen cells was no higher than on A.TH control cells, the results are consistent with the specificity being present mainly on B cells, as well as on a subpopulation of thymocytes.

By staining spleen cells with fluorescent anti-Thy 1.2 antibody it was possible to more directly demonstrate whether any of the cells which rosetted for the new antigen were T lymphocytes. Two A.TL spleen cell preparations were compared, an Ig-negative and an Ig-capped population, and the results of one such experiment are tabulated in Table 5. Cells were incubated with the monoclonal anti-Thy 1.2 antibody-biotin conjugate and then reacted with FITC-avidin. This procedure, which specifically stained T cells, detected 43% of unfractionated, and 90% of Ig-negative spleen cells as Thy 1.2⁺. While only a maximum of 10% of Ig-negative spleen cells carried the new antigenic specificity, none of these cells were Thy 1.2⁺, i.e. T cells. Similarly, while 48% of unfractionated spleen cells carried the new antigen, none of these rosetting cells stained positively with fluorescence.

The pattern of reactivity of this antigen on BALB/c *nu/nu* spleen cells, is also consistent with the new specificity being present on B cells, as well as on null cells (see Table 4). Anti-D^d serum, AS954, reacted with 50% of both BALB/c *nu/nu* and normal BALB/c spleen cells. However, 20% of Ig-negative BALB/c *nu/nu* spleen cells were reactive compared with only 5% of normal Ig-negative spleen cells, suggesting that the specificity is also present on an expanded population of null cells in nude mice, possibly macrophages or immature T cells.

Antigenic Activity Detectable on SDS-PAGE

Since the immunoprecipitation experiments described above gave variable results, other approaches were used to confirm that the

extraneous peak detectable on SDS-PAGE (see Figure 1) corresponded to the newly defined antigen. This was demonstrated by running an NP-40 lysate of B10.A spleen cells on SDS-PAGE, slicing the gel crosswise into 4 mm segments, extracting material from each fraction, and then testing the ability of material from each fraction to inhibit the binding to B10.A spleen cells of an anti-D^d serum (AS45), which had been preabsorbed with A.TH spleen cells. Using this method, virtually all H-2 antigenic activity can be recovered (manuscript in preparation). Antigenic activity detected in a lysate seen under reducing and non-reducing conditions is shown in Figures 3A and 3B, respectively. When the cell lysate was run under reducing conditions, antigenic activity was detectable in two regions of the gel. Greatest activity was detectable in three gel fractions in the molecular weight range of 34,000 to 42,000 daltons, with most activity in the 36,000 to 39,000 dalton fraction. A second smaller peak of activity was detected in just one fraction with a molecular weight range of 55,000 to 59,000 daltons. When the B10.A lysate was electrophoresed under non-reducing conditions, most activity was then detectable in the higher molecular weight region. Whilst this activity was spread over four fractions in the range 46,000 to 63,000 daltons, most activity was present in two fractions in the range 55,000 to 63,000 daltons. Traces of antigenic activity were also detectable in the molecular weight ranges of 38,000 to 42,000 as well as 24,500 to 28,500 daltons. The shift to high molecular weight molecules under non-reducing conditions suggests that the new antigen represents a two chain molecule that, at least after detergent solubilisation, is held together by disulphide bonds.

DISCUSSION

During studies involving the use of anti-H-2 sera to analyse H-2 antigen expression, previously undetected antibody activity in an anti-H-2D^d serum was found to immunoprecipitate a 36,000 dalton Ia-like molecule on SDS-PAGE. Genetic mapping studies using this same anti-serum revealed the existence of a new antigen defined by the *d* haplotype which mapped between the *S* and *D* regions of the murine *MHC*. The existence of this new locus was confirmed with a second anti-H-2D^q serum which was also found to contain antibodies specific for determinants mapping to this same region which was defined by the *k* haplotype. Experiments involving measurement of antigenic activity in cellular lysates extracted from SDS-polyacrylamide gels confirmed that the serologically defined antigens were indeed present on a 36,000 dalton molecule. As a result of these findings, the region coding for these antigens has been designated "*I'*", since the new antigenic specificities appear to be carried on a cell surface molecule resembling the currently defined Ia antigens by virtue of molecular weight and cellular distribution.

Molecular weight characterisation of these molecules warrants a more detailed discussion. Antigenic activity was detectable on molecules in a molecular weight range of 34,000 to 39,000 daltons which resembles the α -chain of previously described Ia antigens (Cullen *et al.* 1976, Cook *et al.* 1978). A β -chain equivalent molecule was not obvious, but the gel electrophoresis results of Figures 1 and 3 do not exclude the possibility of a second molecule of slightly lower molecular weight, indistinguishable in these experiments from the major (α) chain. While there is some indication of a 25,000 dalton molecule in Figure 3, such a molecule could exist and be undetectable because it is weakly labeled or serologically silent.

Further evidence for the 'Ia-like' nature of this molecule is indicated by the existence of a high molecular weight molecule in the molecular weight range of approximately 55,000 to 60,000 daltons (Figures 1 and 3) which is more readily detectable under non-reducing conditions of electrophoresis. This molecule could be a dimer of the 36,000 dalton molecule, or could comprise the 36,000 dalton molecule together with a second smaller chain of approximately 25,000 daltons. This larger molecule resembles the dimer of the α and β Ia chains previously detected by SDS-PAGE which has been characterised for Ia antigens mapping to both *I-A* and *I-E* subregions, and which can also be more easily demonstrated under non-reducing conditions of electrophoresis (Cullen *et al.* 1976, Cook *et al.* 1978).

Further evidence that the new specificities described here resemble Ia antigens, is indicated by their predominant expression on B cells, apparent by the reaction of the antisera with 50-55% of Ig-positive spleen cells and 35% of lymph node cells. While normal splenic T cells do not carry the antigen, its presence on thymocytes (30%) as well as on Ig-negative BALB/c *nu/nu* spleen cells (20%) suggests that the antigen may be present on certain immature T cells. Conventional Ia antigens have also been detected on these T cell populations (McKenzie and Potter 1979, Frelinger *et al.* 1974).

By using two antisera with activity for different allelic variants at this locus, it has been possible to assign different *I'* alleles in different strains, either by detection of positively reacting strains, or by deducing the haplotype origin of the specificity using various recombinant strains derived from the $H-2^d$ and $H-2^k$ strains. A summary of these results is given in Table 6. Since C3H.OH is *k* in the *I'* region, the recombination event which occurred between the *d* and *k*

strains giving rise to the recombinant haplotype must have occurred between *S* and *I'*. Similarly, B10.T(6R) which derives from *q* and *a*, involves a recombination event proximal to *I'*, as does A.TFR2 which is derived from *f* and *t2*. Further evidence that this new region is separate from the *D* region is that mutations which are known to affect the expression of H-2D (Dishkant *et al.* 1973, Klein *et al.* 1976, Brown *et al.* 1978) and H-2L (McKenzie *et al.* 1977, Hansen and Sachs 1978) antigens do not influence the *I'* specificities in B10.D2-H-2^{dm1} and BALB/c-H-2^{dm2} mice, respectively.

The specificities defined for the *I'* region so far represent a simple genetic system since no crossreactive determinants have been recognised with these two antisera. However, crossreactive determinants cannot be excluded until more antisera have been analysed. Detection of the antigen may also be difficult because it appears that only anti-H-2 sera contain the activity, and antibodies may only be generated in the presence of a concurrent strong antibody response to a *D* region difference. Furthermore, since the activity was not detected by complement-mediated lysis, the rosetting assay is needed to detect these new antigenic specificities.

The existence of a new *I*-like region, closely associated with H-2D raises the possibility that the postulated gene duplication event that gave rise to the *K* and *D* regions of the H-2 complex (Shreffler *et al.* 1971) may also have resulted in the duplication of a primordial *I* region. Since the recombination distance between the *K* and *S* regions is of a similar magnitude to that between *S* and *D* (Klein 1975), the new *I'* region represents only a part of the large, unmapped, genetic region proximal to *D*. There also appears to be a correlate of this second *I*-like region in the MHC complex in man, namely the HLA complex. Besides

the *HLA-D* locus, which maps proximal to *HLA-B* and is the human homologue of the murine *I* region, there is recent evidence for a second B cell antigen locus associated with weak mixed lymphocyte reactivity, which maps very close to the *HLA-A* locus and between *HLA-A* and *HLA-B* (van Rood *et al.* 1976, 1977). The association of *I*-like regions with either end of the *MHC* represents a further structural similarity between the *HLA* and the *H-2* complexes.

In line with current terminology, designation of this new region as *I*-like is dependent on association of the new region with an immune response gene (*Ir* gene) effect. While *Ir* gene effects on cytotoxic T cell responsiveness have been mapped to the *D* region, insufficient genetic data exists to determine whether these effects map to the new *I'* region (Simpson and Gordon 1977, von Boehmer *et al.* 1977, Doherty *et al.* 1978, Zinkernagel *et al.* 1978, Kurrle *et al.* 1978, Mullbacher and Blanden 1979). In the case of the *D*-linked *Ir* gene effect on autoimmune thyroiditis described by Kong *et al.* (1979), the *I'* region does not appear to be involved, since B10.AKM and B10.BR, which are both *k* at the *I'* region, differ in their responsiveness. Since this *D*-linked effect determines infiltration of mononuclear cells into the thyroid, this event could involve *H-2D* restricted cytotoxic T cells. Further functional studies are in progress to determine whether this new region plays any role in immune responsiveness and controls lymphocyte activating determinants.

SUMMARY

A new genetic region, mapping within the *H-2* complex, has been serologically defined with several alloantisera raised in mice which differ at the *D* region. When these antisera were absorbed to remove anti-*H-2D* antibodies, residual antibody activity remained that reacted

in a strain-specific manner, and the antigens involved mapped to a new genetic region between the *S* and *D* regions. Two allelic variants relating to the *d* and *k* haplotypes have been defined by genetic mapping studies. This new region has been designated *I'* since the antigens it controls appear to resemble Ia antigens in their cellular distribution and molecular weight. The new antigen is primarily expressed on B cells, and is carried on protein molecules with approximate molecular weights of 36,000 and 60,000 daltons which resemble the α -chains and α - β chain dimers characteristic of Ia antigens.

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Fig. 1 SDS-polyacrylamide gel electrophoresis of radioiodinated cell surface antigens immunoprecipitated from an NP-40 lysate of B10.A spleen cells by a (B10.AKM x 129) F_1 anti B10.A antiserum (AS285) or by normal mouse serum (NMS). The migration distance of bovine serum albumin (BSA), ovalbumin (OV), as well as heavy (H) and light chains (L) is shown in the upper graph, and the dye front in each gel is shown by \downarrow . The samples were run in a 10% polyacrylamide gel under reducing conditions.

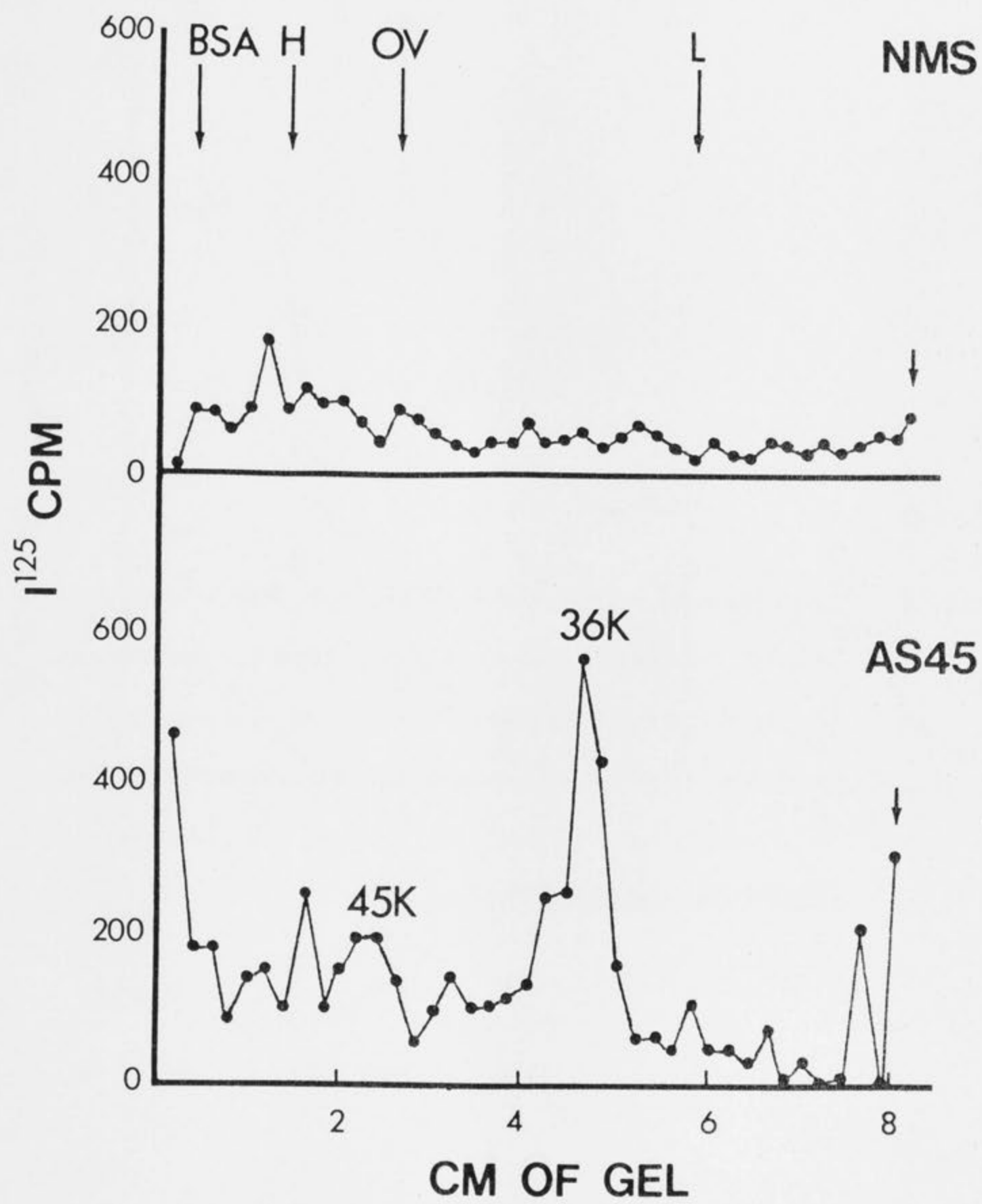


Fig. 2 Titration of a (B10.AKM x 129) F_1 anti B10.A antiserum (AS285) on B10.A spleen cells and thymocytes followed absorption of the antiserum with A.TH (\square), A.TL (Δ) or B10.T(6R) (\circ) spleen cells. Unabsorbed antiserum is included as a control (\bullet). Tube 1 represents a 1/100 dilution of antiserum.

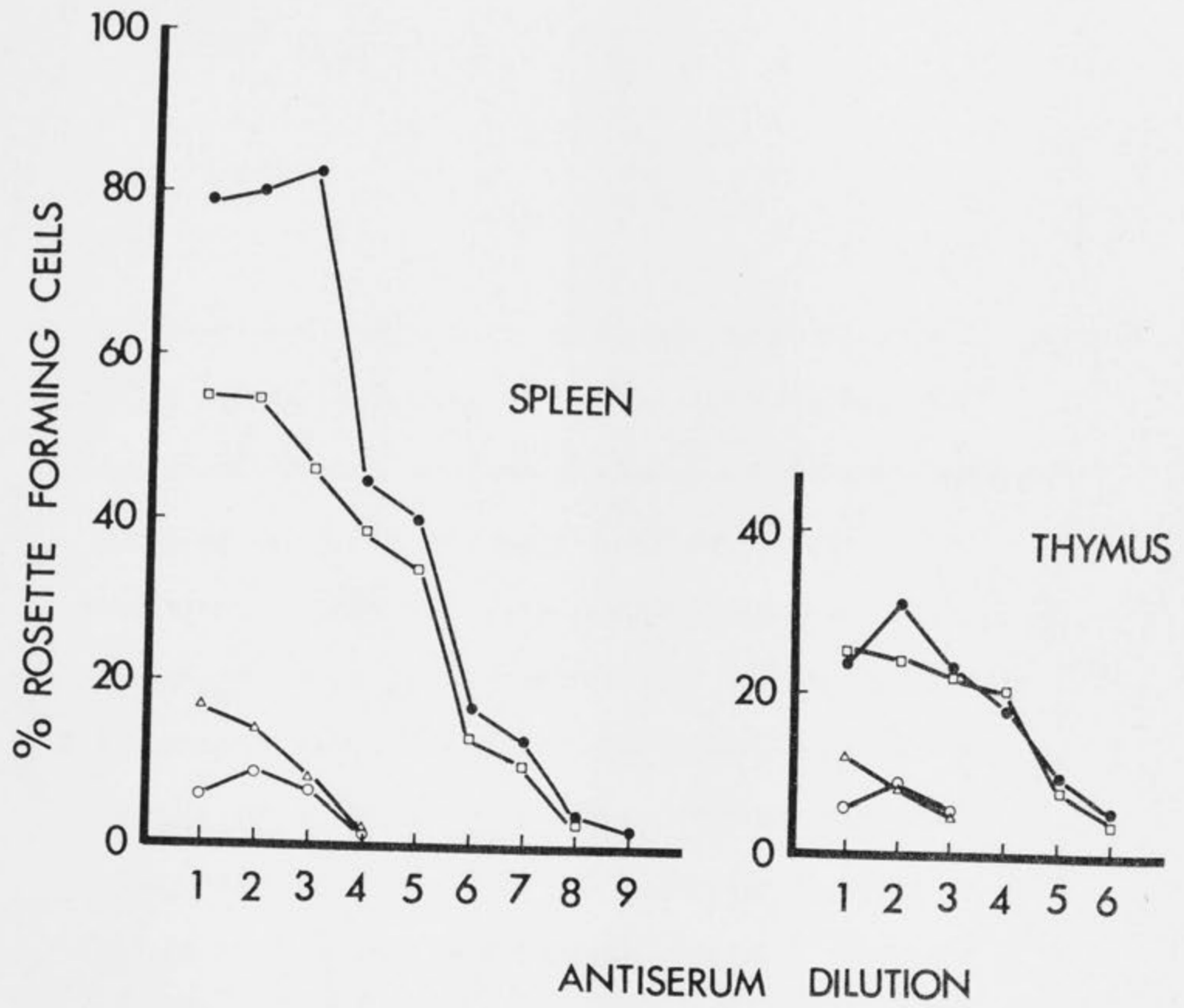


Fig. 3 Distribution of antigenic activity detectable with a (B10.AKM x 129) F_1 anti-B10.A antiserum (AS45), following electrophoresis under (A) reducing and (B) non-reducing conditions, of an NP-40 lysate of B10.A spleen cells on a 10% SDS-polyacrylamide gel. Material was extracted from the sliced gel and SDS removed by extensive dialysis. Antigenic activity in each fraction was measured by a rosette-inhibition assay, and represents the highest dilution of extract which gave a 25% inhibition of rosetting. Data is expressed as activity per ml of extract ($\times 10^{-3}$). In order to estimate the molecular weight of material contained in the different fractions, standard ^{125}I -labelled proteins were run on an accompanying gel. The standards used are described in Figure 1.

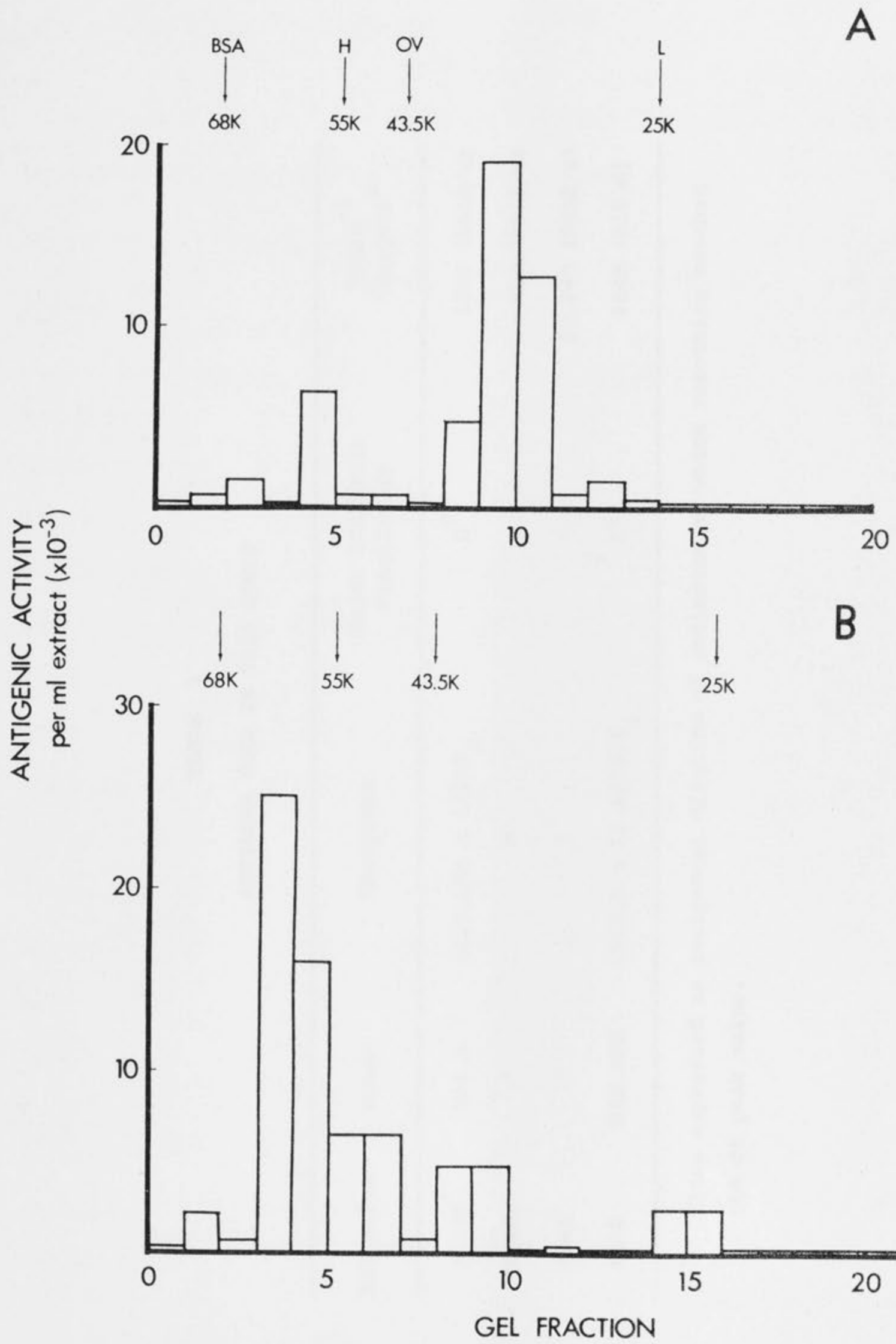


TABLE 1
ANTISERA USED IN THIS STUDY

Antiserum	Donor	Recipient	Major Antigenic Specificity	Titre ⁻¹ (target) ^a
AS285	B10.A	(B10.AKM x 129)F ₁	D ^d	1200 (BALB/c)
AS954	"	"	"	800 (BALB/c)
AS45	"	"	"	10,240 (BALB/c)
AS30	B10.AKM	(B10.A x LP.RIII)F ₁	D ^q	2048 (B10.G)

a Titre expressed as reciprocal dilution of antiserum at which rosetting reached 50% of peak value.

TABLE 2

REACTION OF ANTI-D^d (AS285) SERUM ABSORBED WITH A.TH SPLEEN CELLS
ON SPLEEN CELLS FROM DIFFERENT H-2 RECOMBINANT AND MUTANT MOUSE STRAINS

Strain	H-2 Haplotype ^b								Titre ⁻¹ _a	Reaction	Designated Haplotype
	K	I						S			
		A	B	J	E	C					
B10.G	q	q	q	q	q	q	q	q	<50	-	q
C57BL/10	b	b	b	b	b	b	b	b	<50	-	b
B10.BR	k	k	k	k	k	k	k	k	<50	-	k
BALB/c, B10.D2	d	d	d	d	d	d	d	d	1600	+	d
SJL	s	s	s	s	s	s	s	s	<50	-	s
D2.GD	d	d	d	b	b	b	b	b	<50	-	b
B10.A	k	k	k	k	k	d	d	d	1600	+	d
B10.A(2R)	k	k	k	k	k	d	d	b	<50	-	b
C3H.OH	d	d	d	d	d	d	d	k	<50	-	k
C3H.OL	d	d	d	d	d	d	k	k	<50	-	k
B10.T(6R)	q	q	q	q	q	q	q	d	800	+	d
A.TL	s	k	k	k	k	k	k	k	1600	+	d
A.TH	s	s	s	s	s	s	s	s	<50	-	s
A.TFR2	f	f	f	f	f	f	f	d	<50	-	s
A.TFR5	f	f	k	d	1600	+	d
BALB/c-H-2 ^{dm2}	d	d	d	d	d	d	d	dm2 ^c	1600	+	d
B10.D2-H-2 ^{dml}	d	d	d	d	d	d	d	dml ^c	1600	+	d

a See Table 1.

b Haplotype origin of strains according to Klein *et al.* (1978).

c Symbols represent mutations in the D region.

TABLE 3

REACTION OF ANTI-D^q (AS30) SERUM ABSORBED WITH B10.G ON
SPLEEN CELLS FROM DIFFERENT *H-2* RECOMBINANT MOUSE STRAINS

Strain	H-2 Haplotype								Titre ⁻¹	Reaction	Designated Haplotype	
	K	I						S				D
		A	B	J	E	C						
B10.G	q	q	q	q	q	q	q	q	<40	-	q	
C57BL/10	b	b	b	b	b	b	b	b	<40	-	b	
B10.BR	k	k	k	k	k	k	k	k	320	+	k	
BALB/c	d	d	d	d	d	d	d	d	<40	-	d	
SJL	s	s	s	s	s	s	s	s	<40	-	s	
C3H.OH	d	d	d	d	d	d	d	k	320	+	k	
C3H.OL	d	d	d	d	d	d	k	k	320	+	k	
A.TL	s	k	k	k	k	k	k	d	<40	-	d	
B10.AKM	k	k	k	k	k	k	k	q	320	+	k	

Footnotes as in Table 2.

TABLE 4
 REACTION OF ANTI-D^d SERUM ABSORBED WITH
 A.TH SPLEEN CELLS ON DIFFERENT CELL POPULATIONS

Cell Type	Maximum % RFC ^a	
	<u>A.TL</u>	<u>A.TH</u>
Spleen	50 (800)	10
Ig-negative spleen	10	6
Thymus	30 (800)	4
Bone marrow	12	5
Lymph node	35 (800)	8
	<u>BALB/c nu/nu</u>	<u>BALB/c littermate</u>
Spleen	50 (800)	50 (800)
Ig-negative spleen	20 (400)	5

a When a clearly positive reaction was obtained, end point titres are given in brackets. This represents the reciprocal dilution of antiserum AS954 which gave 20% RFC.

TABLE 5

DISTRIBUTION OF NEW ANTIGEN ON THY 1.2⁺ CELLS

Cell Preparation	% Thy 1.2 ⁺ Cells ^a	% RFC ^b	% Thy 1.2 ⁺ RFC ^c
A.TL Ig-negative spleen	90	10	<0.2
A.TL Ig-capped spleen	43	48	<0.2

a Cells were absorbed with monoclonal anti-Thy1.2-biotin conjugate, and then reacted with FITC-avidin. % fluorescent cells was calculated.

b Percentage of cells which reacted with anti-D^d (AS954) serum, preabsorbed with A.TH spleen cells, was estimated by the rosetting assay.

c Percentage of RFC which carry fluorescent label.

TABLE 6

DESIGNATION OF *I'* HAPLOTYPE IN MOUSE STRAINS USED IN THIS STUDY

Strain	H-2 Haplotype ^b							[<i>I'</i>] ^a	D
	K	IA	IB	IJ	IE	IC	S		
C57BL/6, C57BL/10	b	b	b	b	b	b	b	b	b
CBA/H, B10.BR	k	k	k	k	k	k	k	k	k
BALB/c, B10.D2	d	d	d	d	d	d	d	d	d
B10.G	q	q	q	q	q	q	q	q	q
SJL	s	s	s	s	s	s	s	s	s
D2.GD	d	d	d	b	b	b	b	b	b
B10.A	k	k	k	k	k	d	d	d	d
B10.A(2R)	k	k	k	k	k	d	d	b	b
B10.T(6R)	q	q	q	q	q	q	q	d	d
B10.AKM	k	k	k	k	k	k	k	k	q
C3H.OH	d	d	d	d	d	d	d	k	k
C3H.OL	d	d	d	d	d	d	k	k	k
A.TL	s	k	k	k	k	k	k	d	d
A.TH	s	s	s	s	s	s	s	s	d
A.TFR2	f	f	f	f	f	f	f	s	d
A.TFR5	f	f	k	d	d
BALB/c <i>H-2</i> ^{dm2}	d	d	d	d	d	d	d	d	dm2 ^c
B10.D2 <i>H-2</i> ^{dml}	d	d	d	d	d	d	d	d	dml ^c

a Haplotype origin and map location of the new *I*-like region designated *I'*, is deduced from the patterns of reactivity in Tables 2 and 3.

b,c See Table 2.

A MODEL FOR IMMEDIATE-EARLY AND LATE-INDUCED T CELL ACTIVATION
IN T CELL RESPONSE

1. Introduction
2. A new concept for the T-H system
3. Characteristics of the T cell response
4. A model for immediate T cell activation
5. A model for late activation of the T cell pool

CHAPTER 9

GENERAL DISCUSSION

1. Implications
2. Clinical application of T cell response
3. Antigen-specific response in peripheral T cells
4. The anti-viral response

A ROLE FOR CARBOHYDRATE-DEFINED AND PROTEIN-DEFINED H-2 ANTIGENS
IN T CELL RECOGNITION

1. Introduction.
2. A new concept for the *H-2* region.
3. Carbohydrate-defined H-2 antigens.
4. A model for cytotoxic T cell recognition.
5. A model for thymic selection of the T cell pool.
6. Implications of the models.
 - 6.1 Clonal expression of T cell receptors.
 - 6.2 Antigen-specific receptors on cytotoxic T cells.
 - 6.3 The anti-self receptor.

1. Introduction

The original idea that cellular interactions important for the regulation of biological phenomena may be mediated by highly specific protein-carbohydrate interactions on adjacent cells was suggested by Roseman in 1970. He proposed that such interactions might occur via glycosylation of an appropriate carbohydrate determinant on one cell by a glycosyltransferase enzyme on another cell, and that after attachment of a new monosaccharide residue, the enzyme substrate complex would dissociate and the cells separate. With subsequent demonstrations that glycosyltransferases can be detected on intact cells of many kinds, these enzymes now seem to be logical candidates for eliciting interactions between cells known to occur via protein-carbohydrate interaction at the cell surface (reviewed by Shur and Roth 1975).

There is now considerable evidence which suggests that glycosyltransferases are involved in a diversity of recognition functions, such as the specific adhesion between egg and sperm during fertilisation, recognition and synaptogenesis in nervous tissue, adherence of blood platelets to collagen during hemostasis, cellular interactions during morphogenesis and embryonic development, contact inhibition of cell growth in culture as well as adhesion and repair of cell surfaces. Most of these phenomena have been reviewed by Shur and Roth (1975). Studies on the loss of contact inhibition of growth in tumour cells and cell lines transformed with oncogenic viruses, have implicated a biochemical basis for malignancy which also rests on glycosyltransferase function (Shur and Roth 1975, Hakomori 1975). Such cells exhibit no cell density-dependent synthesis of complex glycolipids, as do normal cells, and express aberrant glycolipids, as well as low levels of

surface glycosyltransferases (Hakamori 1975) and higher than normal levels of glycosidases (Flowers and Sharon 1979).

With evidence for the existence of both carbohydrate-defined and protein-defined H-2 antigens, as well as the need for involvement of glycosyltransferases in the synthesis of the carbohydrate antigens (discussed in Chapter 7), cell surface glycosylation becomes an attractive model for T cell recognition. In the following pages, variability in the expression of the different H-2 antigens is discussed, and their possible role in T cell recognition is considered.

2. A new concept for the H-2 region

The most significant finding to emerge from this thesis is the demonstration that H-2 antigenicity can reside in carbohydrate determinants present on molecules which appear to be glycolipids. While the data presented in Chapter 7 concerns antigens controlled by the *K* region, at a late stage in the writing of this manuscript, similar results were also obtained for antigens controlled by the *D* region (results not shown). Such results have only been possible with the use of monoclonal antibodies, and followed as a result of their use in demonstrating the existence of two classes of H-2K^k molecules (Chapter 6). Subsequent biochemical characterisation of the antigenic determinants recognised by several different anti-H-2K^k antibodies has led to the fortuitous finding that chemically different H-2 antigenic determinants exist, and that these are carried on very different molecules (Chapter 7).

2.1 Cell surface glycolipids

The proposal that glycolipid molecules carry carbohydrate-defined H-2 antigens is not unusual, since glycolipids are found ubiquitously on cell surfaces, and are often responsible for antigenic activity.

For example, many human blood group antigens, including ABH, Lewis, P group, and others, are defined by carbohydrate determinants present on glycolipid carriers (reviewed by Hakamori and Kobata 1974). The Thyl.2 antigen, the Forssman antigen, as well as certain Ia antigens (Alving 1977, Milewicz *et al.* 1976, Parish and McKenzie 1980) are also carbohydrate-defined and are present on cell surface glycolipids. The principle glycolipids of mammalian tissues are the glycosphingolipids, and those which contain sialic acid residues, which are always terminating sugars on the oligosaccharide chain, constitute the 'gangliosides'. Both H-2 and Ia glycolipids would fall into this category since both have been shown to carry sialic acid residues (Chapter 7, Parish *et al.* 1976c). Gangliosides are composed of a hydrophobic N-acylsphingosine moiety and one or more sugar units attached to the terminal hydroxyl group of sphingosine. Preliminary assays have indicated high levels of sphingosine in low molecular weight H-2 molecules extracted from gels after electrophoresis (results not shown). The carbohydrate moieties so far detected on H-2K^k glycolipids, i.e. sialic acid, D-mannose, and D-galactose (see Chapter 7), are also common components of the oligosaccharide side-chains of glycoprotein H-2 molecules (Muramatsu and Nathenson 1971). The presence of D-mannose in a glycolipid is unusual (Sharon 1975), but may reflect functional specificity associated with H-2 molecules.

Gangliosides have also been characterised as the receptor sites for many regulatory molecules including cholera toxin and tetanus toxin (van Heyningen 1974), botulinum toxin (Simpson and Rapport 1971), serotonin (van Heyningen 1974), interferon (Vengris *et al.* 1976), and thyroid stimulating hormone (Mullin *et al.* 1978). Their importance as receptors for immunoregulatory molecules has also been demonstrated

since the receptor site on macrophages for migration inhibition factor appears to be a ganglioside (Higgins *et al.* 1978).

2.2 Synthesis of carbohydrate H-2 antigens

The simplest interpretation for the functional linkage between carbohydrate-defined and protein-defined H-2 antigenic determinants is that the *H-2* region contains the structural genes for glycosyltransferase enzymes which are involved in the synthesis of carbohydrate H-2 antigens, i.e. H-2 glycoproteins are glycosyltransferases. Optionally, *H-2* may contain regulators of glycosyltransferase function. These proposals were outlined only briefly in Chapter 7. The mechanisms by which glycosyltransferases synthesize complex carbohydrates are well understood, and were discussed in Roseman's original review article in 1970. These enzymes act by sequentially attaching different nucleotide sugar units (e.g. UDP-galactose, GDP-mannose or CMP-sialic acid) to the terminal carbohydrate unit of a growing oligosaccharide chain, and each enzyme involved is both specific for one particular carbohydrate unit on the chain, as well as for the saccharide unit which it attaches. A battery of glycosyltransferases must therefore cooperate in the building of oligosaccharide chains in a specific sequential manner. In the case of H-2 glycolipids, molecules would have to be synthesized and then transported to the external cell membrane and if the enzymes involved in synthesis of carbohydrate H-2 antigens are themselves glycoprotein H-2 molecules, then these are also expressed on the cell membrane. A proposed mechanism for the synthesis of carbohydrate H-2 antigens is depicted in Figure 1 for the situation where the *H-2* gene actually encodes a glycosyltransferase enzyme which is subsequently expressed on the cell surface. Carbohydrate antigenic determinants

could be either synthesized intracellularly on glycolipid carriers and then transported to the cell membrane, or they could be synthesized on glycolipids on the surface of a cell by an *H-2* encoded glycosyltransferase present on an adjacent cell.

If interactions between glycolipids and glycoproteins, involving glycosyltransferase activity, are the process by which lymphocytes recognise *H-2* and *Ia* antigens on cells as was outlined in Chapter 7, then restrictions will have to be placed on the repertoire of enzymes located on the cell surface, as well as on the nature of the terminating sugars present on glycolipid *H-2* molecules. This matter is discussed further in Section 4, where a model is proposed for cytotoxic T cell (*Tc* cell) recognition involving the different *H-2* molecules.

2.3 Glycosyltransferases and the *H-2* complex

Of more direct relevance to the postulate that *H-2* genes control cell-surface glycosyltransferase activity is the finding that cell surface antigens controlled by the *H-2*-linked *T/t* locus may be functionally related to glycosyltransferases (Shur and Bennett 1979, Shur *et al.* 1979). This locus is known to control morphogenetic interactions during embryonic development and many *t* alleles have been defined which have diverse effects on embryonic development as well as male sterility (Klein and Hammerberg 1977). Spermatazoa from mouse strains carrying certain *t* alleles have recently been shown to have increased cell surface galactosyltransferase activity over wild type strains (Shur and Bennett 1979). This increase has been related to the absence of a specific enzyme inhibitor, and it has been proposed that the *T/t* genetic region regulates glycosyltransferase activity, and in this respect, influences sperm function, fertilisation and subsequent embryonic development.

As a result of this finding, it is interesting to speculate on the strong linkage disequilibrium which exists between the *H-2* and *T/t* regions (Klein 1975). Since evidence for the existence of *H-2* glycolipids suggests that *H-2* also controls glycosyltransferase enzymes, this may reflect a selective mechanism aimed at maintaining a close linkage between many genes in the vicinity of *H-2* which code for or regulate cell surface glycosyltransferases. Since the antigenic determinants of the *T* locus are now known to be carbohydrate-defined (Cheng and Bennett 1979), it is interesting to speculate that the *H-2* region contains a string of genes which code for cell surface glycosyltransferases involved in cellular interactions during embryogenesis and immune recognition.

This hypothesis is attractive in the light of current evidence for molecular similarity between antigens controlled by *K*, *D*, *T1a*, *T/t*, as well as the region between *H-2* and *T1a* which codes for the *Qa* antigens. Their respective *K*, *D/L*, *TL*, *F9* and *Qa-2* antigens are all detectable on molecules of approximately 45,000 daltons which are either associated with β_2 -microglobulin, or a molecule which resembles it (Silver and Hood 1974, Ostberg *et al.* 1975, Vitetta *et al.* 1975a,b, Michaelson *et al.* 1977, Hansen and Sachs 1978). Of further significance is the fact that these different *H-2* coded antigens appear to be 'alternatives' as differentiation antigens. While the *F9* antigens are detectable during embryogenesis, their disappearance correlates with the appearance of *H-2* antigens on maturing cells (Artz and Bennett 1975). Similarly there appears to be an inverse relationship between the expression of *TL* and *H-2D* antigens on thymocytes (Boyse *et al.* 1968), as well as between *TL* and *Qa-2* antigens on T cells (Flaherty 1976).

Fundamental chemical similarity between various molecules controlled by the *H-2* region is also evident from the fact that besides the *K*, *D*, *Ia* and *L* molecules, several other molecules carrying antigens which map to the *Qa/T1a* region, can also act as targets for alloreactive Tc cells without being restricted by *K* or *D* (Wernet and Klein 1979, Forman and Flaherty 1978, Lindahl 1978, Kastner and Rich 1979, Hansen and Levy 1978). This, of course, contrasts with the strict functional specificity associated with different *H-2*-controlled molecules, indicated by the fact that only *K*, *D* and probably *L* can act as restriction antigens in *H-2* restricted Tc cell responses (reviewed by Zinkernagel and Doherty 1979).

A common role for *H-2* region gene products in cellular interactions could serve as an explanation for the fact that the *H-2* complex is highly pleiotropic, influencing a wide range of biological phenomena ranging from immune responsiveness to self-recognition and differentiation. Among these effects are not only immune recognition regulated by *K*, *D* and *I* regions (outlined in Chapter 1), but also spermatogenesis and embryogenesis (Klein and Hammerberg 1977), susceptibility to a number of viruses, and diseases (reviewed by Zinkernagel 1979), cellular interactions such as the honing of lymphocytes controlled by the *K* and *D* regions (Degos *et al.* 1979), the autorosetting phenomenon believed to be controlled by the *L* locus (Sia and Parish 1980a,b), cell adhesion (Bartlett and Edidin 1978, Zeleny *et al.* 1978) as well as aging (Smith and Walford 1977) and mating preference (Yamazaki *et al.* 1979).

3. Carbohydrate-defined H-2 antigens

With evidence now for the existence of carbohydrate-defined H-2 antigens, current serological and functional information on H-2 expression needs to be reinterpreted. In principle, alloantiserum

preparations, used in the past for serological studies with H-2 antigens, should contain antibodies specific for both carbohydrate-defined and protein-defined H-2 antigens.

Many variables could however affect the nature and production of antibodies in any alloantiserum preparation specific for the two types of antigens. These could include differences in the immunogenicity of carbohydrate and protein determinants, the relative number of glycolipid and glycoprotein molecules on the priming cells, as well as genetic differences in the mouse strains used to produce the antisera. While carbohydrate-defined and protein-defined H-2K^k molecules, defined by the 27R9 and 30R3 monoclonal antibodies, are known to be expressed equally on spleen cells (Chapter 6), similar monoclonal antibody analysis of the relative numbers of the two types of molecules carrying the Ia.17 specificity has indicated a four-fold excess of glycolipids (results not shown). It is not yet known whether these two examples are representative of the relative numbers of glycolipid and glycoprotein molecules controlled by all *H-2* and *I-A* alleles, or whether different relative numbers exist in different strains of mice.

With evidence for the existence of both carbohydrate- and protein-defined H-2 antigens, further explanations must also be included for the variation in expression of H-2K and H-2D molecules in F₁ hybrids which was presented in Chapters 2, 3 and 5. While these results are still significant in their own right, mechanisms for *H-2K*- and *H-2D*-linked control of antigen expression in F₁ hybrids (Chapter 5) will have to be reconsidered. While only quantitative differences have been detected so far (Chapter 3), qualitative differences cannot yet be excluded as the cause of variation in antigen expression. Furthermore, any model for the genetic control of H-2 expression in F₁ hybrids must

also account for the dominance pattern observed between different *K* and *D* alleles (Chapter 5), as well as the existence of two chemically different types of H-2 molecules (Chapter 7).

One interesting possibility is that F_1 hybrids can generate specific glycolipid molecules which differ from their counterparts on parental strain cells. Based on the previously described mechanism for synthesis of carbohydrate H-2 antigens, hybrid determinants could be generated if at least one glycosyltransferase derived from one of the parents could substitute at one step in the synthesis of the carbohydrate chain constructed by glycosyltransferases derived from the other parent. Depending on the amount of 'interplay' possible, so a variable number of parental and hybrid carbohydrate H-2 antigens could be generated. Such a mechanism could account not only for differences in the expression of some antigens between parent and F_1 hybrid cells, but also for the dominance pattern which has been described. There would have to be no interplay between *K*- and *D*-controlled glycosyltransferases, since there appears to be no *K*-linked control of *D* antigen expression, and vice versa (Chapters 4 and 5).

Such a model predicts heterogeneity amongst H-2 antigens present on F_1 hybrid glycolipid molecules, and an indication of such a phenomenon has already been presented in Chapter 6. The 30R3 monoclonal antibody, specific for a carbohydrate determinant, detected only one third of the total number of H-2K^k molecules detectable in two different F_1 hybrids with anti-H-2K^k alloantisera, compared with a half for parental strains. Such a loss of H-2K^k glycolipid molecules is consistent with heterogeneity amongst F_1 hybrid H-2K^k molecules. However, there was also a concurrent loss of glycoprotein H-2K^k molecules defined by the 27R9 monoclonal antibody. A possible explanation for variability

attributable to glycoprotein H-2 antigen expression in F_1 hybrids is considered in Section 5.2.

Positive indication of F_1 hybrid-specific H-2 antigenic determinants could be obtained with the use of monoclonal antibodies raised in parent anti- F_1 hybrid mouse combinations. Furthermore, by the use of limiting dilution analysis, clones of parent anti- F_1 hybrid allo-reactive Tc cells could be screened for the existence of clones with specificity for determinants present on F_1 hybrid target cells which are not present on parental strain targets.

Similar proposals could also be used to explain the existence of F_1 hybrid-specific Ia antigens, already described by Fathman (1978), in mixed lymphocyte reactions, and by Lafuse *et al.* (1980) by serological means. For Ia antigens, interpretations would also have to incorporate interplay between glycosyltransferases controlled by the *I-A* and *I-E* subregions, in order to explain complementation between these genes in the synthesis of glycoprotein Ia dimers (Jones *et al.* 1978, Cook *et al.* 1978).

Previous interpretations of the role that quantitative differences in H-2K and H-2D expression might play in Tc cell responsiveness (Chapters 2, 3, 4 and 5), must also be reconsidered in the light of subsequent evidence for H-2 carbohydrate-defined antigens (Chapter 7). While the influence that quantitative differences in H-2 expression on target and stimulator cells have on Tc cell responsiveness cannot be refuted (Chapters 2 and 3), differences in the number of H-2 antigens on the responding T cell population could have much more profound effects on Tc cell responsiveness if these molecules are, in fact, found to be the actual T cell receptors. Without knowledge of the exact role of different types of molecules, no further interpretation can be given at this point.

Further unanswered questions concern the expression of the two different types of H-2 molecules on cells of different tissues as well as on different classes of lymphoid cells with different functions and activation states. Also, the effect of interferon on enhancing H-2 antigen expression on T cells is made more interesting by these findings (Lonai and Steinman 1977, Vignaux and Gresser 1977), and studies on the ontogeny and turnover of the two types of molecules should give clues to the mechanisms which control their synthesis and expression. Expression of the two types of antigens on various H-2 mutant mouse strains is also under investigation. One might predict that a mutation in a *K* or *D* gene may not only affect the relevant H-2 glycoprotein itself, but could also lead to aberrant glycosyltransferase function and perhaps the synthesis of altered carbohydrate H-2 antigens. Such a proposition can be easily tested as appropriate monoclonal antibodies become available.

Any changes in the expression of the different types of H-2 molecules on both virus-infected cells, as well as tumour cells, will also be of interest, especially since aberrant synthesis of carbohydrate sidechains could easily generate new antigenic determinants which could represent an allogeneic stimulus to any responding T cell (see Section 4). The expression of abnormal carbohydrate sidechains on cell surface glycolipids of both naturally occurring tumour cells as well as cell lines transformed with oncogenic viruses such as polyoma, SV40 and Rous sarcoma virus, is already well documented (reviewed by Hakamori 1975). Many accounts of the existence of quantitative as well as qualitative differences in H-2 antigen expression on tumour cells (reviewed by Parmiani *et al.* 1979) could also relate to blocked or aberrant carbohydrate H-2 synthesis, as could

the presence of foreign H-2 determinants on tumour cells detected both serologically and by Tc cell recognition (Garrido *et al.* 1976a,b, Meschini *et al.* 1977, Festenstein *et al.* 1979, see also *Journal of Immunogenetics* 7, 1980).

4. A model for cytotoxic T cell recognition

With evidence for the existence of a second type of H-2 antigenic determinant defined by carbohydrate, new potential is given to the old problem of defining the role of *MHC* antigens in T cell recognition. With need for the intervention of *H-2*-controlled glycosyltransferases in the synthesis of these antigens, the possibility that such enzymes present on the surface of T cells are actually involved in the generation of oligosaccharide chains on the surface membrane of another cell, raises the possibility that such a mechanism constitutes H-2 antigen involvement in T cell recognition. This idea is based on Roseman's (1970) original hypothesis for cellular interaction which was outlined in Section 1, and involves recognition of a carbohydrate determinant on one cell by a specific glycosyltransferase present on an adjacent cell. Although it has not yet been determined whether or not the glycoprotein H-2 antigens are actually the glycosyltransferases themselves, a model for T cell recognition can still be proposed involving interaction between an *H-2*-controlled enzyme receptor on a T cell and a carbohydrate H-2 determinant on a target cell which constitutes the T cell recognition site. Since the two components of the system are controlled by the same gene, the model has inherent self specificity not previously described by any other model. Furthermore, the following model also offers a satisfactory explanation for the role of *MHC* antigens in both allogeneic and self recognition capacities, a feature not easily incorporated into previous models for T cell recognition.

4.1 Recognition of self

Formulation of the model relies upon the assumption that *H-2*-controlled glycosyltransferases on the surface of T cells are the anti-self receptors which mediate active recognition of 'self', by binding to appropriate carbohydrate determinants present on target cells which they can subsequently glycosylate. This mechanism is depicted in the first section of Figure 2. (This figure represents a general mechanism for lymphocyte recognition involving any *MHC*-controlled antigens.) Such a process of self recognition would lead to dissociation of the cells after involvement of an appropriate nucleotide sugar (e.g. UDP- \square) in the attachment of a carbohydrate unit to an incomplete oligosaccharide sidechain of the target antigen. Such a process will lead to the generation of new antigenic determinants, which would then require glycosyltransferases with different specificity for any subsequent T cell interactions. If these interactions are the process by which lymphocytes recognise self *H-2* antigens on other cells, then restrictions will have to be placed on the repertoire of enzymes which T cells can express, as well as on the nature of the target antigens.

Several assumptions are required:

1. There must be continual synthesis of incompletely glycosylated target antigens, followed by loss or degradation of these molecules after synthesis of their carbohydrate sidechains is completed. One interesting possibility is that only core carbohydrate structures are present on the glycolipid molecules when they are first detectable on the cell surface and these are synthesized by specific intracellular glycosyltransferases which may not be under the control of the *H-2* gene complex.

2. There would have to be at least one, or a specific repertoire of *H-2*-controlled glycosyltransferases which are uniquely cell surface antigens. If a number of enzymes exist, then natural selection would favour conservation of a bank of genes which control the synthesis of a battery of enzymes whose functions are interrelated. A further role for the thymus in this selection process is outlined in the following section.

4.2 Alloantigen recognition

The same anti-self receptors could also be involved in alloantigen recognition. The specificity of glycosyltransferases is reflected in both the nucleotide sugar which they attach, as well as in the carbohydrate unit which they utilise as a substrate. However, they may also bind to carbohydrate structures which have incorrect orientation for glycosylation and such sites may constitute an internal or inappropriately linked terminal unit on an oligosaccharide chain. Glycosyltransferase recognition of any such crossreactive sites on an allogeneic target could lead to enzyme binding with no subsequent glycosylation. Such a mechanism is depicted by the second diagram in Figure 2, and should not occur in the normal animal where a repertoire of glycosyltransferases would have been selected for their specific functions. Binding with no glycosylation could trigger subsequent cell activation or lytic mechanisms which are linked to the function of the glycosyltransferase itself. As a result, this model proposes a bifunctional role for the anti-self receptor in both self-recognition as well as alloantigen recognition.

4.3 Recognition of foreign antigens

The same anti-self receptor could also cooperate with an antigen-specific receptor (anti-X) in the *H-2* restricted recognition of a foreign (X) antigen. The generation, selection and function of these receptors is discussed in later sections of this chapter. It is proposed that only those anti-X receptors which are in some way linked up to an anti-self receptor can trigger subsequent T cell activation. Such a mechanism involving two linked receptors has already been proposed by Cohn and Epstein (1978), and more recently by Williamson (1980). The mechanism relies on initial anti-X binding to the X antigen, followed by binding of the linked anti-self receptor to an appropriate nearby carbohydrate antigen on the target cell. By this model, which is outlined in the third diagram of Figure 2, the binding of anti-X alone is an insufficient trigger for T cell activation. However, the constraint of concurrent anti-X binding by a linked receptor could prevent anti-self receptors, i.e. *H-2*-controlled glycosyltransferases, from glycosylating self target antigens. This could lead to cellular activation as described for alloreactive recognition (Diagram 2, Figure 2), and the generation of clones of Tc cells carrying appropriate anti-X receptors which are specifically reactive for target cells carrying self *H-2* antigens as well as the particular X antigen. One further possibility is that membrane perturbation or exposure of core carbohydrate determinants as a result of anti-X binding could also constitute foreign target antigens for anti-self receptors.

4.4 Features of the model

The model presented here differs from any other previous models for H-2 antigen involvement in T cell recognition (see Chapter 1) by the use of H-2 glycoproteins on T cells as receptors for self H-2 carbohydrate determinants and its dependence on an active self recognition mechanism. Salient features of the model can be summarised as follows:

1. The model incorporates an active immune surveillance mechanism mediated by self recognition and based on the continuous synthesis of carbohydrate-defined H-2 antigens. Aberrant antigen expression on glycolipid H-2 molecules, e.g. in virally infected or tumour cells, could lead to *in vivo* activation of Tc cells and elimination of aberrant cells.
2. Unlike previous models, this model incorporates a biochemical role for the H-2 target antigen as well as for the anti-self receptor on Tc cells.
3. The same anti-self Tc cell receptor can function both in self recognition and in alloantigen recognition.
4. Since every T cell receptor has potential alloreactivity, this model can also explain the existence of a much larger number of alloreactive precursor Tc cells (Skinner and Marbrook 1976, Lindahl and Wilson 1977, Marbrook *et al.* 1978), compared with H-2 restricted precursors (Komatsu *et al.* 1978, Teh 1979, Ashman and Mullbacher - personal communication).

5. By this model, *H-2* restricted Tc cell recognition can be accommodated by either a 'dual recognition' mechanism via an anti-X as well as an anti-self receptor, or an 'altered self' mechanism involving allogeneic recognition by the anti-self receptor alone. Further consideration of these two possible mechanisms is outlined in Section 6.2.
6. The mechanism for X recognition can also accommodate both *H-2* restricted recognition of X, as well as recognition of X plus alloantigen. Apart from the normal alloreactive component of the response, the generation of these responses may depend upon the potential crossreactivity inherent in the glycosyltransferase - sugar composition of the strain combinations involved and perhaps, too, in the nature of the X antigen association with *H-2* carbohydrate determinants. The model can therefore provide an explanation for the 'aberrant recognition' phenomenon described in negative selection experiments by several workers (Wilson *et al.* 1977, Doherty and Bennink 1979), as well as the only reported example of crossreactivity in an *H-2* restricted response involving normal animals responding to the HY antigen (Simpson *et al.* 1978).
7. *Ir* gene effects can also be incorporated at the level of target cell recognition by this model. Such effects could relate to the relative number of *H-2* and X antigens expressed on target cells as was discussed in Chapters 4 and 5. By this model, such effects will depend on whether anti-X and anti-self receptor binding can occur concurrently.

8. Based on this same model for self-non-self discrimination, a mechanism for thymic selection of the T cell pool can also be invoked (see Section 5).

While the model has been proposed for Tc cell recognition, its principles should be equally applicable to the appropriate activation of T cell precursors for help, suppression and proliferation. Since each of the *K*, *D* and *I* regions appear to function similarly in the synthesis of antigenic determinants (Parish and McKenzie 1980, Chapter 7), the same basic mechanism could also apply irrespective of whether *K*, *D* or *Ia* antigens are involved.

At the moment, we have no clear idea at the molecular level of how receptor binding of antigen at the lymphocyte surface leads to cell activation, but several findings on the physiological requirements for Tc cell recognition support the proposal for enzymic intervention (reviewed by Henney 1977, Zinkernagel and Doherty 1979). For example, while effector cells have to be metabolically active, only antigen presentation is required of target cells. Divalent cations such as Ca^{++} and Mg^{++} are mandatory for the reaction, but DNA, RNA and *de novo* protein synthesis are not necessary to trigger subsequent target cell destruction or lymphocyte activation, and while these mechanisms are undescribed, they could involve subsequent enzyme-mediated steps.

5. A model for thymic selection of the T cell pool

In order to account for the proposed model for T cell recognition, it is necessary to eliminate T cells carrying self-reactive glycosyltransferase receptors. It is expected that preselection of T cells in

the thymus for their self recognition capacity must therefore serve four main roles. These can be listed as follows:

1. Elimination of cells bearing *K* gene-controlled receptors which are reactive with self *D* antigens, and vice versa.
2. Elimination of F_1 hybrid cells bearing parent 1 receptors which are reactive with parent 2 antigens, and vice versa. This feature will have importance for outbred populations.
3. Rapid selective loss of thymocytes carrying unwanted mutations in genes which code for, or control, glycosyltransferases.
4. A secondary role for the thymus may also be required to select out T cells carrying anti-X receptors which are reactive with self H-2 antigens.

As outlined in Chapter 1, a functional role now seems apparent for *K*, *D* and *I* region antigens expressed on some radioresistant thymic cell in the selection of the repertoire of T cell receptors. Although these antigens are known to be expressed confluenty on epithelial cells of the thymic medulla, as well as variably on the dendritic cells of the cortex (Rouse *et al.* 1979), how these antigens are involved is unknown. Although there is no information currently available on the chemical nature of the H-2 antigens expressed on thymocytes, the carbohydrate-defined antigens appear to be appropriate antigenic candidates for the selection of anti-self receptors on T cells.

A model for thymic selection involving both carbohydrate-defined and protein-defined H-2 antigens has therefore been devised, based on the previously described model for Tc cell recognition (Section 4). At this point no consideration is given to the selection of anti-X receptors, since in terms of the model proposed for Tc cell recognition, they do not determine H-2 restriction specificity. Their requirement in T cell recognition, as well as their generation, are discussed in Section 6.2.

5.1 Outline of the basic mechanism

Immature lymphocytes entering the thymus which express H-2-controlled glycosyltransferases, i.e. anti-self receptors, will be subject to interaction with carbohydrate-defined H-2 antigens expressed on glycolipid molecules present on some resident cell in the thymus. According to the proposed model for T cell recognition involving interaction between carbohydrate-defined and protein-defined cell surface antigens depicted in Figure 2, several possible outcomes will be expected:

1. Those cells bearing receptors which bind to a substrate, but do not glycosylate it, may bind irreversibly and be eventually degraded and lost. Such a process will eliminate potentially destructive self-reactive T cell clones.
2. Cells expressing receptors which bind and glycosylate target cell antigens will be released from the target cell and will survive, unless they go on to bind irreversibly to a subsequent target cell antigen which they can not glycosylate. Such a process will select

out only those T cells which have specific anti-self receptors. Furthermore, it is expected that only these receptors will become linked up to appropriate cell activation pathways in the mature T cell.

3. Some cells carry receptors which do not bind target antigens. These receptors may be expressed on surviving thymocytes, but will remain inactive.

For such a mechanism to operate and to accommodate such a large number of infiltrating thymocytes, specialised screening cells in the thymus will have to be actively synthesising glycolipid H-2 molecules. Only those cells which survive the selection process will differentiate further and enter the peripheral T cell pool. Cells selected on the basis of either *K* and *D* region-controlled receptors, or *I* region-controlled receptors would have to develop into functionally different cells, but this would appear to be either a secondary function of the thymic environment or a post-thymic process. Such a selection mechanism should generate T cells with specific anti-self receptors which can function in one step of the process of glycosylation and synthesis of carbohydrate H-2 antigens on other self cells. In accordance with the previously described model for Tc cell recognition (Section 4), these same cells will have an effective immune surveillance capacity since they can also function in allogeneic recognition.

Despite many unknowns, the model has basic simplicity, but involves a selection mechanism which is probably more stringent than any other previously proposed mechanism based on the somatic mutation of germline *V* genes for the generation of T cell receptors (Jerne 1971, Langman 1978, Blanden and Ada 1978, von Boehmer *et al.* 1978). T cell receptors are

selected on the basis of their ability to bind to as well as glycosylate self target antigens.

5.2 The diversity of T cell receptors

At the present time, it is difficult to predict how many cell surface glycosyltransferases are encoded by *H-2*-controlled genes, and how many of each of the enzymes specific for the *K* and *D* regions are ever expressed on the one cell. For the purposes of thymic selection, there would be no advantage to the individual to express every one of its receptors on every cell, and one, or perhaps a small number, may be tolerated. A method for generation of diversity in the expression of different receptors could be invoked in terms of the original derepression hypothesis (Bodmer 1973, Martin 1975, Festenstein 1978). For example, an individual cell could express just one of its many *K* or *D* gene-controlled receptors. Codominant expression of both parentally-derived gene products would have to be invoked for F_1 hybrids, if *H-2* glycoprotein molecules are found to be the actual receptor molecules.

If *H-2* glycoprotein molecules are, in fact, the glycosyltransferases, such a proposal for thymic selection implicates heterogeneity in the expression of *K* and *D* antigens, either at the level of the individual cell, or on different cells in the T cell pool. While such heterogeneity has already been detected by two-dimensional gel electrophoresis (Jones 1977, Krakauer *et al.* 1980), it appears to go unnoticed by serological testing. The following reasons can be considered:

The different molecules could be very similar. Most serological analysis of *K* and *D* expression has been carried out with alloantisera, and such preparations

may neither detect any heterogeneity, nor reveal a small subpopulation of different molecules. The different cell surface molecules could be functionally distinct, though serologically silent. A precedence already exists amongst various $H-2K^b$ mutant strains of mice (McKenzie *et al.* 1977a, Klein 1978), which exhibit unique T cell specificity, but are, in general, serologically identical. An interesting possibility is that variation in the amino acid sequence of the different molecules could reside only in the active site region of the molecule, which may be hidden. These differences would probably have to involve single amino acid changes, since no major heterogeneity amongst molecules encoded by any particular K or D allele has been reported by workers involved in sequencing K and D molecules.

One further prediction which stems from the argument that K and D glycoprotein molecules are the actual T cell receptors is that if generation of diversity in the T cell pool involves expression of only one or a small number of glycosyltransferases on any one cell, then thymic selection could eliminate cells carrying particular K and D antigens from the T cell pool. In this respect, F_1 hybrid T cells may not express certain K and D antigens derived from one parent which are potentially capable of binding to carbohydrate antigens derived from the other parent. The variability in expression of different K and D molecules in F_1 hybrids compared with parental cells reported in Chapters 5 and 6 is consistent with this hypothesis. However, definitive results will only be possible when appropriate monoclonal antibodies can be used for serological analysis.

5.3 Predictions of the model

By invoking the existence of *H-2*-controlled T cell receptors, several other features of *H-2* genes can now be discussed in terms of their relevance to the thymic selection mechanism. These can be listed as follows:

1. An explanation may be possible for the high mutation rate associated with the *H-2* gene complex (Klein 1975). The thymic selection mechanism proposed here has one unique advantage, in that operative, though aberrant *H-2*-controlled glycosyltransferases can be rapidly incorporated into the T cell receptor pool. Many new mutations in glycosyltransferase genes could be tolerated, without being of direct selective disadvantage to the host.
2. *H-2*-linked immune response gene (*Ir*-gene) effects can also be incorporated at the level of thymic selection by this model. For example, since anti-self *K*-controlled T cell receptors will be selected for their lack of reactivity to D antigens and vice versa, this could lead to effects on the size of the T cell receptor pool involved in *K* or D recognition. Similar effects could occur in F_1 hybrids, and could also involve *I* region-controlled antigens. The establishment of self tolerance could therefore lead to variability in the number of specific precursors and could explain several examples of *H-2*-linked *Ir* gene effects on Tc cell responsiveness (Zinkernagel *et al.* 1978, Doherty *et al.* 1978, Simpson and Gordon 1977, von Boehmer *et al.*

1977, Kurrle *et al.* 1978, Mullbacher and Blanden 1978). The effect of *Ir* gene complementation on Tc cell responsiveness in F_1 hybrids could also be explained in terms of thymic selection of the T cell receptor pool (Simpson and Gordon 1977, von Boehmer *et al.* 1978, Schmitt-Verhulst and Shearer 1975).

5.4 Thymic selection in chimeric mice

This model also provides an explanation for the generation of the T cell pool in chimeric mice which becomes restricted to the *H-2* type of the host thymus (Bevan 1977, Zinkernagel *et al.* 1978a,b). Stem cells from bone marrow or fetal liver which are used to reconstitute a lethally irradiated host, will enter the host thymus and undergo the same selection process outlined for normal animals in Section 5.1. Only T cells which express no host reactive receptors, and have anti-self receptors which can glycosylate and synthesize carbohydrate antigens on target cells will survive and become part of the T cell pool. Such stringent selection is adaptive differentiation and could explain the variability in survival rates of chimeras made between different strains of mice, as well as the great difficulty in making completely allogeneic chimeras e.g. A \rightarrow B, compared with semi-allogeneic chimeras e.g. F_1 hybrid \rightarrow parent 1 ($F_1 \rightarrow P_1$) (for example, Zinkernagel *et al.* 1980b).

Chimeras which survive will express glycosyltransferases controlled by the *K* and *D* genes of the donor, which can specifically bind and glycosylate carbohydrate *H-2* antigens present on allogeneic cells. Such a situation could lead to the following predictions on the *H-2*

restriction phenotype of chimeras, for which there is already some precedence in the literature:

1. The first prediction is that in an $F_1 \rightarrow P1$ chimera, peripheral T cells could carry both anti-P1 and anti-P2 receptors which can participate in the synthesis of P1 carbohydrate antigens on other cells. It is expected that active anti-P1 receptors will comprise most of the receptor repertoire, together with a smaller variable number of active anti-P2 receptors. Depending on the strain combinations used to make the chimera, mature T cells in $F_1 \rightarrow P1$ chimeras will have self recognition capabilities which are not necessarily restricted only to P1. This may explain several reported examples of the lack of absolute restriction to the *H-2* type of the host thymus in $F_1 \rightarrow P1$ chimeras (Bevan 1977, Blanden and Andrew 1979).
2. The second prediction involves completely allogeneic $A \rightarrow B$ chimeras. Any A strain T cells which survive thymic selection will carry anti-self, e.g. anti-A receptors which can cross-reactively participate in glycosylation of carbohydrate *H-2* antigens on B strain cells. However, this same T cell pool may still contain some cells that can glycosylate A antigens, such that the *H-2* restriction specificity of the chimera is not entirely restricted to the host *H-2* type. Such a result has been reported by Matzinger and Mirkwood (1978), where Tc cells generated in allogeneic chimeras against minor H antigens were found to be reactive against appropriate targets of both donor and host type.

6. Implications of the models

6.1 Clonal expression of T cell receptors

One of the earliest findings reported by the proponents of the *H-2* restriction phenomenon was the existence of subpopulations of Tc cells in an infected animal with specificity for the viral antigen as well as the restricting K or D antigen (reviewed by Zinkernagel and Doherty 1979). While many different workers have since confirmed the basic finding that *H-2* restricted Tc cells were monospecific for either K + X or D + X (see Chapter 1), no evidence has been obtained which determines whether clones of monospecific T cells exist prior to contact with target antigens, or whether the described specificity is acquired upon antigen stimulation.

Interpretation of this phenomenon has been influenced greatly by Jerne's (1971) original prediction of the role of the thymus in the selection of T cell specificities. More recently, this idea has received impetus from the finding that the thymus itself determines the T cells' specificity for self *MHC* antigens (reviewed by Zinkernagel 1978b, Bevan and Fink 1978). As a result, more recent theories on the nature of T cell receptors have incorporated explanations for the thymic selection of T cell clones with restricted specificity for self K or self D antigens, as well as for X antigens (Langman 1978, Cohn and Epstein 1978, Blanden and Ada 1978, Schwartz 1978, Williamson 1980). In terms of the model proposed in Section 5 for thymic selection of T cell precursors, monospecificity for self K and D antigens can be imposed on the selection mechanism if the following two premises are invoked:

1. Only one *K*- or *D*-controlled anti-self receptor (i.e. glycosyltransferase) on any one cell is linked up to the cell activation pathway.
2. Only one of the *K*- or *D*-controlled anti-self receptors is linked up to an anti-X receptor.

The mechanism of linkage to a T cell activation pathway could occur during the thymic selection process, and could, for example, involve the first anti-self receptor which makes contact with target antigens in the thymus.

While clonal expression of T cell receptors has been easily demonstrable at the effector cell level, many workers have tried with little success to deplete unprimed cell populations of precursor T cells by absorption on monolayers of appropriate target cells. One group of workers has successfully defined conditions for depletion of alloreactive Tc cell precursors (Schnagl and Boyle 1979), but there seems to have been no report of depletion of *H-2* restricted or self-reactive Tc cell precursors. If the possibility that clonal expression is established upon antigen contact has to be considered, then the proposed model is flexible with respect to the point in time that clonal expression of anti-*K* and anti-*D* restricted T cells is established.

6.2 Antigen specific receptors on cytotoxic T cells

A role for anti-X receptors in *H-2* restricted recognition was largely adopted to account for the early evidence which indicated that *H-2* restricted Tc cells were highly specific for the particular virus or foreign antigen used to stimulate a response (reviewed by Zinkernagel and Doherty 1979). As a result, any theory on the nature of T cell receptors has incorporated clonally expressed antigen specific (anti-X) receptors which have specificity either for X alone, or for some

neoantigenic determinant formed by association of X with a K or D antigen. Since the nature of the association between X and *MHC* antigens is poorly understood (see Chapter 1), a major quandary exists for immunologists regarding the number and nature of T cell receptors required for *H-2* restricted recognition. According to the model for Tc cell recognition proposed in Section 4, anti-X receptors may or may not function in *H-2* restricted responses, depending on whether X antigens are presented on the cell surface, or effect a modification of carbohydrate *H-2* antigens, which could lead to alloreactive recognition.

6.2.1 The generation of anti-X receptors

According to the model, there is no requirement for anti-X receptors in the recognition of self *H-2* determinants on target cells. As a result, the generation of diversity amongst these receptors could be completely unrelated to the establishment of T cell clones by the thymic selection process. Anti-X receptors could still be derived by somatic mutation of germline *V* genes as is the accepted view (see Chapter 1), but some mechanism for establishment of self tolerance by elimination of cells bearing anti-X receptors which are reactive with self *H-2* determinants must be invoked. Such a process could operate either at thymic selection, at some stage in T cell differentiation prior to thymic infiltration, or development of self-reactive clones could be checked by a peripheral suppression mechanism (Blanden and Ada 1978).

6.2.2 The requirement for anti-X receptors

While it was first thought that the strong crossreactivity of TNP-specific Tc cells with alloantigens (Shearer and Schmitt-Verhulst 1977), as well as with virally-infected cells (Mullbacher and Blanden 1979b)

was an exception rather than the rule, more and more evidence is accumulating which suggests that *H-2* restricted Tc cells are not absolutely specific for the priming X antigen. Several examples of Tc cell crossreactivity between serologically distinct viruses can now be cited.

1. Effector Tc cells generated against one of the serologically distinct influenza A strain viruses can crossreact on target cells infected with another A strain virus (Biddison *et al.* 1977, Braciale 1977, Ada and Yap 1977, 1979).
2. Serologically different alphaviruses, namely Sindbis, Bebaru and Semliki Forest virus, all crossreact in terms of Tc cell recognition (Mullbacher *et al.* 1979).
3. Different murine leukemia viruses also induce crossreactive Tc cells (Gomard *et al.* 1978, Plata and Lilly 1979).

Evidence such as this raises the possibility that there may be either none or a minimal requirement for anti-X receptors in the generation of *H-2* restricted Tc cells. Further to this point is the lack of available evidence for the generation of anti-idiotypic antibodies specific for T cell receptors involved in *H-2* restricted recognition, despite the fact that the methodology has been well documented (reviewed by Binz and Wigzell 1979a, Eichmann 1978). Also of relevance is the difficulty involved in blocking target cell lysis with anti-serum specific for foreign or viral antigens present on the target cells, compared with specific anti-*H-2* serum, and this problem was discussed in Chapter 1.

The mode of presentation of X antigens on the cell surface probably varies for cells infected with different viruses or carrying different foreign antigens. While both viral and exogenously added antigens are usually detectable on the cell surface, whether such antigens are directly involved in anti-X recognition is unknown. At this point, it would be difficult to predict whether the degree of specificity documented for *H-2* restricted responses which in the case of viruses relates at least to virus groups, could be generated by some specific effect of the viral infection on the synthesis of target *H-2* antigens. If such antigens are carbohydrate-defined, then viral infection could result in some loss of glycosyltransferase function, or sequestration of nucleotide sugar units leading to blockage of synthesis, and production of specifically altered carbohydrate sidechains which mimic alloantigenic determinants. One further possibility is that Tc cell responses to certain minor H antigens may be directed at small, but specific background gene effects on *H-2* antigens themselves. While there is evidence which indicates that HY and *H-2* antigens do not cocap (Geib *et al.* 1977), a contradictory report by van Leeuwen *et al.* (1979) shows the existence of a human antiserum which is specific only for HLA determinants in males, suggesting that HY may actually be a modified *MHC* antigen.

Probably the best evidence in favour of involvement of an anti-X receptor in *H-2* restricted Tc cell recognition is the fact that either exogenous addition of haptens such as TNP (Shearer and Schmitt-Verhulst 1977), as well as UV-inactivated Sendai and influenza viruses (Schrader and Edelman 1977, Braciale and Yap 1978), or the fusion of purified viral components to cell membranes (Gething *et al.* 1978, Sugamura *et al.* 1978, Braciale and Yap 1978, Hapel *et al.* 1978, Kurrle *et al.* 1979)

are sufficient alone to render cells capable stimulators or targets for Tc cell responses. One interesting proposition is that the requirement for anti-X receptors, and the demonstration of exquisite specificity of Tc cells for X, may occur only when X is presented on the cell surface and has no direct effect on H-2 antigen expression. The relative number of specific versus crossreactive Tc cells generated in any response may vary according to the relative antigen effect derived from within (e.g. inherent H-2 differences, or viral infections), versus the antigen effect which results from antigen presentation on the cell membrane (including inactivated viruses, purified viral proteins, type of TNP-modification, etc.). Such a proposition may explain differences in the relative specificity of Tc cells generated with live versus inactive influenza A virus strains (Braciale and Yap 1978), and a similar proposal may also explain differences in the specificity of Tc cells to TNP-modified cells which are related to the concentration of trinitrophenylbenzene sulphonic acid used to modify targets (Polisson *et al.* 1980). Different concentrations may effect modification of different cell surface molecules, e.g. high concentrations may modify amino sugars present on carbohydrate H-2 antigens leading to alloantigen recognition and crossreactive Tc cells, while low concentrations could specifically modify free amino groups on cell surface proteins leading to a specific Tc cell response.

6.3 The anti-self receptor

With respect to the nature of the T cell receptor, the following two assertions were made in the development of the Tc cell recognition model described in Section 4.

1. An anti-self receptor which has glycosyltransferase function exists on T cells.
2. The H-2 glycoprotein molecule is the anti-self receptor.

Adoption of these two proposals stemmed from the need for a glycosyltransferase in the synthesis of carbohydrate H-2 antigens, and while experimental work is in progress to test their validity, it is important to reconcile these proposals with the current evidence for the immunoglobulin-like nature of the Tc cell receptor.

Early genetic mapping studies in both mice and rats have indicated that the expression of idiotypes on Tc cells is linked to the *Ig-1* locus, and thus appears to be controlled by heavy chain allotype genes (V_H genes) (Krammer and Eichmann 1977, Binz and Wigzell 1976a). However, there is also evidence in mice, though not in rats, which suggests that the expression of idiotypic on alloantigen reactive Tc cells is also linked to the *MHC* complex (Krammer and Eichmann 1977). While the combined data from several laboratories indicates that the T cell receptor is, in part, composed of an immunoglobulin-like molecule controlled by V_H genes, this latter result suggests that receptors on Tc cells may also be controlled by *MHC* genes.

While evidence for linkage of T cell receptor idiotypes to the *MHC* complex favours the theory proposed here for the nature of T cell receptors, linkage to *V* region genes poses a contradiction. The following possible explanations can be given:

1. The *H-2* gene complex controls the expression of glycosyltransferases which are encoded by *V* genes present on a different chromosome.

2. The *H-2* gene complex may encode anti-self receptors, and *V* genes may encode anti-X receptors.
3. Anti-idiotypic antibodies raised against Tc cells may bind crossreactively to both immunoglobulin molecules as well as *H-2* controlled T cell receptors. Evidence in favour of some crossreactive binding site comes from amino acid sequencing data which indicates that HLA molecules may have some sequence homology with immunoglobulins (Tragardh *et al.* 1980).
4. Tc cell receptor molecules isolated by immunoprecipitation with anti-idiotypic antibodies and which resemble Ig but not H-2 antigens (Bing and Wigzell 1976b), could be anti-X receptors.

Studies on the analysis of Tc cell receptors have involved the use of anti-idiotypic antiserum raised in F_1 hybrid mice or rats against T cell receptors present on parental (P1) lymphocytes sensitised either against alloantigens of P2 cells or against TNP-modified syngeneic cells (Binz and Wigzell 1975, 1976b, Binz *et al.* 1978, 1979a, Krammer and Eichmann 1977, Rehberger *et al.* 1979, Krammer 1978, Aguet *et al.* 1978). In view of the evidence for carbohydrate-defined H-2 antigens, and the likelihood of qualitative as well as quantitative differences in H-2 antigen expression in F_1 hybrids (Section 3, also Chapters 3 and 5), anti-idiotypic antisera raised in F_1 hybrid mice against parental H-2 antigens could be contaminated with antibodies directed against parent-specific H-2 determinants. The presence of any contaminating anti-H-2 antibodies in anti-idiotypic antiserum preparations would suggest different interpretations for the following reported experimental results:

1. Blocking of T cell functions using anti-idiotypic antibodies (Krammer 1978, Aguet *et al.* 1978, Binz *et al.* 1979b, Rehberger *et al.* 1979, Rubin *et al.* 1979).
2. Isolation of T cell receptors with anti-idiotypic antiserum, and interpretation of the ability of these receptors to subsequently bind *MHC* glycoprotein molecules (Binz *et al.* 1979a).
3. Use of such antisera to isolate reputed T cell receptor material from serum or culture supernatant (Binz and Wigzell 1975, 1976b), in view of evidence for serum-borne, carbohydrate Ia antigens (Parish and McKenzie 1980).

While anti-idiotypic analysis has provided the most promising evidence for the nature of the T cell receptor, it now seems likely that further chemical analysis of H-2 antigens, and of their role in T cell recognition may provide more answers. Work is in progress to isolate and characterise the reputed T cell receptor described in this review, and the author is hopeful that (to quote Jan Klein (1975)):

'Clearly the most exciting era of the H-2 studies is yet to come!'

Fig. 1 The role of glycosyltransferase enzymes encoded by the *H-2* gene in the synthesis of carbohydrate H-2 antigens. The substrate for the enzyme is a trisaccharide side-chain on a glycolipid carrier. By binding to the terminal sugar unit (\diamond) on the glycolipid, the enzyme catalyses the transfer of a monosaccharide (O) from a uridine diphosphate (UDP) donor to the terminal sugar unit. This process could occur on the cell surface of adjacent cells or intracellularly before the two molecules are transported to the cell membrane.

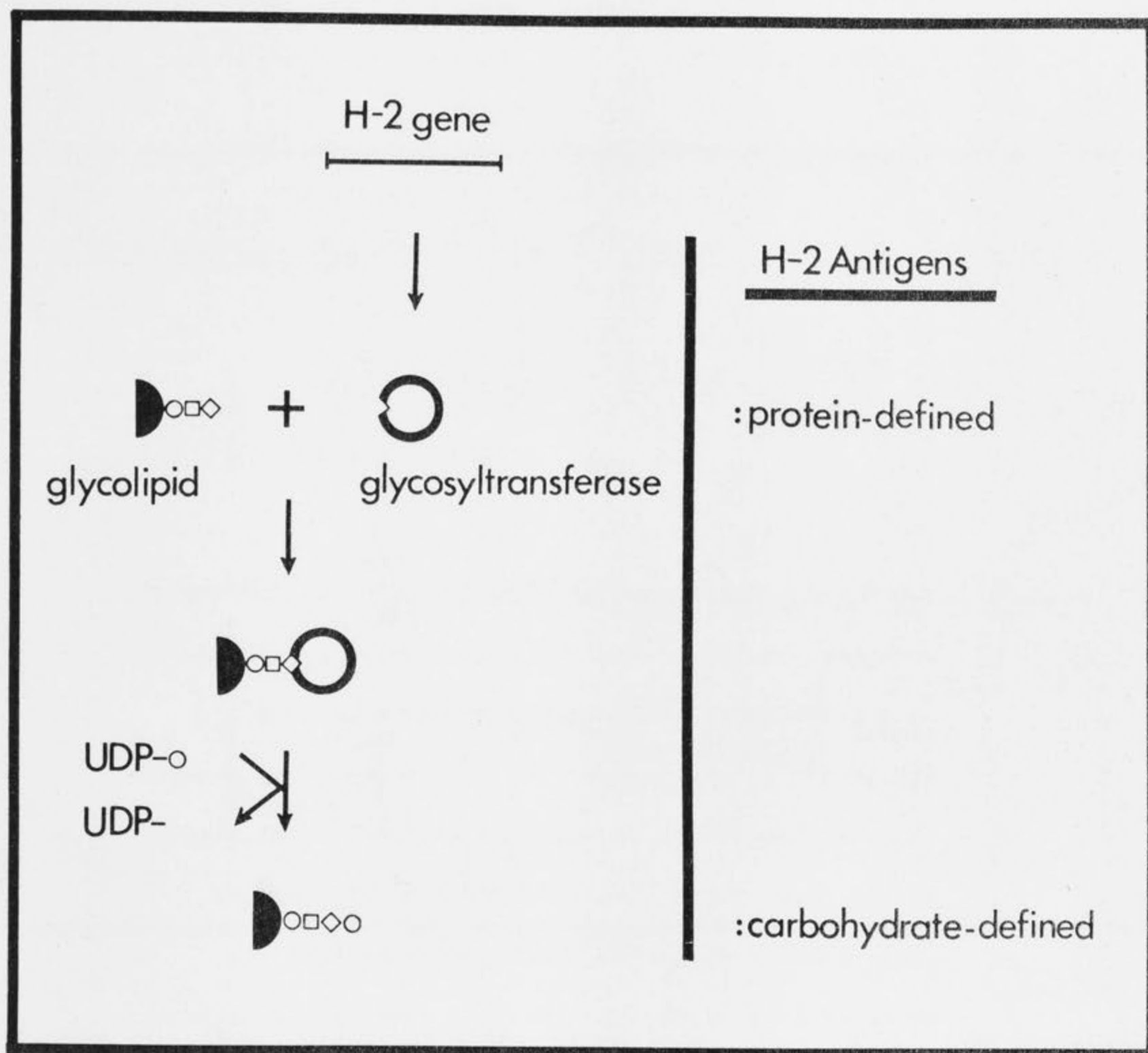
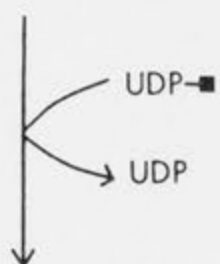
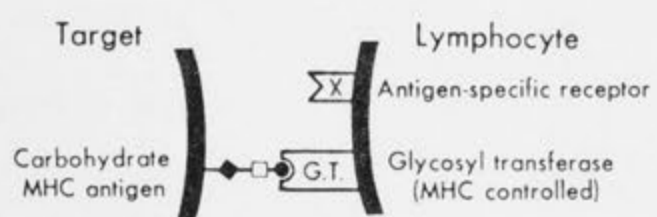


Fig. 2 A model for T cell recognition involving an anti-self receptor, an *MHC*-controlled glycosyltransferase (G.T.) on a lymphocyte, and a carbohydrate-defined *MHC* antigen on a target cell. An antigen-specific receptor (X) is involved in recognition of foreign antigens. Recognition of *MHC* antigens involves binding and glycosylation of an *MHC* carbohydrate antigen *via* transfer of a monosaccharide unit (■) from a uridine diphosphate donor to a terminal sugar unit.

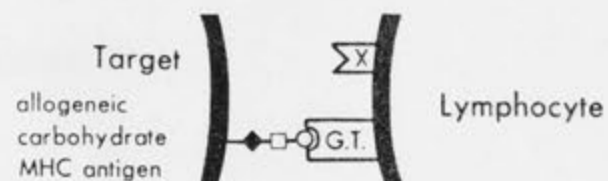
1. NO ANTIGEN: Binding, glycosylation → release



2. ANTIGEN: Binding, no glycosylation → activation



3. ALLOANTIGEN: Binding, no glycosylation → activation



INTRODUCTION

The study of cell surface antigens and their relationship to cell function is a rapidly expanding field. This approach has been particularly successful in the study of the relationship between cell surface antigens and cell function. In the past, several procedures have been used to analyze the relationship between cell surface antigens and cell function. The approach is to correlate cell surface antigens with cell function. This involves the study of the relationship between cell surface antigens and cell function. The approach is to correlate cell surface antigens with cell function. This involves the study of the relationship between cell surface antigens and cell function.

APPENDIX

A NEW PROCEDURE FOR ANALYSING THE
RELATIONSHIP BETWEEN DIFFERENT CELL SURFACE ANTIGENS

Although this report is a preliminary report, there have been several studies of cell surface antigens in the past. The approach is to correlate cell surface antigens with cell function. This involves the study of the relationship between cell surface antigens and cell function. The approach is to correlate cell surface antigens with cell function. This involves the study of the relationship between cell surface antigens and cell function. The approach is to correlate cell surface antigens with cell function. This involves the study of the relationship between cell surface antigens and cell function. The approach is to correlate cell surface antigens with cell function. This involves the study of the relationship between cell surface antigens and cell function.

INTRODUCTION

One means of analysing cell differentiation and diversity is to raise antisera to different cell surface antigens. This approach has been used particularly extensively by immunologists in their studies of the major histocompatibility complex (Klein *et al.* 1978) and of lymphocyte differentiation (McKenzie and Potter 1979). Once a number of cell surface antigens have been identified on a single cell, the spatial relationship between these antigens on the plasma membrane is frequently of interest. In the past, several procedures have been used to assess the relationship between different membrane antigens. One approach is to saturate cells with antibodies to one antigen and then measure the binding of antibodies of another specificity. This blocking procedure is a measure of the proximity of cell surface antigens rather than a demonstration of molecular identity. Another approach has been to determine whether different cell surface antigens co-cap (Néauport-Sautès *et al.* 1973, Hauptfeld *et al.* 1975). Although this method is superior to blocking, there have been instances of molecular complexes in the membrane co-capping. Currently the only true test for molecular identity of membrane antigens is sequential immunoprecipitation developed by Cullen *et al.* (1972, 1976). This method entails immunoprecipitating antigens of one specificity from a detergent solubilised preparation of radiolabelled cells and subsequently measuring, via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the ability of a second antibody to immunoprecipitate labelled material from the cell lysate. In this chapter, a modified immunoprecipitation procedure is described which has several advantages over the original method.

MATERIALS AND METHODS

Animals

B10.A mice were bred in the John Curtin School of Medical Research and mice of either sex were used as spleen cell donors when 6-12 weeks old. The mouse strains used for raising the various alloantisera are listed in Table 1.

Antisera

Several mouse antisera directed against different mouse allo-antigens were prepared (Table 1). The sera were produced by giving adult female mice a weekly intraperitoneal injection of a suspension of thymus, lymph node and spleen cells for six weeks, and thereafter bleeding and immunising on alternating weeks. The sera were pooled and stored at -20°C .

Preparation of spleen cell suspensions

Spleen cell suspensions of high viability (85-95%) were prepared as previously described in Chapter 6 in Eagle's minimal essential medium F15 (Grand Island Biological Co., Grand Island, N.Y.) containing 5% fetal calf serum (FCS). The cell suspensions were depleted of red cells and dead cells by centrifugation on a cushion of Isopaque/Ficoll (Davidson and Parish 1975). For alloantisera studies, spleen cells were cleared of surface Ig by a capping procedure (Parish and McKenzie 1978). The procedure consisted of incubating the cells ($10^7/\text{ml}$) for 75 minutes at 37°C in F15/5% FCS containing $1\text{mg}/\text{ml}$ of sheep IgG specific for mouse Ig. During incubation the mixture was gassed with 10% CO_2 , 7% O_2 in N_2 . Following Ig-capping the cells were washed twice with medium prior to use. On some occasions spleen cells were surface labelled with ^{125}I using a lactoperoxidase catalysed iodination procedure (Parish *et al.* 1978, Chapter 8).

Solubilisation of spleen cells

Spleen cells were pelleted by centrifugation and resuspended to a concentration of 2×10^8 cells/ml in a 0.5% (v/v) solution of Nonidet P-40 (NP-40) in phosphate buffered saline (PBS) containing 1.5mM MgCl_2 and 10^{-3} M phenylmethanesulphonyl fluoride (PMSF). Following incubation for 30 minutes at 4°C, nuclei and cell debris were removed from the preparation by centrifugation at 20,000 g for 20 minutes at 4°C. In order to remove NP-40 the cell lysate was then added to an equal volume of packed XAD-8 resin beads (Rohm and Hass Co., Philadelphia, Pa) which had been prewashed with methanol and stored in distilled water as previously described (Holloway 1973). The lysate-resin mixture was agitated for 2 hours at 4°C and the lysate then collected and stored at 4°C in the presence of 0.1% (w/v) sodium azide.

In some experiments, spleen cells were treated with different concentrations of NP-40 solution for 30 minutes at 4°C, pelleted by centrifugation, washed twice with medium and cell viability determined. Viability was assessed by adding ethidium bromide (10 µg/ml final concentration) to cell suspensions and enumerating fluorescent dead cells under the fluorescence microscope. NP-40 concentrations were estimated from absorbance at 275nm ($E_{1\text{ cm}}^{1\%} = 16.2$).

Immunoprecipitation of antigens from cell lysates

Spleen cells were lysed with NP-40 and nuclei and cell debris removed as described above. An appropriate dilution of antiserum was then added to the cell lysate and the mixture incubated overnight at 4°C. Antibody was then cleared from the lysate by a 30 minute absorption at 4°C with protein-A bearing *Staphylococcus aureus* (Cowan 1 strain) bacteria (20 µl packed bacteria/0.1 ml of cell lysate).

The *S. aureus* bacteria were grown, harvested, formalin fixed and stored as previously described (Kessler 1975). After removal of *S. aureus* bacteria from the lysate by centrifugation, the lysate was depleted of NP-40 by the XAD-8 resin as described above. Each preparation was then tested initially for complete removal of allo-antibody by the rosetting assay and subsequently for alloantigen content by the rosette-inhibition assay.

Rosetting assay for alloantibodies

The binding of alloantibodies to mouse spleen cells was detected by a rosetting procedure which has been described in detail elsewhere (Chapter 6, Parish and McKenzie 1978). Briefly, 10 μ l of ice-cold Ig-capped spleen cells (4×10^6 /ml in F15/5% FCS) were absorbed for 30 minutes with 10 μ l doubling dilutions of antiserum in microtitre plates (Linbro Chemical Co., New Haven, Conn.). After washing with medium, 10 μ l of a 2% suspension of sheep erythrocytes coated via CrCl_3 with sheep anti-mouse Ig was added and the mixture centrifuged gently to form rosettes. Methyl violet staining solution was then used to determine the percentage of rosette forming cells.

Rosette-inhibition assays

Serial dilutions of various NP-40 lysates of spleen cells were tested for their ability to inhibit the binding of a constant amount of alloantibody to spleen cells, as detected by the rosetting assay described above. To 10 μ l serial dilutions of NP-40 lysate in microplates was added 10 μ l of the appropriate dilution of antiserum. In order to maximise the sensitivity of the inhibition assay, an antiserum dilution was chosen that was one tube greater than that which gave maximal rosetting. The antiserum-lysate mixtures were

incubated overnight at 4°C, 10 µl of Ig-capped spleen cells (4×10^6 /ml) added to each well and the rosette assay for alloantibodies performed as described. In some experiments a control (unabsorbed) treatment was included which consisted of serial dilutions of NP-40 lysate preincubated with medium rather than antiserum.

Microcytotoxicity assays

The ability of alloantisera to induce complement-mediated lysis of spleen cells was measured by a two-stage microcytotoxicity test which was described in Chapter 2. Cell death was estimated by the trypan blue exclusion technique.

RESULTS

Detergent solubilisation of spleen cell membranes

A procedure was developed whereby cell membranes could be detergent solubilised, rapidly freed of detergent, and then assayed for antigen content. The procedure entailed solubilising spleen cells with the non-ionic detergent NP-40 using a method similar to that described by other workers (Schwartz and Nathenson 1971, Cullen *et al.* 1976). This detergent has the advantage that it readily solubilises the plasma membranes of lymphocytes but leaves the nuclear membranes intact. After sedimentation of nuclei, NP-40 was then removed from the spleen cell lysates with XAD-8 resin, a neutral porous copolymer that adsorbs detergents. In preliminary experiments, using 0.5% NP-40 solutions, it was found that after 2 hours at 4°C the XAD-8 resin reduced NP-40 to 0.003 - 0.004%, a detergent concentration that is unable to lyse spleen cells at 4°C (Figure 1) but which is sufficiently high to keep membrane components in solution. Thus, this

membrane preparation can be readily added to spleen cell targets in antigen-antibody inhibition assays. It should be noted, however, that 0.003 - 0.004% NP-40 very efficiently solubilises spleen cells at 37°C.

As an assessment of the recovery of membrane components after treatment of lysates with XAD-8 resin, spleen cells were surface labelled with ^{125}I prior to solubilisation. It was found that 80-85% of membrane bound ^{125}I was recovered in the supernatant harvested from the XAD-8 beads. Furthermore, the 15-20% of ^{125}I counts lost represented lysate trapped in the porous beads rather than specific adsorption of labelled membrane components to the XAD-8 resin.

Inhibition assay for detection of alloantigens

The antigen content of spleen cell lysates was assessed by measuring the ability of serial dilutions of lysate to inhibit the binding of a fixed concentration of antibody to spleen cell targets. To optimise the sensitivity of the assay, an antibody dilution was chosen that was one tube greater than that which gave maximal rosetting.

Typical inhibition data is presented in Figure 2. It can be seen that the binding of anti- K^b antibodies to B10.A(5R) (K^b, D^d) target cells was strongly inhibited by a C57BL/6J ($H-2^b$) lysate but not by a BALB/c ($H-2^d$) lysate. Conversely, the binding of anti- D^d antibodies to the same target cells was efficiently inhibited by a BALB/c lysate but a C57BL/6J lysate lacked inhibitory activity. Thus, the inhibition assay shows a high degree of specificity.

The experiments described above used a rosetting assay to measure antibody binding to targets. In additional experiments the spleen cell lysates were tested for their ability to inhibit antibody binding as measured by complement-mediated lysis (data not shown). Unfortunately

10-20 times higher concentrations of alloantibody were needed in these inhibition assays as microcytotoxicity is generally much less sensitive than rosetting at detecting alloantibodies (Parish and McKenzie 1978). Consequently, only weak inhibition of antibody binding was observed using this technique, i.e. significant inhibition was only obtained with undiluted and $\frac{1}{2}$ diluted lysates. Thus, the rosetting assay was chosen for all subsequent inhibition studies. It should be noted, however, that this technique could be adapted to microcytotoxicity assays if much more concentrated spleen cell lysates were used, i.e. 2×10^9 cells/ml rather than 2×10^8 cells/ml.

Immunoprecipitation procedure

In order to determine whether different antigenic determinants are carried on the same molecule or on different molecules in the cell membrane, spleen cell lysates were subjected to an immunoprecipitation procedure. The method is briefly summarised in Table 2. It consisted of exposing NP-40 lysates of cells to antibody against one antigenic specificity, removing free antibody and immune complexes by *S. aureus* bacteria, and then measuring the inhibitory activity of the lysate for antibody against another specificity.

Using this procedure the relationship between H-2K, H-2D and H-2L antigens was assessed using specific antisera against these three antigens. It was found that anti-K^b antibodies very efficiently cleared K^b inhibitory material from NP-40 lysates of B10.A(5R) spleen cells but were much less effective at absorbing anti-D^d inhibitory activity (Figure 3). Conversely, anti-D^d antibodies readily removed D^d inhibitory activity from lysates but only partially removed the inhibitory antigens for anti-K^b antibodies (Figure 3). These data imply that there are unique molecules carrying H-2K^b and H-2D^d

antigenic specificities in B10.A(5R) mice. However, there are also substantial numbers of molecules in the NP-40 lysates which bind both antibodies (approx. 20-50%), a result which was reproduced in a number of experiments. This is a surprising result as, with rosetting assays using intact cells, there was only a 0.5 - 2.0% crossreaction between these antisera and it is generally accepted that H-2K and H-2D are separate molecules in the cell membrane (Cullen *et al.* 1972, Démant and Néauport-Sautès 1978). The higher degree of crossreaction observed in the inhibition assay may be due to the prolonged incubation of antibody with antigen (16-20 hours compared with 30 minutes in the rosetting assay) which would favour the binding of low affinity antibodies or it may result from internal crossreactive molecules which are not expressed on the cell surface. Experiments are in progress that may resolve this discrepancy. It should be emphasised, however, that when monoclonal anti-H-2 antibodies were analysed in this immunoprecipitation procedure unexpected crossreactions were not observed (see Chapter 6).

Figure 4 depicts the ability of anti- K^k , anti- D^d , anti- L^d and anti- I^k sera to absorb anti- L^d inhibitory activity from NP-40 lysates of B10.A (K^k , $I^{k/d}$, D^d , L^d) spleen cells. It can be seen that only anti- L^d antibodies removed the inhibitory antigens which indicates that the H-2L antigens are carried on different molecules to the H-2K, H-2D and Ia antigens. No crossreactive inhibition was detectable with this antiserum.

Some additional features of this immunoprecipitation method should be emphasised: a) To ensure adequate immunoprecipitation of antigens from the NP-40 lysates a concentration of antibody was added that was 10-20 fold higher than the end point titre of the antiserum.

b) Fresh NP-40 lysates were used in all experiments. Membrane components gradually reaggregate in lysates which have been stored at 4°C for 24 hours or more. c) Before the antibody-treated lysates were used in inhibition assays they were spot tested in the rosette assay for any residual antibody which had not been absorbed by *S. aureus*. d) Protein A on *S. aureus* only binds IgG antibodies. To analyse IgM antibodies the procedure needs to be modified. One approach is to bind anti-Ig antibodies to *S. aureus* and use these organisms as an immunoabsorbant for IgM.

DISCUSSION

This report describes an immunoprecipitation procedure for determining whether different antigenic determinants are carried on the same molecule or on different molecules in cells. The procedure entails exposing NP-40 lysates of cells to antibody against one antigenic specificity, removing free antibody and immune complexes by protein A bearing *S. aureus* bacteria and following adsorption of the NP-40 with a detergent-binding resin, measuring the inhibitory activity of the lysate for antibody against another specificity by rosetting or microcytotoxicity assays. Using this method it was confirmed that unique H-2K, H-2D and H-2L molecules exist in spleen cells, although with the alloantisera used, some unexpected sharing of antigenic specificities between H-2K^b and H-2D^d molecules was observed.

The new method has several advantages over the widely used sequential immunoprecipitation procedure (Cullen *et al.* 1972, 1976). First, no radioactive materials are used. Second, the method is much more sensitive than SDS-PAGE at detecting cell surface antigens, particularly when the rosetting assay is used to detect antibodies. Third, the method can analyse antigens which are difficult to radio-label or cannot be detected on SDS-PAGE (e.g. glycolipids). Fourth,

the procedure can analyse antisera of low affinity that cannot immunoprecipitate antigens for SDS-PAGE analysis. For example, it was found that low affinity monoclonal anti-H-2 antibodies which produce very weak protein peaks on SDS-PAGE can be easily analysed by the new procedure (Chapters 6 and 7). Presumably in the SDS-PAGE method low affinity antigen-antibody complexes are dissociated during the washing of complexes bound to *S. aureus* bacteria. On the other hand, a disadvantage of the new procedure is that it does not estimate the molecular weights of the antigens being examined.

The method described in this paper measures both cytoplasmic and plasma membrane antigens. However, the method can be adapted to analyse only cell surface antigens either by preincubating cells with antibody prior to solubilisation or by isolating from cell lysates molecules which bind to lentil lectin columns as membrane components are usually glycosylated (Cullen *et al.* 1976).

Although the new procedure has been developed to analyse the relationship between different cellular antigens the basic inhibition assay represents an excellent means of following membrane antigens during purification. Membrane components can be separated in the presence of detergents and then different fractions rapidly cleared of detergent by the XAD-8 resin and assayed for antigenic activity. It should be noted that the XAD-8 resin adsorbs detergents other than NP-40 (e.g. Triton X-100) and reduces detergents to their critical micelle concentration (Holloway 1973); a concentration that usually does not lyse cells but which is sufficiently high to keep membrane components in solution.

SUMMARY

An immunoprecipitation-inhibition procedure is described for assessing whether different antigenic determinants are carried on the same molecule or on different molecules on cells. The procedure entails (a) exposing NP-40 lysates of cells to antibody against one antigenic specificity; (b) removing free antibody and immune-complexes by absorption with protein A-bearing *S. aureus* bacteria; (c) adsorption of the NP-40 with a detergent binding resin and (d) measuring the inhibitory activity of the lysates for antibody against another specificity by a rosetting assay. This method has several advantages over the widely used sequential immunoprecipitation procedure.

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Fig. 1 Ability of different concentrations of NP-40 (30 minutes, 4°C) to solubilise mouse spleen cells. Solubilisation of plasma membranes was assessed by the staining of nuclei with ethidium bromide. The arrow represents the percent NP-40 remaining after the treatment of a 0.5% NP-40 solution with XAD-8 resin for 2 hours at 4°C.

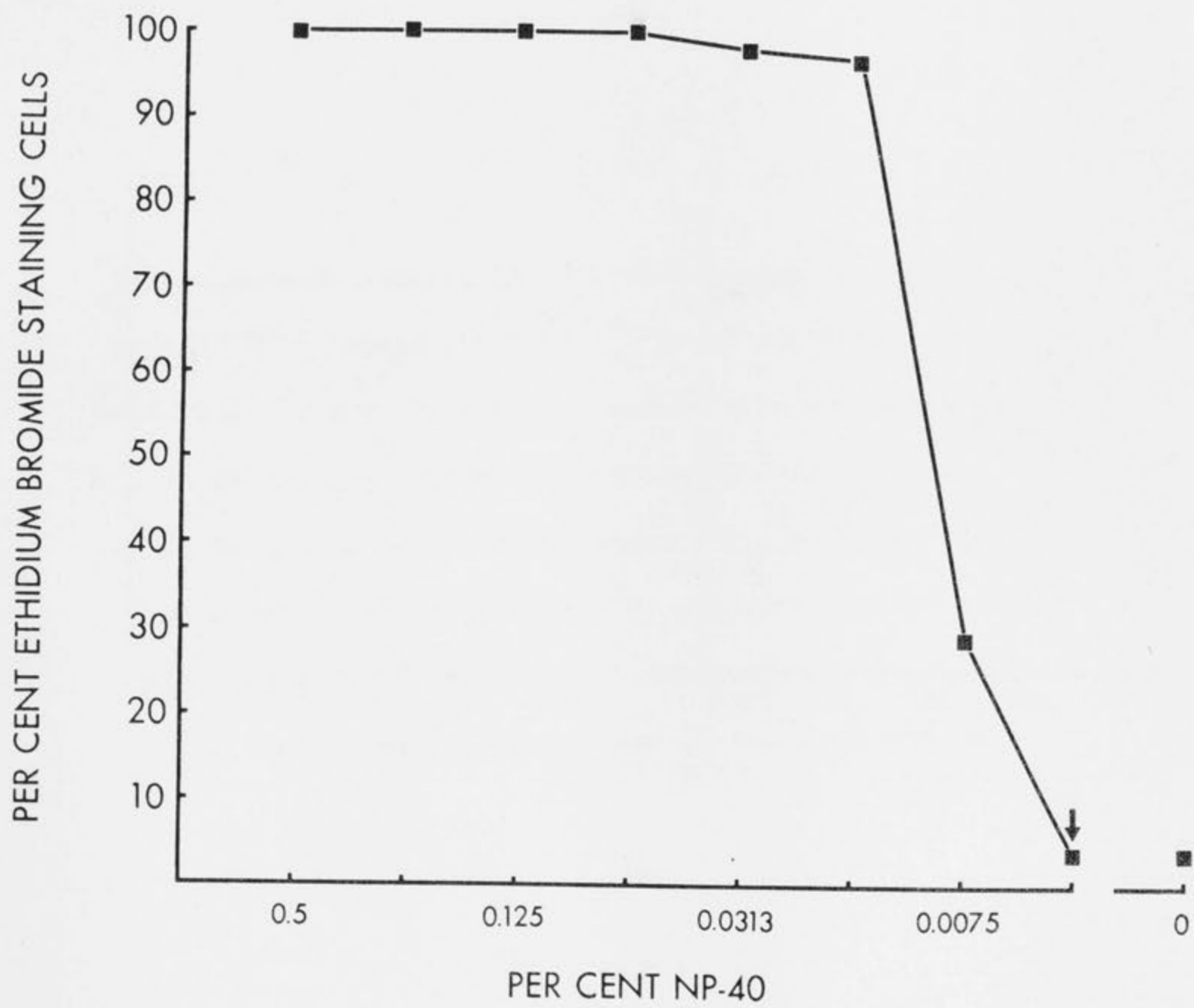
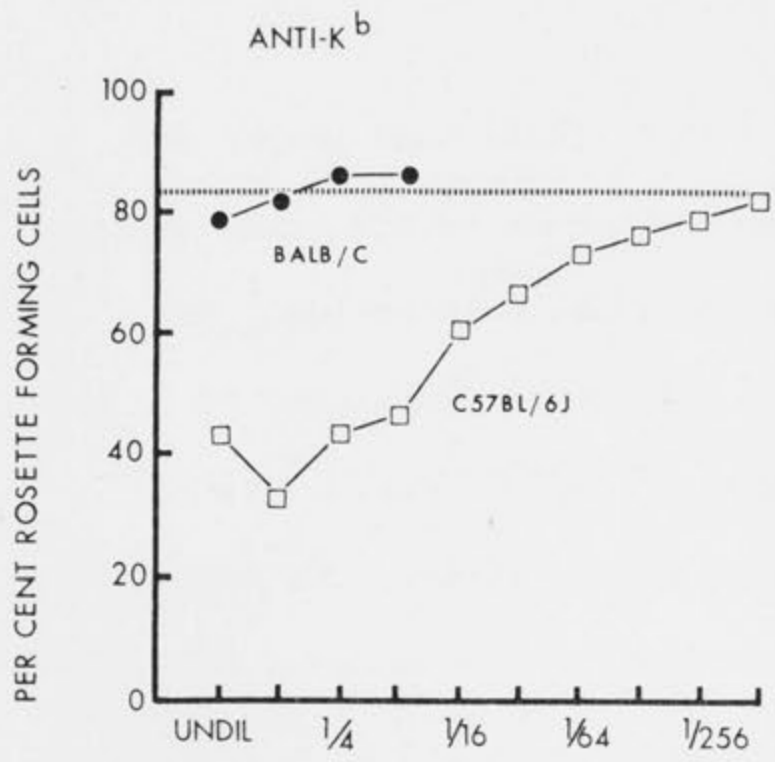


Fig. 2 Ability of serial dilutions of lysates of BALB/c ($H-2^d$) (●) and C57BL/6J ($H-2^b$) (□) spleen cells to inhibit the binding of a constant amount of anti- K^b (left hand graph) or anti- D^d (right hand graph) antibodies to B10.A(5R) (K^b, D^d) spleen cells. The undiluted lysate is derived from 2×10^8 spleen cells/ml. The dotted line represents antibody activity, as measured by the rosetting assay, in the absence of inhibitor.



LYSATE DILUTION

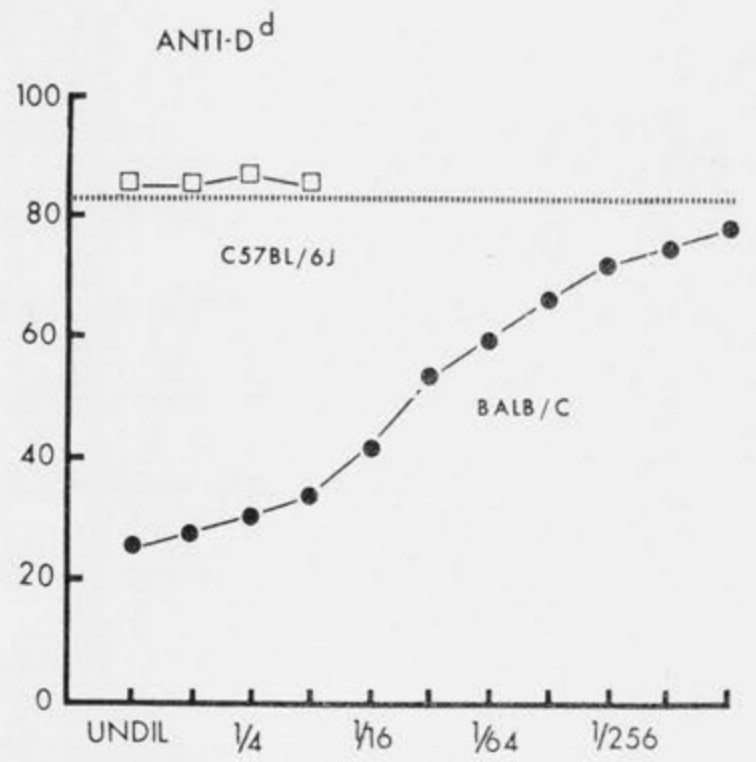


Fig. 3 Inhibition of binding of anti-K^b (left hand graph) and anti-D^d (right hand graph) antibodies to B10.A(5R) spleen cells by NP-40 lysates of B10.A(5R) splenocytes. The lysate used for inhibition was either unabsorbed (O), or immunoprecipitated with anti-K^b (■) or anti-D^d (●) antibodies prior to addition to the assay. The dotted line represents antibody activity in the absence of inhibitor, antibody binding being measured by a rosetting assay.

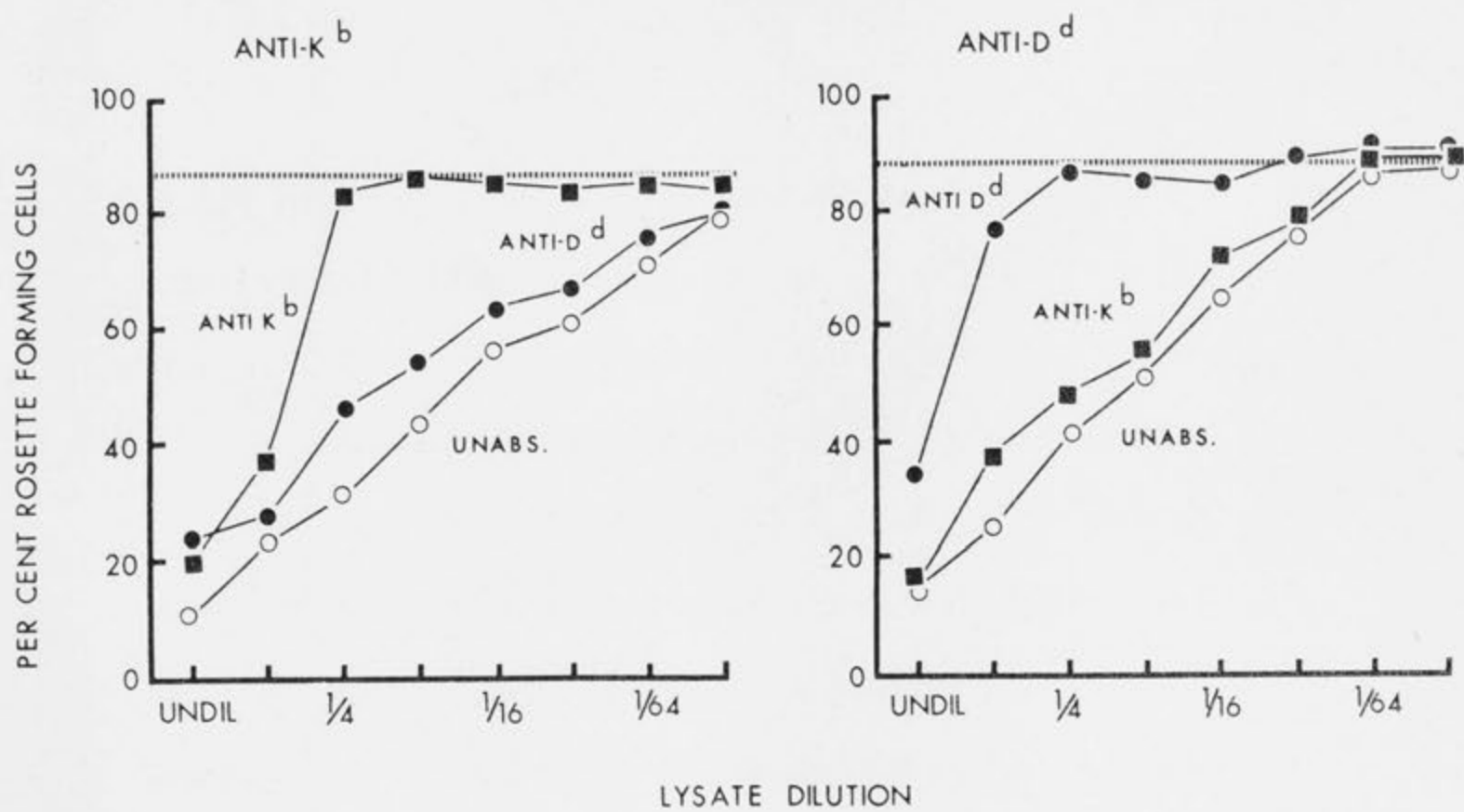


Fig. 4 Inhibition of binding of anti-L^d antibodies to B10.A (K^k, I^{k/d}, L^d, D^d) spleen cells by NP-40 lysates of B10.A splenocytes. The lysate used for inhibition was either unabsorbed (x) or immunoprecipitated with anti-K^k (Δ), anti-I^k (o), anti-D^d (▲) or anti-L^d (●) antibodies prior to addition to the assay. The dotted line represents antibody activity in the absence of inhibitor, antibody binding being measured by a rosetting assay.

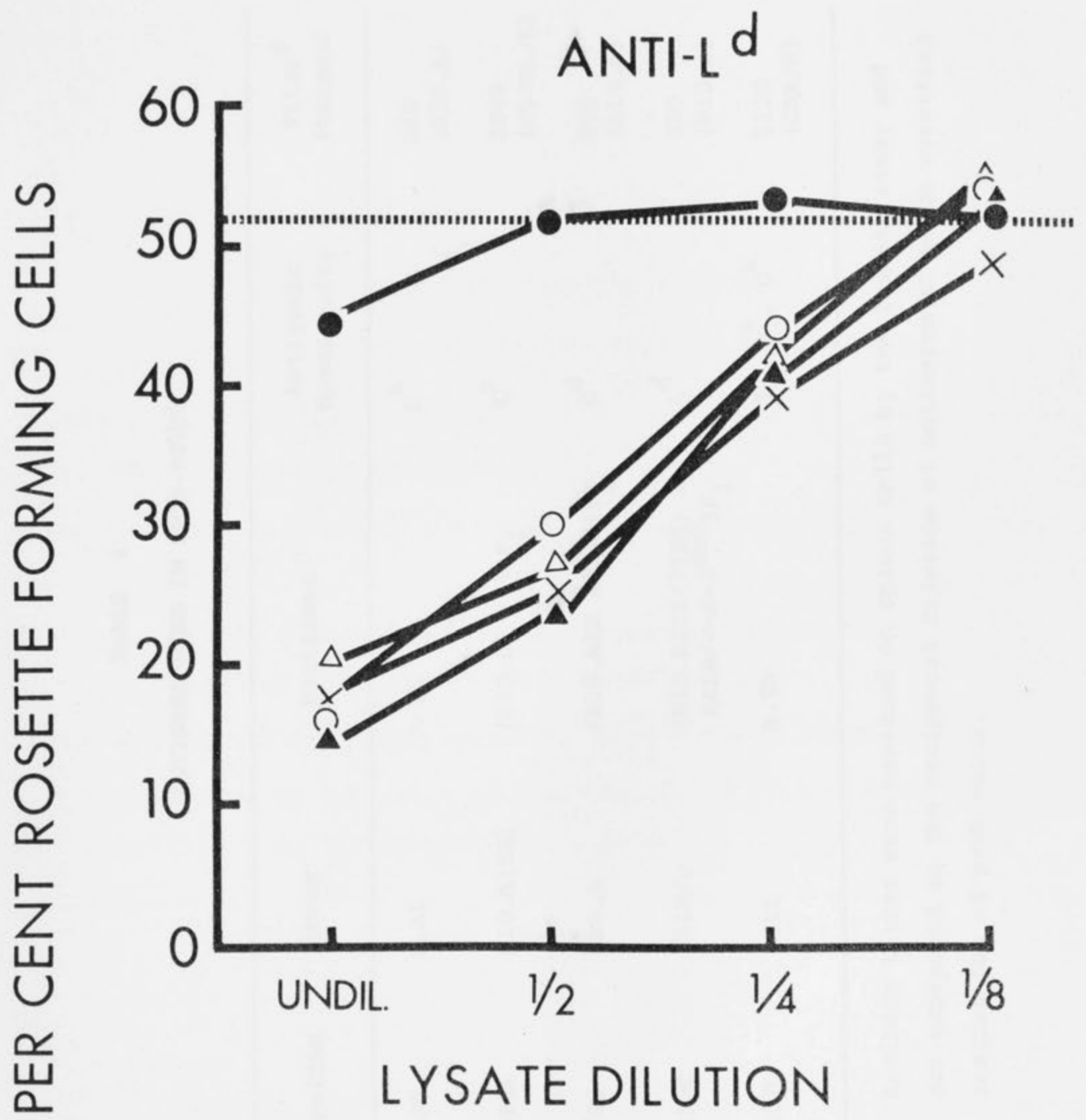


TABLE 1
ANTISERA USED IN THIS STUDY

Designation	Donor	Recipient	Antigenic Specificity	Titre ^a (target)
508	A.AL	A.TL	K ^k	960 (B10.A)
924A	B10.A(5R)	(B10.D2 x A)F ₁	K ^b	2048 (C57BL/6)
285	B10.A	(B10.AKM x 129)F ₁	D ^d	800 (B10.A)
153C	BALB/c	[B10.RIII(71NS) x BALB/c-H-2 ^{dm2}]F ₁	L ^d	320 (B10.A)
1070	A.TL	A.TH	I ^k , S ^k , G ^k	5120 (CBA/H)

a Antibody titres were measured on spleen cells by the rosette assay and are expressed as the reciprocal dilution of antiserum at which rosetting reached 50% of peak value.

TABLE 2

SUMMARY OF IMMUNOPRECIPITATION PROCEDURE USED TO
ANALYSE RELATIONSHIP BETWEEN CELL SURFACE ANTIGENS

-
1. NP-40 solubilisation of cells.
 2. Addition of antibody A to NP-40 lysate and incubation for 16-20 hours at 4°C.
 3. Removal of antibody and antigen-antibody complexes from lysate by protein A bearing *S. aureus* bacteria.
 4. Removal of NP-40 from lysate by XAD-8 resin (2 hours, 4°C).
 5. Assessment of remaining antigen content of lysate specific for antibody B by rosette-inhibition assay.
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The material presented in this thesis will also be presented in the following publications:

- O'Neill, H.C. and Blanden, R.V. 1979. Quantitative differences in the expression of parentally-derived H-2 antigens in F₁ hybrid mice affect T-cell responses. *J. Exp. Med.* 149:724-731.
- O'Neill, H.C. and Blanden, R.V. 1979. Variation in H-2 antigen expression in F₁ hybrid mice: Analysis using monoclonal antibodies. *Aust. J. Exp. Biol. Med. Sci.* 57:627-635.
- O'Neill, H.C. and McKenzie, I.F.C. Quantitative variation in H-2 antigen expression. I. Estimation of H-2K and H-2D expression in different strains of mice. *Immunogenetics*. In press.
- O'Neill, H.C. Quantitative variation in H-2 antigen expression. II. Evidence for a dominance pattern in H-2K and H-2D antigen expression in F₁ hybrid mice. *Immunogenetics*. In press.
- O'Neill, H.C. and Parish, C.R. Detection of two classes of H-2K^k molecules by monoclonal antibodies. Submitted for publication.
- O'Neill, H.C., Parish, C.R. and Higgins, T.J. Identification of protein and carbohydrate H-2K antigens by monoclonal antibodies. Submitted for publication.
- Parish, C.R., O'Neill, H.C. and McKenzie, I.F.C. A new procedure for analysing the relationship between different cell surface antigens. Submitted for publication.
- O'Neill, H.C., Parish, C.R. and Higgins, T.J. Demonstration of carbohydrate-defined and protein-defined H-2 antigens using monoclonal antibodies. Submitted for publication.
- O'Neill, H.C. and Parish, C.R. A new gene at the D end of the murine MHC controlling Ia-like antigens. Submitted for publication.
- O'Neill, H.C. and Parish, C.R. Molecular weight characterisation of carbohydrate and protein-defined H-2 antigens using monoclonal antibodies. Manuscript in preparation.
- Parish, C.R. and O'Neill, H.C. A model for T cell recognition based on MHC-controlled glycosyltransferases. Manuscript in preparation.

Abstracts presented at meetings

O'Neill, H.C. and Blanden, R.V. 1978. The expression of H-2 antigens in F₁ hybrid mice. Australian Society for Immunology Annual Meeting, Melbourne, Australia.

O'Neill, H.C. and Parish, C.R. 1979. The use of monoclonal antibodies to detect 2 classes of H-2K^k molecules. Australian Society for Immunology Annual Meeting, Sydney, Australia.

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