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ABSTRACT

The major histocompatibility antigens are the predominant cell surface molecules recognized by the immune system in the rejection of grafts between individuals of the same species. The genes coding for the histocompatibility antigens are extremely polymorphic; the products of different alleles are defined serologically. The studies described in this thesis were carried out on the major histocompatibility (H-2) antigens of the mouse. The goals of these studies were to define the structure of the H-2 molecule in solution and on the cell surface and to compare the structures of different H-2 molecules to provide some insight into the nature of the genetic polymorphism.

In order to carry out these studies, specific anti-H-2 alloantisera were produced. The sera recognized only one H-2 gene product as shown by antibody mediated cytotoxicity and immunoprecipitation assays. The alloantisera also recognized components of molecular weight 70,000-80,000 on mouse lymphocytes and leukemia cells. These components were also detected by a goat antiserum against the murine leukemia virus (MuLV) glycoprotein (gp70) and are therefore closely related to or identical with that viral protein. Further experiments suggest that factors such as viral antigens on the immunizing cells, aging, and general stimulation of the immune system may contribute to the production of these antibodies in H-2 alloantisera.

The physico-chemical properties of H-2K^b antigens solubilized by detergents and by the protease papain were compared by gel exclusion chromatography, ultracentrifugation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Detergent solubilized H-2 antigens (molecular weight 120,000) consist of two disulfide-linked heavy chains (47,000 daltons) and two noncovalently associated light chains (12,000 daltons). The light chain of histocompatibility antigens is known to be the protein β_2 -microglobulin. Alkylation with iodoacetamide prior to extraction prevented the formation of a disulfide linkage between the two heavy chains. A water-soluble 50,000-dalton molecule (F_S) consisting of a 39,000-dalton fragment (F_H) of the heavy chain and one intact light chain was obtained by papain digestion of cells or

detergent extracts. Experiments using the cross-linking reagent dimethyl-3,3'-dithiobispropionimidate indicated that the heavy and light chains of H-2 antigens are closely associated on the cell surface. Amino-acid sequence analysis of the F_H fragment of H-2K^b by radio-chemical techniques showed that it is identical to the detergent solubilized H-2K^b heavy chain in eight positions for the three amino acids tested. These data indicate that the fragment F_H derives from the amino-terminus of the heavy chain and suggest that it projects outward from the cell surface, while the carboxyl-terminal region is associated with the plasma membrane. From these data a model is proposed for the H-2 antigens that includes the size and arrangement of the subunits on the cell surface and in solution.

The H-2 heavy chains coded by the H-2K and H-2D genes of the haplotypes b, d and k were compared by SDS-PAGE, two dimensional gel electrophoresis and amino terminal amino acid sequence analysis. All of the H-2 gene products tested have similar overall structures. Furthermore, the amino terminal amino acid sequences of the heavy chains display considerable homology, supporting the hypothesis that the K and D genes of the major histocompatibility antigen complex have evolved by gene duplication. The amino acid sequences of the H-2 heavy chains are homologous to those reported for the heavy chains of the major histocompatibility antigens of man (HLA) and guinea pig (GPLA), indicating that the histocompatibility systems of mouse, man and guinea pig have a common evolutionary origin. The H-2 heavy chains also show distinct differences in molecular weight, isoelectric point and amino acid sequence. These differences appear to reflect the genetic polymorphism of the H-2 system. No feature has been defined, however, that unequivocally distinguishes allelic products of the K locus from those of the D locus. All of these findings have certain implications for the evolution and genetic organization of the murine histocompatibility antigens.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	ii
ABSTRACT.....	iii
TABLE OF CONTENTS.....	v
I. INTRODUCTION.....	1
II. HISTORY AND BACKGROUND.....	3
The Genetics of the H-2 Gene Complex.....	8
The Structure of the Histocompatibility Antigens.....	20
The Function of the Histocompatibility Antigens.....	27
III. CHARACTERIZATION OF H-2 ALLOANTISERA.....	36
Materials and Methods.....	36
Results.....	42
Discussion: The Origin of Anti-MuLV Antibodies in H-2 Alloantisera.....	52
IV. THE STRUCTURE OF H-2 ANTIGENS.....	55
Materials and Methods.....	55
Results.....	59
Discussion: A Model for the Structure of H-2 Antigens.	77
V. STRUCTURAL COMPARISONS OF H-2D AND H-2K GENE PRODUCTS.....	82
Materials and Methods.....	82
Results.....	88
Discussion: The Polymorphism of the H-2 Antigens.....	112
VI. BIBLIOGRAPHY.....	122

I. INTRODUCTION

When a graft of tissue or an organ is transplanted from one individual to another the graft is usually rejected. The immune system of the recipient sees the grafted tissue as foreign and is stimulated to produce a response against the foreign cells. This is the allograft reaction and is primarily a cellular immune response. The antigens on the foreign tissue against which this response is directed are known as transplantation or histocompatibility antigens. Although there are usually many histocompatibility antigen differences between the graft and the recipient, the strongest immune response is directed against one class of antigens, the major histocompatibility antigens.

There are systems of major histocompatibility antigens in virtually all vertebrate species examined. The best characterized, however, are the H-2 antigens of mice and the HLA antigens of man. The histocompatibility antigens are cell surface glycoproteins which are found predominantly on lymphoid cells but are probably present on all cells in the body. The genes coding for the antigens in both man and mouse are located in a small segment of the genome known as the major histocompatibility complex. Also located in the complex are genes coding for other cell surface antigens of lymphoid cells and genes controlling various aspects of the immune response.

The most striking feature of the major histocompatibility antigens is their polymorphism, that is, there are many variants of the antigens in the population. These have been extensively characterized by serological methods and also by transplantation. The genes determining each variant are believed to be alleles at a small number of genetic loci. Although the major histocompatibility antigens were detected originally by transplantation of tissues, this is not a natural biological event and the role of these antigens in the allograft reaction is probably not their true physiological function. Recent data, however, indicates that the major histocompatibility antigens may be involved in the recognition and destruction of abnormal cells.

The major questions concerning the histocompatibility antigens are the nature and origin of the polymorphism and the function of these molecules. In order to approach these questions it is necessary to understand their structures. This thesis describes, first, characterization of one type of murine histocompatibility (H-2) antigen in terms of its size and subunit structure in solution and on the cell surface and, second, structural comparisons of several different H-2 antigens by gel techniques and by amino acid sequence analysis. The results obtained from these experiments allow certain conclusions to be made about the nature of the polymorphism and its genetic origin.

II. HISTORY AND BACKGROUND

Antigens of the mouse H-2 histocompatibility system were first defined by Peter Gorer in London in 1936. He used human group A serum to detect differences in the haemagglutination patterns of erythrocytes of different inbred strains of mice (Gorer 1936a). Cells from one strain, ZS, showed a stronger reaction than those of other strains, such as the strain now called C57BL. Breeding experiments using these strains clearly indicated that a strong reaction to the human serum was determined by a single dominant gene.

In succeeding papers Gorer further defined this antigenic system with rabbit antisera prepared against mouse erythrocytes (Gorer 1936b, 1937). By means of suitable absorptions he showed that the antisera would agglutinate cells of one strain but not of other strains. For example, one antiserum prepared against cells of A strain mice reacted with A strain cells but not with cells from C57BL mice. Gorer deduced that the differences in the reactions of cells of various strains were due to the presence of different antigens on their erythrocytes. The antigen originally defined by the rabbit antiserum against A strain cells, which Gorer named antigen II, is probably the one now known as H-2.4, the private antigen of H-2D^d.

A vital factor in the initial detection of the H-2 antigens was the use of genetically homogeneous inbred strains of mice. These had been developed early in the century for the study of the genetics of tumour transplantation. It was soon found that tumours could be readily transplanted between mice of the same strain but not between mice of different strains. Little (1914) proposed that the susceptibility of a mouse to a tumour was determined by several dominant genes. Later, Little showed that the transplantation of normal tissue, such as spleen fragments, between mice of different strains, was also determined by a similar genetic system. At the time that Gorer began his experiments with H-2 antigens it was not known whether tumours were rejected because they differed antigenically from normal tissue or because they carried the same antigens as normal tissue and were thus rejected in the same way as normal tissue.

Gorer attempted to resolve this question. He tested the ability of a tumour that arose in an A strain mouse to grow in A and C57BL mice and the F_1 , F_2 and backcross mice obtained by breeding these strains (Gorer 1938). He also tested the mice for possession of antigen II, present in A but not C57BL mice. All mice carrying antigen II were killed by the tumour and the results were consistent with the hypothesis that two dominant genes were required in the host for tumour growth, one of these was the gene for antigen II. Furthermore, he found that sera from mice carrying a regressing tumour contained haemagglutinating antibodies with a reaction similar to that of the rabbit antiserum defining antigen II. For the first time the ability of a host to accept a graft was correlated with the presence of a particular serologically detectable antigen. The H-2 antigens were now truly defined as transplantation antigens. In Gorer's own words: "Normal and neoplastic tissues contain iso-antigenic factors which are genetically determined. Iso-antigenic factors present in the grafted tissue and absent in the host are capable of eliciting a response which results in the destruction of the graft." (Gorer 1938).

In 1948, George Snell of the Jackson Laboratories at Bar Harbor suggested that the genes determining the susceptibility of an animal to a tumour be named histocompatibility genes (Snell 1948). He found that one such histocompatibility gene in A and DBA mouse strains was linked to the gene Fu (fused) which caused a structural abnormality of the tail. In collaboration Gorer and Snell demonstrated that this gene was identical with the gene determining Gorer's antigen II (Gorer et al 1948). The name of the gene was changed to H-2. In addition, Gorer and Snell found that the DBA strain possessed an antigen which was similar but not serologically identical to the antigen II of strain A. They suggested that the gene determining this antigen was an allele of H-2, designated H-2^d.

Snell was able to define further alleles of the H-2 gene by using the linkage of H-2 with the dominant gene Fu: progeny from the cross (MxF)xN, where M and N are two different inbred strains and F is a strain carrying Fu, were tested for their susceptibility to a tumour from strain M and scored for the fused tail phenotype. By simple genetic

reasoning if M and N possess different alleles at the H-2 locus then resistance to the tumour is linked to possession of a fused tail. If the two strains have the same H-2 allele then all mice will be susceptible to the tumour. By this simple test Snell was able to define alleles H-2^d, H-2^b, H-2^p, H-2^k, H-2^q and H-2^r (Snell and Higgins 1951, Snell 1951, Snell et al. 1953).

Snell also proposed methods for the identification of other histocompatibility alleles. These required the production of mice which carried an H-2 allele from one inbred mouse strain but were otherwise genetically identical with another inbred strain. The introduction of a single gene from one strain into the genome of another required a complex breeding system. Progeny mice were tested for possession of the required histocompatibility gene by inoculation with a tumour that would only grow in the background strain: only mice homozygous for the gene would survive to be used for further breeding. For this reason, these mice are called congenic resistant (originally isogenic resistant) strains. Because the congenic resistant strains differ from their background strain only in the particular allele which they carry at the H-2 locus, mice of this type were to prove enormously important in the subsequent analysis of H-2 genetics and serology.

While Snell was defining the various alleles of the H-2 gene by transplantation methods, Gorer continued to characterize the H-2 antigens serologically. His progress was furthered by two technical advances: the development of a reproducible and sensitive haemagglutination technique for antibodies using human serum and dextran (Gorer and Mikulska 1954) and a cytotoxicity assay for antibodies involving dye exclusion (Gorer and O'Gorman 1956). Although mouse antisera had been used in Gorer's earlier studies (Gorer et al. 1948), these new techniques were particularly suitable for the analysis of the weak antibodies produced by mice. The use of antisera produced by mice of one strain against tissues or tumour cells from mice of another strain avoids the problem of antibodies against species-specific antigens. Allogenic sera or alloantisera have therefore formed the basis of H-2 serology.

Using these techniques and a wide variety of alloantisera it was soon apparent that the H-2 antigens were not only genetically polymorphic

as Snell had demonstrated by tumour transplantation, but were also extremely complex serologically: each H-2 allele appeared to determine several antigens (Hoecker et al. 1954). In addition, intra-H-2 recombinant mice were detected by serological techniques (Gorer and Mikulska 1959) suggesting that the H-2 antigens were controlled by more than one closely linked gene. This conclusion had been anticipated some years earlier by Snell who showed that a strain A tumour (H-2^a) would grow in an H-2^d/H-2^k heterozygote but not in either parent (Snell 1951). The H-2^a allele was subsequently found to be a naturally occurring recombinant between the H-2^d and H-2^k alleles.

The H-2 system was considered to be a complex of several closely linked genes or genetic regions each determining one or a few antigens. The pattern of antigens displayed by mice of a particular strain was referred to as a haplotype. Thus, C57BL mice possessed the haplotype H-2^b characterized by H-2 antigens 2, 5, 6, 27, 28, 29 and 33. Each number represented an antigenic determinant defined by reaction with a particular alloantiserum and assumed to be determined by a particular region of the H-2 complex. The antigenic determinants appeared to be of two types: some were widely distributed among different H-2 haplotypes, these were known as public antigens (Klein 1971a); whereas others were restricted to a single haplotype, these were called private antigens (Hoecker et al. 1954). The private antigens generate high titered, non-cross-reacting antisera and are characteristic of a particular haplotype.

In addition, two unrelated traits were mapped in the H-2 complex. In 1964 Shreffler found that a gene determining the quantity of the serum protein, Ss, was located inside the H-2 region: mice of different H-2 haplotypes had either high or low serum levels of this protein (Shreffler 1964). In 1965, McDevitt and Sela discovered that the immune response of mice to synthetic polypeptide antigens was under the control of a single gene and later showed that this gene was linked to the H-2 complex (McDevitt and Sela 1965, McDevitt and Tyan 1968). To account for these genes and for the serological patterns of recombinant mice, the number of genetic regions required in the H-2 complex had increased to eight by 1970 (Shreffler and Klein 1970).

As more intra-H-2 recombinant mice were analysed, inconsistencies began to appear in the multi-locus genetic map of the H-2 complex: the serological patterns of particular recombinants could only be explained by rare or bizarre genetic mechanisms. This problem was resolved by a new, two locus model of the H-2 system requiring only two multi-allelic loci, H-2K and H-2D, each coding for several antigenic determinants (Klein and Shreffler 1971, Stimpfling 1971, Snell et al. 1973). The keys to this hypothesis were the discovery that the same public antigen could be coded for by different regions of the H-2 complex (Shreffler et al. 1966) and that the private H-2 antigens could be arranged in two mutually exclusive series, K and D (Snell et al. 1971). Thus, each allele at either the K or D locus determines several public antigen specificities and one private antigen characteristic of that allele. Furthermore, the antigenic cross-reactivity between the products of the K and D loci led to the suggestion that these genes and possibly other genes mapping in the H-2 complex may have arisen during evolution by duplication of an ancestral gene (Klein and Shreffler 1971).

The Genetics of the H-2 Gene Complex

The two locus model is consistent with the current knowledge of the genetics of the H-2 antigens and forms the basis of the present genetic map of the H-2 complex. The linkage experiments of Gorer and Snell (Gorer et al. 1948) had placed the H-2 gene in linkage group IX which was subsequently found to be on the 17th chromosome of the mouse (Klein 1971b). A large number of genes have now been mapped both within the H-2 complex and in closely linked regions of the 17th chromosome. The products of several of these genes have structural and possibly functional features in common with the H-2 antigens. I shall first discuss the genes of the H-2 complex itself and then turn to a discussion of the other loci on the 17th chromosome.

The H-2 complex (Fig. 1) is considered to be bounded by the structural loci for the H-2 antigens, H-2K and H-2D, and subdivided into five regions: K, I, S, G and D. Most recently, the I region has been further subdivided into I-A, I-B, I-E, I-J and I-C subregions. The genes that have been mapped in each region are shown in Figure 1. The map distance between the K and D loci is approximately 0.5 cM (Klein 1975), sufficient to accomodate as many as 2000 cistrons (Bodmer 1972). It is likely that each region contains more than one gene and that the different traits mapped to each region could be controlled by separate genes. The resolution of H-2 genetic fine structure is limited by the availability of appropriate intra-H-2 recombinant mouse strains which define the boundaries of each region. The genetic maps of various inbred and recombinant strains are shown in Table I. Different alleles at each locus or region are distinguished by superscripts derived from their haplotype of origin. For example, the I-C region of the H-2^b haplotype is represented as I-C^b. The most extensive and experimentally useful series of congenic resistant strains are those with the H-2 complex on a C57BL/10 (B10) genetic background: the strain symbols for these mice begin with the code B10.

The K and D loci form the boundaries of the H-2 complex and code for the major histocompatibility antigens. The distribution of antigenic

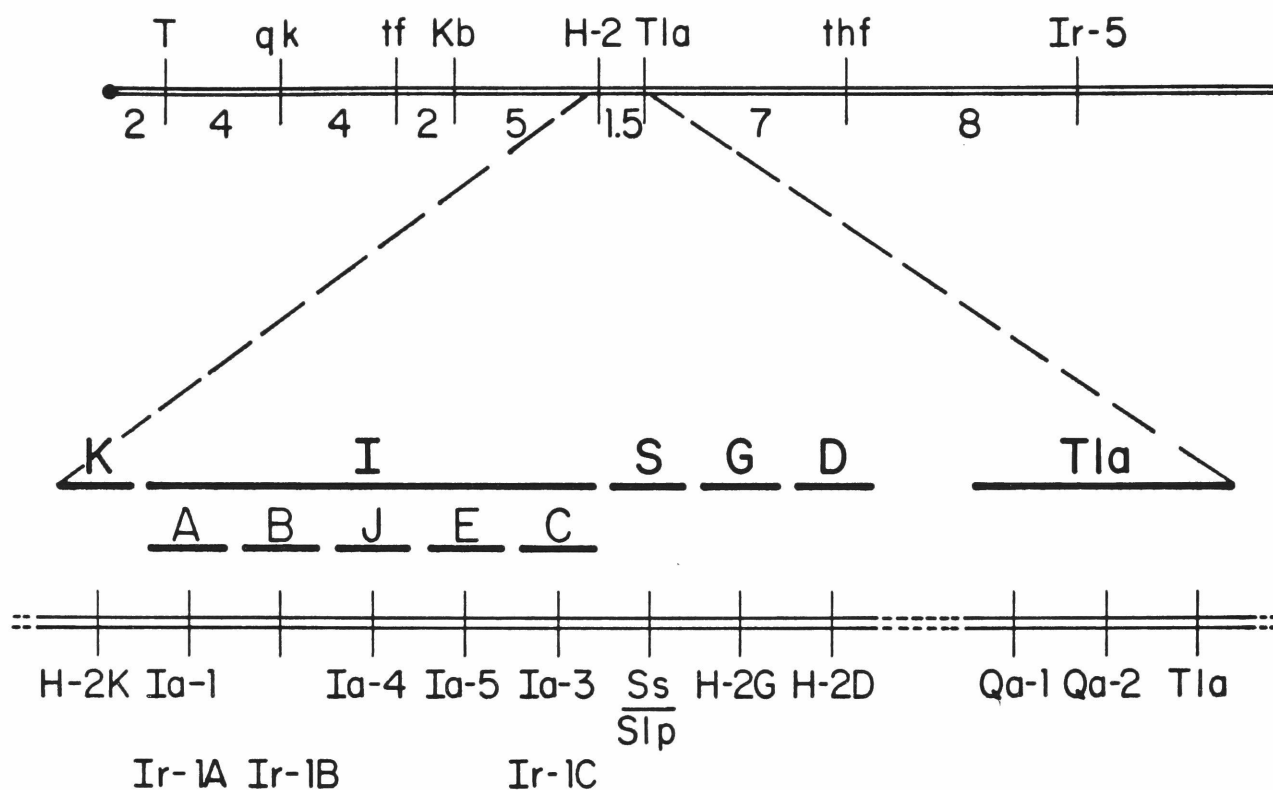


Fig. 1. Genetic map of the 17th chromosome of the mouse and the H-2 gene complex. The upper map shows the 17th chromosome. The centromere is to the left and the numbers are map distances in centimorgans. The lower map shows the fine structure of the H-2 gene complex and Tla region. The heavy bars indicate regions of the H-2 complex.

Table I
H-2 Haplotypes of Mouse Strains

<u>Mouse Strain</u>	<u>H-2 Region</u>								
	<u>K</u>	<u>A</u>	<u>B</u>	<u>J</u>	<u>E</u>	<u>C</u>	<u>S</u>	<u>G</u>	<u>D</u>
C57BL/10	b	b	b	b	b	b	b	b	b
B10.D2	d	d	d	d	d	d	d	d	d
B10.BR	k	k	k	k	k	k	k	k	k
B10.A	k	k	k	k	k	d	d	d	d
B10.A(2R)	k	k	k	k	k	d	d	d	b
B10.A(4R)	k	k	b	b	b	b	b	b	b
B10.A(5R)	b	b	b	k	k	d	d	d	d
B10.RIII	r	r	r	r	r	r	r	r	r
C3H	k	k	k	k	k	k	k	k	k
C3H.OH	d	d	d	d	d	d	d	d	k
AKR.M	k	k	k	k	k	k	k	k	q

Adapted from Klein (1975a).

determinants in the independent haplotypes b, d and k is shown in Table II. Although the two-locus model for the arrangement of the serological specificities remains consistent, most recently a third locus has been postulated. In mice of the haplotype H-2^d, both co-capping (Lemmonnier et al. 1975) and sequential immunoprecipitation (Hansen et al. 1977a) studies indicate that the lymphocytes of these mice possess H-2 antigens which carry the H-2.28 public specificity but not the H-2.4 private specificity of H-2D^d. This conclusion is supported by the discovery of a mutant, BALB/c-H-2^{db}, that appears to lack this gene (McKenzie et al. 1977). The third H-2 locus has been tentatively mapped into or to the right of the H-2D gene (Hansen et al. 1977b).

The H-2G region contains a gene coding for an antigen, H-2.7, found predominantly on erythrocytes and assumed to be an H-2 public specificity. This antigen was later shown, however, to be controlled by a separate locus (David et al. 1975, Klein et al. 1975). It has not been characterized biochemically.

The S region contains genes determining the quantitative expression of a serum protein, Ss (Shreffler and Owen 1963) and the expression of a variant of this protein, Slp (Sex-limited protein). The Slp antigen was detected originally only in male mice of strains positive for Ss (Passmore and Shreffler 1970) but subsequently, a female mouse was found that normally expressed the Slp antigen (Klein 1975b). In 1973, Demant demonstrated a correlation between Ss type and the serum level of haemolytic complement (Demant et al. 1973). The Ss protein was later positively identified as the C4 component of mouse complement by its structural similarities to human C4 (Lachman et al. 1975, Meo et al. 1975). There is also evidence suggesting that the levels of complement components C1 and C2 may be influenced by genes in the S region (Goldman and Goldman 1976). The complement components each consist of several polypeptide chains and it has not been established which, if any, of the chains are coded by genes in the H-2 complex.

The I region contains genes that appear to influence almost every aspect of the immune response (Shreffler and David 1975). The first gene mapped in this region, Ir-1, now called Ir-1A, determines the quantitative antibody response to the synthetic polypeptide antigens

Table II

Public and Private H-2 Antigens

H-2 gene	H-2K Public Antigens																Private Antigen
	1	3	5	8	11	25	27	28	29	34	35	36	39	45	46	47	
K ^b			5				27	28	29		35	36	39		46		33
K ^d		3		8			27	28	29	34					46	47	31
K ^k	1	3	5	8	11	25								45		47	23

	H-2D Public Antigens																
	1	3	5	6	13	27	28	29	35	36	41	42	43	44	49		
D ^b				6		27	28	29									2
D ^d		3		6	13	27	28	29	35	36	41	42	43	44	49		4
D ^k	1	3	5												49		32

Antigenic determinants are defined by specific H-2 alloantisera and are designated by arbitrary numbers. Public antigens are antigenic determinants common to products of both D and K genes; private antigens are unique for one gene product.

Adapted from Klein (1975a)

poly-L-(tyrosine, glutamic acid)-poly-DL-alanine-poly-L-lysine, abbreviated to T,G-A--L (McDevitt et al. 1972). This antigen consists of a poly-lysine backbone with poly-DL-alanine side branches to which are attached glutamic acid and tyrosine containing peptides. Antigens in which the terminal tyrosine residues are replaced with histidine, (H,G)-A--L, or phenylalanine, (Phe,G)-A--L, are also used. The Ir-1 gene determines the level of the secondary IgG response to the antigen (McDevitt and Benacerraf 1969): for example, H-2^b mice produce a high response to (T,G)-A--L whereas H-2^k mice produce a low response. The genetic regulation of the response is quite specific: for the antigen (H,G)-A--L the response pattern is reversed, H-2^k mice are high responders and H-2^b mice are low responders.

The immune responses to a wide variety of other antigens are under the control of genes in the I region. The antigens tested include linear synthetic polypeptides such as GAT¹⁰ (a random copolymer containing 60% L-glutamic acid, 30% L-alanine and 10% tyrosine), proteins such as IgG or S. aureus nuclease, and cellular antigens such as Thy-1 or H-2 itself. Using recombinant strains the genes controlling the responses to these antigens have been mapped to subregions within the I region. Thus, the I-A subregion contains genes controlling the response to (T,G)-A--L and (H,G)-A--L (McDevitt et al. 1972) and ovalbumin (Dunham et al. 1973). The I-B subregion contains the immune response genes for mouse IgG allotypes (Lieberman et al. 1972) and lactate dehydrogenase B (Melcher et al. 1974). Recently, however, the existence of I-B as a separate subregion has been brought into doubt (Shreffler et al. 1976). The immune response to some linear synthetic antigens such as GLØ (copolymer of glutamic acid, lysine and phenylalanine) appears to be controlled by two interacting genes, one located in the I-A region and the other in the I-C region (Benacerraf and Dorf 1976). It has been suggested that the response to (T,G)-A--L may also be controlled by two genes, both mapping in the I-A subregion (Munro and Taussig 1975).

Genes in the I region also code for the Ia antigens, a class of cell surface molecules found mainly on lymphoid cells, particularly B lymphocytes and macrophages (Shreffler and David 1975). These antigens are detected by specific alloantisera and display an extensive

antigenic polymorphism similar to that of H-2 antigens. The Ia antigens coded by the genes Ia-1 in the I-A subregion and Ia-3 in the I-C or I-E subregions have been most extensively characterized. Both genes code for molecules which consist of two polypeptide chains of molecular weight 33,000 and 25,000 and which are found largely on B lymphocytes (Cullen et al. 1976, Murphy et al. 1977). It is not known whether both or only one of the two chains carries the antigenic specificities nor whether both chains are coded by genes in the I region. The I-E subregion is defined by the Ia-5 gene coding for the Ia antigenic specificity Ia.22, found on B lymphocytes and a subpopulation of T lymphocytes (Shreffler et al. 1970) and having a similar structure to the products of Ia-1 and Ia-3 (Murphy et al. 1977). Another gene, Ia-4, in the I-J subregion codes for Ia antigens found on soluble factors and T lymphocytes having immune suppressive properties (Tada et al. 1976, Murphy et al. 1976). The structure of this antigen has not yet been characterized. No Ia antigen genes have been located exclusively in the I-B subregion.

The physiological function of the Ia antigens and their relationship to the products of the Ir genes remains largely unknown. Partial identity of the Ia and Ir genes is suggested by the detection of Ia antigens on soluble factors believed to mediate cell-cell interactions during the immune response (Munro and Taussig 1975, Amerding et al. 1974). In addition, the strain distribution of some Ia antigens parallels that of Ir genes: for example, the expression of Ia.2 correlates with the ability to produce a high response to (H,G)-A--L (Shreffler and David 1975).

There does appear to be a relationship, however, between the Ia antigens and the antigens causing stimulation in the mixed lymphocyte reaction (MLR), a proliferative reaction occurring when lymphocytes from mice of different genotype are cultured together. Usually the cells from one donor are irradiated or pretreated with mitomycin C to prevent proliferation of one cell type. Genes, termed *Lad* loci, which are believed to code for the stimulating antigen, have been mapped in various regions of the H-2 complex. The antigens causing the strongest stimulation are coded by a gene, LD-1 in the I-A region

(Meo et al. 1973). Other, weaker loci map in the I-C (Meo et al. 1973) and I-J regions (Okuda et al. 1977). The mixed lymphocyte reaction is inhibited if the stimulating cell population is pretreated with alloantisera directed against the Ia antigens coded by genes mapping in the same subregion as the stimulating Iad loci, suggesting that the MLR is a response to foreign Ia antigens (Meo et al. 1975b, Okuda et al. 1977).

Another class of genes located in the H-2 complex determines susceptibility or resistance of the animal to viral infection, particularly viral leukemogenesis. Viral leukemogenesis is measured by the incidence of leukemia after inoculation with virus. For most leukemia viruses, a number of genes appear to control the leukemogenic process (Lilly and Pincus 1973). Genes that map in the H-2 complex, however, appear to be mainly concerned with the immune response to leukemic or virally infected cells.

There appear to be two types of H-2 linked genes controlling leukemogenesis and this may reflect two different mechanisms of action. For example, the genes Rgv-1 (Lilly and Pincus 1973), X-1 (Sato et al. 1973) and Rrv-1 (Lonai and Haran-Ghera 1977) are located in the K or I regions, and are probably immune response genes. The immune response to AKR leukemia antigens has been linked to the I-J region (Meruelo et al. 1977b) suggesting that I-J region genes may actively suppress the response to viral or tumour antigens.

Genes located in the H-2D regions (Blank et al. 1976, Meruelo et al. 1977a) probably influence the cellular response. Recent evidence indicates that the H-2D linked control of radiation virus leukemogenesis does not influence initial infection or replication, but alters virus proliferation and spread (Meruelo et al. 1978). Furthermore, increased expression of H-2D antigens occurs after infection of thymocytes in resistant strains but not susceptible strains. These results indicate that the H-2 antigen may have a direct effect on leukemogenesis, possibly by influencing the immune response to the virus.

Finally, the genes determining a number of non-immunologically related traits have been linked to the H-2 complex. These include genes affecting androgen metabolism and the serum levels of testosterone-

binding protein (Ivanyi et al. 1972), cyclic AMP levels in the liver (Meruelo and Edidin 1975) and the levels of cortisol-binding protein (Goldman et al. 1977, Saloman and Pratt 1976).

There is considerable evidence that the H-2 complex contains a variety of loci that arose during evolution by gene duplication (Klein and Shreffler 1971). In addition there are a number of genes linked to the complex which code for proteins with structural features similar to those of the H-2D and H-2K antigens (Fig. 1). The Tla region contains two such genes. The Tla locus codes for the thymus leukemia antigen (TL) which is found on the thymus cells of some strains and on the leukemia cells of mice including those that do not normally express it on their thymus cells (Boyse and Old 1969). The structure of the TL antigen resembles that of the H-2K and H-2D antigens (Vitetta et al. 1976b) and its expression is reciprocally related to the expression of the H-2D antigen on cell surfaces (Boyse and Old 1969). Also in the Tla region are the genes Qa-1 and Qa-2 (Stanton and Boyse 1976, Flaherty 1976) which code for cell surface antigens found on lymphoid cells. One of the products of the Qa-2 locus is structurally similar to the H-2 and TL antigens and shows reciprocal expression with the TL antigen (Michaelson et al. 1977). The H-2, Tla and Qa-2 gene products all have a large subunit of molecular weight 43-46,000 in association with the 12,000 dalton β_2 -microglobulin (Vitetta et al. 1976b, Michaelson et al. 1977).

The t complex, located in the T-qk region of the 17th chromosome (Fig. 1) contains genes affecting embryonic development, fertility, transmission ratio and recombination in the t-H-2 interval (Bennett 1975). Genes mapping in the t complex also code for cell surface antigens found on sperm and embryonic cells and it has been suggested that these molecules may control cell-cell interactions during embryonic development (Bennett 1975). One cell surface antigen, the F9 antigen, also has a similar structure to the H-2 antigens, consisting of a heavy chain of molecular weight 45,000 and a light chain of 12,000 daltons (Vitetta et al. 1975). The light chain, however, has been shown not to be β_2 -microglobulin (Dubois et al. 1976). The expression of the F9 antigen on embryonic cells in vitro appears to be inversely related to the expression of H-2 antigens (Jacob 1977). There is also linkage disequilibrium in

wild mice between alleles at the t complex and alleles at the H-2 complex; that is, in the wild population particular t alleles are predominantly associated with the same H-2 haplotype (Hammerberg and Klein 1975). This is probably due to suppression of recombination by the t complex.

In addition to the strong histocompatibility antigens coded by the H-2 complex, there are at least four loci on the 17th chromosome which determine minor histocompatibility antigens: H-31 and H-32, in the Tla region (Flaherty and Wachtel 1975), H-33 located between H-2K and the t complex (Flaherty 1975), and H-39 closely linked to the t complex (Artzt et al. 1977). These are only a few of the nearly 40 minor histocompatibility loci (Graff and Bailey 1973) which are scattered throughout the murine genome and their linkage to the H-2 complex probably has no special significance.

There are also a number of loci on the 17th chromosome which determine traits that are probably unrelated to the H-2 complex. These include genes affecting tail structure (Fu and Kb), genes affecting hair growth (thf and tf) and quaking (qk), a gene affecting myelin formation (Klein 1975a). In addition, there are genes controlling polymorphic variants of the enzymes glyoxylase, Glo-1 (Meo et al. 1977), and kidney catalase, ce-1 (Grieshaber and Hoffman 1976). The ce-1 locus appears to be closely linked to the H-2 complex, probably in the Tla region, and affects post-translational modification of the kidney catalase, possibly by affecting the degree of sialation.

The H-2 complex of the mouse is probably the most extensively characterized genetic region of any higher animal. Several genes with separate but possibly interrelated functions are closely linked together in a relatively small segment of the genome. This organization, however, is not unique to the mouse and has been found in every vertebrate species so far investigated.

In man and rhesus monkey, the best studied species, the genes for the major histocompatibility antigens, B cell (Ia) antigens and complement components are closely linked in a major histocompatibility complex (MHC) similar to that of the mouse (Albert and Gotze 1977, Balner 1977). In other species, not all of these components have so far been associated with the MHC. Many species, however, show linkage

between genes determining the major histocompatibility antigens and genes controlling the mixed lymphocyte reaction (Table III). Furthermore, biochemical characterization of the major histocompatibility antigens in man (Strominger et al. 1976, Snary et al. 1976), mouse (Cunningham et al. 1976a), guinea pig (Schwartz et al. 1976), and chicken (Ziegler and Pink 1975) has revealed that all consist of a 43-48,000 dalton heavy chain non-covalently associated with a light chain of molecular weight 12,000. Preliminary sequence analysis has also shown considerable homology between the antigens of these species (Silver 1976). Similarly, Ia (B cell) antigens from man (Strominger et al. 1976), mouse (Cullen et al. 1976), and guinea pig (Schwartz et al. 1976) showed marked similarities in subunit structure and N-terminal sequence (Silver 1976).

The similarity of the major histocompatibility complexes in different species implies that strong selection pressures must have maintained the various genes of the MHC as a single unit during long evolutionary periods. Exactly why these genes appear to function better in a single complex is open to speculation. It seems that linkage only is essential because the detailed genetic organization of the complex may vary from species to species. In fact, the H-2 complex has a rather different genetic map from that of man or rhesus monkey. In the mouse the complement (Ss) and Lad loci map between the genes for the major histocompatibility antigens H-2K and H-2D, whereas in man and in the rhesus monkey, complement (C2 or Bf) and MLC genes map outside loci for the major antigens. This may only be a function of our present ignorance of the detailed genetics of the MHC in different species.

Table III

The Major Histocompatibility Complex in Different Species

Species	MHC	Genes known to map in the major histocompatibility complex					Reference
		Histocomp-ability antigens	MLC loci	Ia antigens	Complement components	Immune response genes	
mouse	H-2	H-2K,D	LD ₁ , LD ₂	Ia-1,3,4,5	C4	Ir-1A,1A,1C	Klein 1975a
man	HLA	HLA-A,B,C	HLA-D	HLA-D	C2, C4, Bf	(+)	Albert and Gotze 1977
rhesus monkey	RhLA	SD ₁ , SD ₂	LD ₁	Ia ₁ , Ia ₂	Bf	+	Balner 1977
chimpanzee	ChLA	SD ₁ , SD ₂	(+)				Balner 1977
dog	DLA	DLA-A,B,C	DLA-D			+	Vriesendorp et al. 1977
pig	SL-A	SL-A	PL-A				Ivanyi 1977
rabbit	RL-A	RL-A	+			+	Ivanyi 1977
guinea pig	GPLA	B, S	+	+		+	Geczy and de Weck 1977
rat	Ag-B/H-1	Ag-B/H-1	+			+	Gunther and Stark 1977
chicken	BL-A	B	+				Hala 1977

+ indicates that a gene has been located in the MHC but not named.

The Structure of the Histocompatibility Antigens

Histocompatibility antigens are integral membrane proteins (Walsh and Crumpton 1977). They are present in highest concentration on lymphoid cells, particularly spleen lymphocytes, but only to the extent of $10^5 - 10^6$ molecules per cell. Structural studies of the histocompatibility antigens have therefore had to overcome two major problems; solubilization of the antigens from the cell membrane, and detection of trace amounts of the antigens. These considerations apply to both H-2 and HLA antigens but classical protein purification techniques have been more successfully applied to the isolation of HLA antigens, due mainly to the availability of larger amounts of starting material. The application of classical methods to the isolation of H-2 antigens has had only limited success and it has been necessary to devise new approaches.

Of the wide variety of agents that have been used to solubilize cell membrane components, detergent extraction and protease treatment have been most successfully applied to the H-2 antigens. The nonionic detergent NP40, used at low (0.5%) concentration solubilizes H-2 antigens with almost 100% efficiency (Schwartz and Nathenson 1971), and has the advantage of not affecting nuclear membranes. In aqueous solution, however, NP40 forms micelles of molecular weight 300-400,000 causing problems for purification of the solubilized antigens. For solubilization of HLA antigens, the detergents Brij 98 and Brij 99 (Springer et al. 1974) and sodium deoxycholate (Snary et al. 1975) have been most successful. Treatment of cells or detergent extracts with the protease papain generates a large water soluble fragment of the H-2 antigen (Shimada and Nathenson 1969). This technique has also been applied to HLA antigens (Cresswell et al. 1974).

Detection of H-2 or HLA antigens has depended largely on the use of specific alloantisera. Solubilized histocompatibility antigens are most commonly assayed by their inhibition of complement mediated cytolysis by alloantisera (Sanderson 1965, Wigzell 1965). Because the antisera are highly specific, trace amounts of antigens in a complex extract may be detected by this means. Alternatively, histocompatibility

antigens that have been radiolabeled can be isolated directly from an extract by immunoprecipitation (Schwartz and Nathenson 1971) and detected by determination of radioactivity. This simple and efficient technique exploits the high specificity of the alloantisera and is the basis of modern H-2 antigen biochemistry. Strictly, the most biological assay for H-2 or HLA antigenicity should involve an effect on graft rejection. Assays of this type, however, are not suitable for monitoring antigenic activity during fractionation.

Different combinations of solubilization and detection techniques have been used to characterize the histocompatibility antigens. One of two strategies has usually been adopted: purification of the antigens from large quantities of source material using classical protein fractionation techniques or characterization of the antigens in extracts of small quantities of material using immunoprecipitation and assays for H-2 or HLA antigenicity.

H-2 antigens solubilized by papain treatment from large quantities of source material (4,000 mouse spleens) have been purified by Nathenson and co-workers by ammonium sulfate precipitation, gel filtration, ion-exchange chromatography and gel electrophoresis (Shimada and Nathenson 1969, Yamane and Nathenson 1970). Two classes of fragments carrying H-2 antigenic activity were produced by papain treatment: Class I fragments having a molecular weight of 60,000 and Class II fragments having a molecular weight of 33,000. These fragments were subsequently found to correspond to the antigens H-2K^b and H-2D^d (Class I) and H-2D^b and H-2K^d (Class II) (Schwartz et al. 1973). The final products were homogeneous as judged by SDS gel electrophoresis and were purified 300-800 fold with 1 to 2% yield. The Class I fragments contained 90% protein and 10% carbohydrate. Both classes of fragments were further characterized by amino acid composition and peptide mapping but little difference was found between H-2^d and H-2^b alloantigens.

The oligosaccharide side chain was also isolated from the purified antigens and found to consist of mannose, galactose, fucose, galactosamine and sialic acid with a total molecular weight of 3,300 (Muramatsu and Nathenson 1971). Each H-2 heavy chain probably carries two oligosaccharide side chains (Nathenson et al. 1978). The carbohydrate side

chains isolated from different H-2 antigens are identical in size and carbohydrate composition suggesting that the carbohydrate component of H-2 antigens does not directly determine the antigenic polymorphism. Furthermore, removal of terminal sialic acid residues or penultimate galactose residues had no effect on H-2 antigenicity (Nathenson and Muramatsu 1971).

The detergent solubilized H-2 antigens are assumed to represent the intact structure of the molecules. In NP40 solution H-2 antigens, assayed by inhibition of immune cytotoxicity, appeared to have a molecular weight of 380,000, as determined by gel filtration (Schwartz et al. 1973). The high molecular weight may have been due to aggregation of the antigens or to the micelle size of the detergent. The technique of immunoprecipitation (Schwartz and Nathenson 1971) enables radiolabeled H-2 antigens to be isolated directly from detergent solution and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). By this means the H-2 antigens were found to have a molecular weight of 43-47,000 in the presence of a reducing agent, some 2,500-6,000 daltons larger than papain solubilized antigens isolated by the same technique.

Characterization of papain solubilized HLA antigens, the human homologues of the H-2 antigens, revealed that these molecules consisted of two polypeptide chains, one of 31,000 daltons and the other 12,000 daltons (Cresswell et al. 1973). The smaller peptide was subsequently shown to be identical to β_2 -microglobulin (Nakamuro et al. 1973, Grey et al. 1973, Peterson et al. 1974), a protein previously isolated from urine (Berggård and Bearn 1968) and shown to have amino acid sequence homologies with immunoglobulins (Peterson et al. 1972). H-2 antigens were also found to contain a small subunit (Silver and Hood 1974, Rask et al. 1974) which has been identified as the mouse homologue of β_2 -microglobulin (Appella et al. 1976).

Analysis of the structures of both H-2 and HLA antigens has progressed significantly during the time covered by this thesis. Studies in a number of laboratories and the experiments described in this thesis have defined the overall structure of the H-2 molecule in terms of the number and organization of the polypeptide chains on the cell surface and in solution. The structures of different H-2 heavy chains have also

been compared by molecular weight, peptide maps, isoelectric point and amino acid sequence. These studies are discussed in later sections.

In man, the light chain of the HLA antigens has been shown to be identical with the protein β_2 -microglobulin (Nakamuro et al. 1973, Grey et al. 1973, Peterson et al. 1974). In the mouse, the homologous protein has been isolated and identified as the light chain of the H-2 antigens (Silver and Hood 1974, Appella et al. 1976). Similar proteins from a variety of other species have now been isolated and characterized. All β_2 -microglobulins so far examined show structural homologies with immunoglobulins. This is the most striking feature of β_2 -microglobulin and is the strongest indication that there is an evolutionary relationship between the histocompatibility antigens and immunoglobulins.

Human β_2 -microglobulin was first isolated from urine by Bergg rd and Bearn (1968). It was found to be a protein of molecular weight 11,600, containing a single intrachain disulfide bond and no carbohydrate. The amino terminal amino acid sequence of the molecule indicated that β_2 -microglobulin might be related to the immunoglobulins and it was suggested that the gene for β_2 -microglobulin arose as a result of a large deletion in an immunoglobulin-like gene (Smithies and Poulik 1972a). The complete amino acid sequence of human β_2 -microglobulin shows striking homologies with the sequences of immunoglobulin light and heavy chains (Peterson et al. 1972, Cunningham et al. 1973). The molecule contains exactly 100 residues and an intrachain disulfide bond which forms a loop of 57 residues. The structure is therefore very similar to that of a single immunoglobulin domain (Edelman 1970).

The sequence of human β_2 -microglobulin has been compared to the known sequences of immunoglobulins G, M and E. The strongest homology in each case is with the carboxyl-terminal domain, i.e. the domains $C_{\gamma}3$, $C_{\mu}4$ and $C_{\epsilon}4$ (Peterson et al. 1972, Cunningham 1976). The fact that there is uniform homology with constant region domains of three different classes of immunoglobulin suggests the gene specifying β_2 -microglobulin was derived from the immunoglobulin precursor gene before duplication of this gene to generate genes coding for the various immunoglobulin classes (Peterson et al. 1972, Cunningham 1976).

Similar proteins have been isolated from a number of other species and identified as the homologues of β_2 -microglobulin by the criteria of molecular weight and amino acid sequence homology with human β_2 -microglobulin. The mouse and guinea pig proteins have also been identified on the cell surface in association with the heavy chains of the major histocompatibility antigens (Vitetta et al. 1976b, Bjork et al. 1978). The sequences of the proteins from rabbit, dog, mouse, cow, and guinea pig are compared with the sequence of human β_2 -microglobulin for the first 35 residues in Figure 2. Over this range, any β_2 -microglobulin differs from the human protein in from five to eight positions. From residues 1-7 there is considerable variation. Among these residues, the leucine at position 2 is unique to the guinea pig, the lysine at position 3 is unique to the mouse, and the asparagine at position 6 is unique to the rabbit. From residues 8-35, however, there is little variation in sequence. As a basis for comparing H-2 and HLA heavy-chain sequences, it is worth noting that the human and mouse proteins differ in only 5 out of the first 27 residues.

Lactollin, the bovine analogue of β_2 -microglobulin, has been crystallized and preliminary X-ray diffraction studies have recently been reported (Becker et al. 1977). The protein crystallizes in the orthorhombic space group $P2_12_12_1$ and the data suggest that the asymmetric unit contains one polypeptide chain. Unlike immunoglobulin domains which are always paired, β_2 -microglobulin therefore exists both in the crystal and in solution as a monomer.

A possible homologue of β_2 -microglobulin has been isolated from chicken serum (Winkler and Sanders 1977). This protein has a molecular weight of 11,400 and its amino acid composition is similar to that of human β_2 -microglobulin. It is probably identical with the light chain of chicken histocompatibility antigens (Ziegler and Pink 1976). Using a heterologous radioimmunoassay, homologues of β_2 -microglobulin have also been tentatively identified in a variety of other species including three species of marsupials, two species of birds and a chondrichthian (Gordon and Kindt 1976).

Within a species, no convincing evidence for any genetic variant of β_2 -microglobulin has been obtained. This is in striking contrast to

	1	5	10	15	20															
Human	Ile	Gln	Arg	Thr	Pro	Lys	Ile	Gln	Val	Tyr	Ser	Arg	His	Pro	Ala	Glu	Asn	Gly	Lys	Ser
Rabbit	<u>Val</u>	Gln	Arg	<u>Ala</u>	Pro	<u>Asn</u>	<u>Val</u>	Gln	Val	Tyr	Ser	Arg	His	Pro	Ala	Glu	Asn	Gly	Lys	<u>Asp</u>
Dog	<u>Val</u>	Gln	<u>His</u>	<u>Pro</u>	Pro	Lys	Ile	Gln	Val	Tyr	Ser	Arg	His	Pro	Ala	Glx	Asx	Gly	Lys	<u>Pro</u>
Mouse	Ile	Gln	<u>Lys</u>	Thr	Pro	<u>Gln</u>	Ile	Gln	Val	Tyr	Ser	Arg	His	Pro	<u>Pro</u>	Glu	Asn	Gly	Lys	<u>Pro</u>
Cow	Ile	Gln	Arg	<u>Pro</u>	Pro	Lys	Ile	Gln	Val	Tyr(Ser)	Arg	His	Pro	<u>Pro</u>	Glu(Asx)	Gly	Lys	<u>Pro</u>		
G. Pig	<u>Val</u>	<u>Leu</u>	<u>His</u>	<u>Ala</u>	Pro	<u>Arg</u>	<u>Val</u>	Gln	Val	Tyr	-	Arg	His(Pro)	Ala	Glu	Asn(Gly)	-	-		
	21	25	30	35																
Human	Asn	Phe	Leu	Asn	Cys	Tyr	Val	Ser	Gly	Phe	His	Pro	Ser	Asp	Ile					
Rabbit	Asn	Phe	Leu	Asn	Cys	Tyr	Val	Ser	Gly	Phe	His	Pro	Ser	Asp	Ile					
Dog	Asx	Phe	Leu	Asx	Cys	Tyr	Val	Ser	Gly	Phe	His	Pro	-	<u>Glx</u>	Ile					
Mouse	Asn	<u>Ile</u>	Leu	Asn	Cys	Tyr	Val	<u>Thr</u>	<u>Glu</u>	Phe	His	Pro	<u>Pro</u>	-	Ile					
Cow	Asn	Tyr	Leu	Asn	Cys	Tyr	Val	-	(Gly)	Phe	His	Pro	-	-	-					

Fig. 2. Comparison of the amino terminal amino acid sequences of β_2 -microglobulins from different species. The data for the human protein are from Cunningham et al. (1973); for the rabbit protein from Cunningham and Berggard (1975); for the dog protein from Smithies and Poulik (1972); for the mouse protein from Appella et al. (1976); for the cow protein from Groves and Greenberg (1977) and Becker et al. (1977); and for the guinea pig protein from Cigen et al. (1978). Residues differing from the human protein are underlined.

the extensive polymorphism of the histocompatibility antigens. The region of the heavy chain that interacts with the β_2 -microglobulin may therefore be more conserved than other parts of the molecule.

In man, the gene coding for β_2 -microglobulin has been mapped on the 15th chromosome (Goodfellow et al. 1975) while the genes for HLA antigens are located on the 6th chromosome (van Someren et al. 1974). Although the genes for the two chains are not linked, the expression of HLA antigens appears to be dependent on the expression of β_2 -microglobulin. The human cell line Daudi, which lacks the gene for β_2 -microglobulin and does not express HLA antigens, will re-express its HLA antigens after fusion with mouse cells (Fellous et al. 1977a). In this case the HLA antigens have been shown to be in association with mouse β_2 -microglobulin on the surface of the hybrid cells (Fellous et al. 1977b). This suggests that the structure of the heavy chain region, which is in contact with β_2 -microglobulin, is conserved between species.

Despite the detailed knowledge about the amino acid sequence of β_2 -microglobulin its function remains unknown. Although it is assumed to be an intrinsic polypeptide chain that makes up the H-2 and HLA antigens, it is possible that it could have a function separate from that of the histocompatibility antigens. Human β_2 -microglobulin inhibits rosette formation by guinea pig macrophages and human IgG coated erythrocytes (Painter et al. 1974). This suggests that it has some of the properties assigned to the $C_{\gamma}2$ and $C_{\gamma}3$ domains with which it is homologous. There is some evidence for a role for β_2 -microglobulin in immune recognition phenomena. For example, anti- β_2 -microglobulin antiserum inhibits the mixed lymphocyte reaction (Bach et al. 1973) but only when bound to the responding cell population (Ostberg et al. 1976). Anti- β_2 -microglobulin is also mitogenic for mouse B lymphocytes (Moller and Persson 1974). Alternatively, the function of β_2 -microglobulin may be related to the function of the histocompatibility antigens. This will be discussed in the next section.

The Function of the Histocompatibility Antigens

Histocompatibility antigens by definition are those components of a grafted organ or tissue which are recognized as foreign by the recipient of the graft. At the cellular level, the process of graft rejection appears to depend on the generation of cytotoxic T cells. Once stimulated, these cells are specific in that they can destroy other cells of the same genetic type as the graft, either in vivo or in vitro, but are not active against cells derived from an animal genetically different from the donor of the graft. In the mouse, nearly 40 histocompatibility antigen systems have been described (Klein 1975a), each controlled by a separate gene and each capable of causing graft rejection. But, of these forty systems, differences in the H-2 complex result in the strongest immune response and the fastest rejection of the graft (Graff and Bailey 1973).

This unique feature of the H-2 system is probably due to the fact that an unusually large number of T cells are capable of responding to alloantigens coded by the H-2 complex. Experiments using combinations of congenic mice have demonstrated that the antigens on the target cell specifically recognized by the killer cell are (or are closely linked to) the H-2K and H-2D antigens (Alter et al. 1973). It has been estimated that 1 to 2% of the cytotoxic T cell precursors in mouse lymph nodes will respond to a particular H-2 alloantigen (Lindahl and Wilson 1977). In immune cell populations this figure may be much higher: for example, the frequency of cytotoxic lymphocytes specific for a particular alloantigen in peritoneal exudate has been estimated at 35% (Berke et al. 1975). In rats 6% of normal T cells were found to react with anti-idiotypic antibodies which were assumed to react with B and T cell receptors for a particular alloantigen (Binz and Wigzell 1975). This frequency is similar to previous estimates obtained by other techniques (Ford et al. 1975).

The immune system therefore appears to be particularly adapted to respond to foreign alloantigens. But the transplantation of tissues from one individual to another of the same species is not a natural

biological event. There are certain circumstances, such as pregnancy, where the immune system of one individual can encounter foreign allo-antigens. In this case, however, an immune response against the foetus would probably be detrimental. In fact, the mother does produce antibodies against paternal histocompatibility antigens on the foetus but these have no effect on its development (Bodmer 1972). There are also rare examples of contagious cancers in dogs and hamsters (Cooper et al. 1964, Weber et al. 1965) and it has been suggested that the polymorphism of the histocompatibility antigens evolved to protect the individual against infection by these cancers (Burnet 1962, 1973).

A more general hypothesis has been proposed by Jerne (1971). His proposal attempts to provide a role for histocompatibility antigens in the generation of antibody diversity. He assumes that the repertoire of antigen-binding immunoglobulin variable regions are generated from a small set of germ-line genes (v-genes) by somatic mutation. He then proposes, however, that the v-genes are the structural genes for antibodies directed against all the major histocompatibility antigens in the species. The v-genes may be of two types: a subset S (self) that determines variable regions directed against the individual's own histocompatibility antigens and a subset A (allo) that determines variable regions directed against all other histocompatibility antigens. During the diversification of variable region genes by somatic mutation, cells expressing antibody-variable regions that recognize self antigens are suppressed. Therefore, the cells that express variable regions coded by the v-gene subset S will be suppressed unless the v-gene has mutated so that the immunoglobulin binding site which it determines no longer recognizes self antigens. These cells, however, will probably bind antigens with a structure similar to the self antigens, i.e., "not-quite-self". The cells which express the A subset of v-genes are not suppressed and continue to express a variable region directed against foreign alloantigens. In this way, Jerne suggests that the range of antibody diversity in any one individual is determined by that individual's pattern of histocompatibility antigens.

Bodmer has objected to this hypothesis on the grounds that it requires strict parallel evolution at the population level of histocompatibility

antigen genes and immunoglobulin v-genes (Bodmer 1972). He further argues that an individual possessing a mutant histocompatibility antigen would be at a selective disadvantage if none of the available v-genes corresponded to that antigen. There would therefore be selection against new histocompatibility antigens. This does not seem likely in view of the extensive polymorphism of the histocompatibility antigens. Bodmer offers the alternative hypothesis that the S subset of v-genes is directed against differentiation antigens which are common to all individuals of a species and which function in cell-cell interactions during embryonic development (Bodmer 1972).

In addition to the response against allogeneic histocompatibility antigens, cytotoxic T cells can also be generated against syngeneic tumour cells and virally-infected cells. Thus, if a mouse is immunized with syngeneic tumour cells or infected with a virus, cytotoxic T cells appear in the mouse that are capable of killing cells infected with the same virus or expressing the same tumour antigens. In these cases, it is possible to demonstrate two types of specificity: only cells expressing the same viral or tumour antigen as the original stimulating cell are lysed, and then only if they express the same histocompatibility antigens as the stimulating cell.

In studies of the lysis of virally-infected cells by cytotoxic T cells from virally-infected mice, the viruses used have included lymphocytic choriomeningitis (Zinkernagel and Doherty 1974), vaccinia (Koszinowski and Ertl 1975), ectromelia (Gardner et al. 1975), Sendai (Schrader and Edelman 1977), herpes viruses (Pfizenmaier et al. 1977), murine sarcoma virus (Doherty and Zinkernagel 1974), and SV40 virus (Trinchieri et al. 1976). Most of the work has been done with ectromelia, Sendai and LCM viruses, which have two important features in common. They have an outer envelope which can incorporate host components and they cause virus-specific changes at the plasma membrane. In all cases tested, cell-mediated lysis of infected cells is highly specific for the virus and cross-reaction has been detected only for closely related viruses. T cell-mediated lysis also was observed only when the cytotoxic cells and infected targets shared at least part of the H-2 complex. Detailed studies (Doherty and Zinkernagel 1974) showed that the cytotoxic cell and target cell must share either H-2K or H-2D genes

and that identity at other genes in the H-2 region was not sufficient for lysis. In addition to restriction in vitro, many of the results have been confirmed in vivo (Doherty and Zinkernagel 1974, Zinkernagel and Welsh 1976, Kees and Blanden 1976).

T cell-mediated responses to tumour cells in H-2 compatible or syngenetic mice presumably involve tumour-specific or viral determinants as the primary foreign determinants. In these systems, however, H-2 restriction is not absolute and cross-reactivity has been observed, for example, T cells stimulated against an H-2^d tumour cell will lyse an H-2^b tumour (Schrader and Edelman 1976). Nevertheless, in all of these systems, the lysis observed for syngenetic targets was consistently stronger than that observed for cross-reactive allogeneic targets.

A similar restriction of lysis is observed in three other systems: the lysis of cells from congenic mice differing in minor histocompatibility antigens (Bevan 1975), the lysis of cells by cytotoxic cells directed against the male specific H-Y antigen (Gordon et al. 1975), and the lysis of cells covalently derivatized with haptens (Shearer et al. 1975). Cytotoxic T cells from mice immunized with H-2 compatible cells that differ at minor histocompatibility loci will lyse targets from the immunizing strain, but not targets from a congenic strain that carries all the minor antigens but differs at H-2 (Bevan 1975). The male specific antigen (H-Y) may also be considered as a minor histocompatibility antigen. Female mice, primed by injection of syngenetic male cells, will produce cytotoxic cells capable of lysing male target cells expressing the same H-2 antigens as the original stimulating cells (Gordon et al. 1975). The cell-mediated lysis of trinitrophenyl (TNP)-modified cells shows less restriction than the lysis of virally-infected cells. In addition, the response to the TNP-modified cells is strong and requires no in vivo priming (Shearer et al. 1975).

More direct evidence that cell surface H-2 antigens are essential to the specificity of the cytotoxic reaction arises from studies of H-2 mutant mice. In particular, characterization of cytotoxic T cells from H-2^b mutants that had been immunized with LCM, vaccinia or ectromelia virus (Doherty and Zinkernagel 1974, Kees and Blanden 1976, Zinkernagel 1976) indicated that a single mutation at H-2K^b is sufficient to limit

lysis. Virus-immune spleen cells generated in H-2^{ba} or H-2^{bf} mutant mice did not lyse virus-infected wild-type H-2^b target cells, and vice versa. The mutation in K^b also abolished the ability of immune T cells to provide anti-viral activity in transfer experiments in vivo. These results indicate that the restriction is very closely linked with H-2K (and H-2D) and likely involves the products of these loci.

Specific recognition, however, appears to involve only the H-2 antigens specified by the target cells. Thus, cells which do not express H-2 antigens, such as the F9 cell line, are not lysed by anti-viral cytotoxic cells, even though the F9 cells expressed the appropriate viral antigen (Zinkernagel and Oldstone 1976). Furthermore, a variety of experiments in which specific H-2 antisera were used to block cell-mediated lysis have indicated that the crucial H-2 antigens are those on the target cells (Schrader et al. 1975, Germain et al. 1975). In addition, studies of chimeric mice have shown that virus-specific cytotoxic T cells could be generated against allogeneic target cells (Pfizenmaier et al. 1976). Irradiated (CBA/J x C57BL/6)F₁ mice were reconstituted with CBA/J-T6 lymphocytes. The resulting chimeric mice were immunized with LCM virus. The cytotoxic T cell populations recovered from these mice attacked LCM-infected CBA/J cells but were also active against LCM-infected C57BL/6 cells. Uninfected cells of either type were not attacked, nor were cells of an irrelevant H-2 type. H-2 identity between the killer and target cells therefore appears not to be required.

The data so far suggest that the cytotoxic T cell must recognize two groups of antigenic determinants on a potential target cell in order to lyse it. The first group consists of the H-2 antigenic determinants, which must be identical to (or strongly cross-reactive with) H-2 antigens expressed by the cells originally used to stimulate the cytotoxic T cell. The second group consists of the "foreign" antigen (virus, tumour antigen, hapten, etc.) and again must be identical to all or part of the foreign antigen expressed by the original stimulating cell. Neither group of antigens need be the same as any antigen expressed by the cytotoxic T cell.

A variety of models have arisen from the results of the studies on H-2 restriction in cell-mediated lysis. Most of these focus on consideration of whether the cytotoxic T cell interacts with separate receptors for H-2 antigens and the foreign antigen on the target cell or with a single receptor (Fig. 3). Single receptor models suggest that the T cell sees either a hybrid antigen composed of preformed H-2 and viral or tumour components or a target cell antigen whose synthesis or expression is modified by the foreign component. Dual recognition models have varied in terms of the nature of the various T cell receptors postulated. An alternative model suggests that the target antigens may be linked by a third molecule at or beneath the cell surface. The evidence for each of these models is generally indirect and circumstantial. None of these has been proven conclusively.

Studies directed towards single receptor models have focussed on the nature of the antigen on the target cell surface. The possibility that the viral or other foreign component modifies the synthesis or expression of H-2 antigens on the target cell (Fig. 3b) has largely been ruled out by experiments using inactivated viruses (Schrader and Edelman 1977). The target cells become susceptible to lysis by virus specific T cells within 30 minutes after the binding of inactivated Sendai virus. No virus specific protein synthesis was observed. Similar results have also been reported by Koszinowski et al (1977). It has been shown that the fusion glycoprotein of Sendai virus is essential for the formation of the target antigen (Sugamora et al. 1977). These experiments place severe restrictions on the nature of any covalent modification of the H-2 antigens on the target cell.

The alternative form of the single receptor model suggests that H-2 antigens and viral antigens may interact on the cell surface to form a hybrid antigen (Fig. 3a). Several studies have shown that this can occur. For example, H-2 antigens can be co-capped with leukemia virus antigens on mouse leukemia cells (Schrader et al. 1975). Similar results have been obtained in this system using the lysostrip technique (Henning et al. 1976b). H-2 antigens have also been shown to co-cap with Friend virus antigens on cells infected with the virus

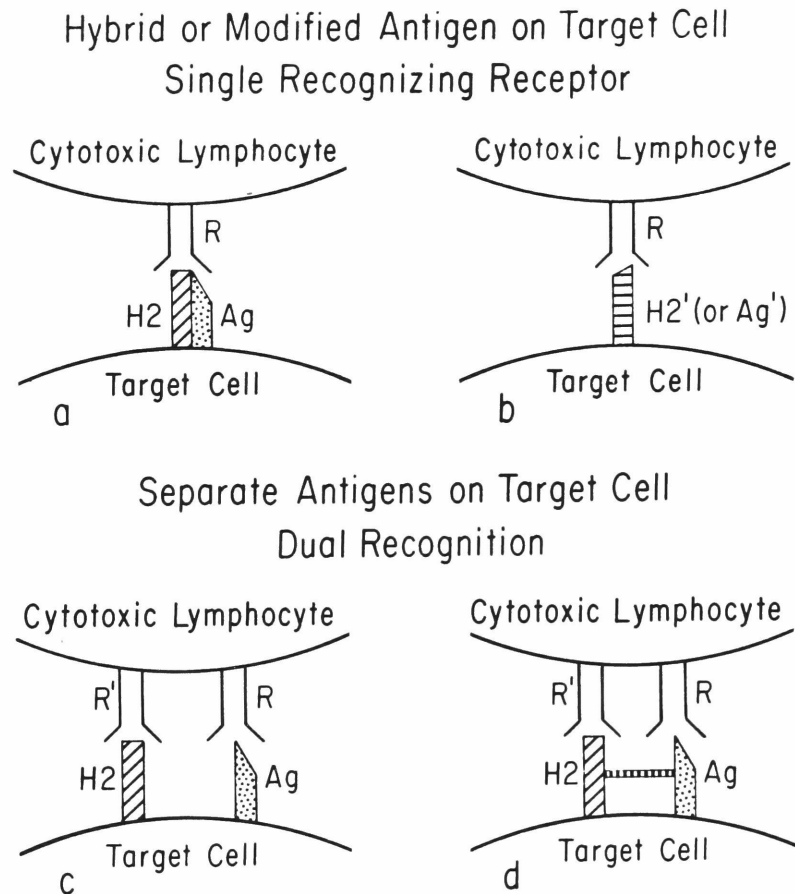


Fig. 3. Possible models for how cytotoxic T cells might recognize H-2 and viral or tumour antigens on target cells. Single receptor models include recognition of hybrid antigens (a) or modified antigens (b). Dual recognition could involve independent target antigens (c) or targets linked by a third molecule at or under the cell surface (d). Reproduced with permission from Cunningham et al. (1978).

(Bubbers and Lilly 1977). In this case, the association was only with the H-2D^b antigen. This is consistent with the finding that lysis of Friend virus-infected H-2^b target cells requires only the recognition of the H-2D^b antigen (Blank and Lilly 1977). H-2D^b and H-2K^k antigens are also incorporated into Friend virus particles whereas H-2K^b, K^d, D^d, or D^k antigens are not (Bubbers et al. 1978). All of these results indicate that H-2 antigens can form associations with viral antigens and hence could be recognized by a single T cell receptor.

Two-receptor models assume that there are two recognition units on the cytotoxic T cell: one receptor recognizes H-2 antigens, the other recognizes viral or tumour antigens. A variant of the model (Zinkernagel and Doherty 1976) involves two different antigen-binding sites (i.e. variable regions) assembled into a single receptor. Dual recognition of H-2 and viral antigens on the target is consistent with all the data so far reported, and provides a more attractive explanation of H-2 restriction in cell-mediated lysis involving minor histocompatibility antigens (Bevan 1975). Furthermore, recent evidence from studies involving chimeric mice suggests that cytotoxic T cells are restricted to a particular H-2 specificity during maturation in the thymus before any contact with other antigens (Bevan 1977, Zinkernagel et al. 1978a, b, c).

Irradiated parental (A) strain mice were reconstituted with bone marrow cells from heterozygous (A x B)₁ donors. The recipients were immunized with minor histocompatibility antigens (Bevan 1977) or with vaccinia virus (Zinkernagel et al. 1978a, c). In both cases the stimulator cells are heterozygous and should express the minor histocompatibility antigen or vaccinia virus antigen in association with the H-2 antigens of both parents (A and B). According to the one receptor model the cytotoxic T cells that are generated should be able to lyse target cells of both parental types. In fact, the cytotoxic response was restricted to the H-2 type of the A parent only. Further experiments showed that the specificity of H-2 restriction was determined by the H-2 type of the thymus in which the precursors of the cytotoxic T cells had matured (Zinkernagel et al. 1978a).

The results of the chimera experiments have been interpreted

in favour of a two-receptor model and it has been suggested, on the basis of these experiments that the T cell must "learn" to recognize the right H-2 antigen (Zinkernagel et al. 1978a). Other cell-cell interactions in the immune system are also restricted by the H-2 gene complex. For example, B cell-T cell interactions require identity in the products of I region genes (Katz and Benacerraf 1975). Similarly, interactions between cytotoxic T cells and helper T cells require I region compatibility (Zinkernagel et al. 1978a,b). These findings have been interpreted in terms of an "adaptive differentiation" of cells of the immune system, resulting in preferential interactions between cells that have matured in similar genetic environments (Katz 1976). The results of the chimera experiments, however, may also be interpreted as evidence for Jerne's prediction that the H-2 antigens of the thymus determine the antigen-binding repertoire (Jerne 1971).

A complete description of the function of the H-2 antigens will require detailed knowledge of the structure of these molecules. The experiments described in this thesis are directed towards, firstly, the detailed characterization of one H-2 antigen and, secondly, comparisons of the properties of different H-2 antigens.

III. CHARACTERIZATION OF H-2 ALLOANTISERA

The production of specific H-2 alloantisera was an essential prerequisite for the structural studies on the H-2 antigens. As these sera were to be used eventually for isolation of H-2 antigens for amino acid sequence analysis it was particularly necessary to characterize the specificity of H-2 alloantisera in terms of their reaction with cellular components. In this chapter I shall describe the production of H-2 alloantisera against various haplotypes and their serological characterization. In particular, I shall describe the anti-murine leukemia virus (anti-MuLV) activity of these H-2 alloantisera and compare the main cell surface component other than H-2 antigens recognized by these antisera on normal and tumour cells with the viral glycoprotein gp70. The experiments suggest that factors such as viral antigens on the immunizing cells, aging, and general stimulation of the immune system may contribute to the production of these antibodies in H-2 alloantisera.

Materials and Methods

Mice and Tumour Cells. All mice were obtained from Jackson Laboratories, Bar Harbor, Maine, or from our own colonies. The lymphoid cell lines EL4, P388 and S49.1 were obtained from the Salk Institute, San Diego, Calif. and maintained either in ascites form in C57BL/6, DBA/2 and BALB/c mice, respectively, or in vitro in Dulbecco's modified Eagle's medium (Gibco, Grand Island, N.Y.) containing 10% fetal calf serum (Microbiological Associates, Bethesda, Md.).

Antisera. The H-2 alloantisera were produced using the immunization schedule described by Snell et al. (1973) (Table IV). Spleen, thymus and mesenteric lymph nodes from donor mice were dissected out and cell suspensions prepared by teasing the organs through steel mesh into Hank's balanced salt solution (HBSS, Gibco, Grand Island, N.Y.). Clumps and aggregated cells were removed by slow speed centrifugation. The cell suspension was centrifuged at 400 g x 3 minutes and resuspended in an appropriate volume of HBSS. For the first two injections the organs from 4-5 week old (weaner) mice were used, each donor mouse providing

Table IV

Production of H-2 Alloantisera: Schedule of Injections

<u>Week</u>	<u>Source of Cells</u>	<u>Recipients/Donor</u>
0	weaner	25
6	weaner	25
7	adult	10
8	adult	10
9	adult	10
10	test bleed	

Table V

Mouse Strain Combinations used in the Production
of H-2 Alloantisera

<u>Alloantiserum</u>	<u>Donor</u>	<u>Recipient</u>
Anti-H-2 ^d	B10.D2	C57BL/10
Anti-H-2 ^k	B10.BR	C57BL/10
Anti-H-2 ^b	C57BL/10	B10.D2
Anti-H-2D ^d	B10.A	(C57BL/10 x AKR.M)F ₁
Anti-H-2K ^d	C3H.OH	(C3H x C57BL/10)F ₁
Anti-H-2D ^b	B10.A(2R)	(B10.D2 x A)F ₁
Anti-H-2K ^b	B10.A(5R)	(B10.D2 x A)F ₁
Anti-H-2K ^k	B10.RIII	(B10.D2 x DBA/1)F ₁

cells for 25 recipients (approximately 10^7 nucleated cells/recipient; for subsequent injections the organs from adult mice were used, each donor providing cells for ten recipients (approximately 4×10^7 cells/recipient). In both cases the recipient mice were injected intraperitoneally with 0.2 ml cell suspension.

Mice were bled from the retro-orbital sinus. In week 10 the mice were bled and the sera tested for anti-H-2 activity by a complement-dependent cytotoxicity assay. If the serum did not show sufficient anti-H-2 activity, then weekly injections of adult cells were continued. Once a sufficient titre had been established the mice were bled and injected with adult cells on alternate weeks.

The mouse strain combinations used are given in Table V. At each bleeding the blood from approximately 50 mice was pooled and allowed to clot overnight at 4°C . The serum was recovered, centrifuged at 15,000 rpm for 15 minutes in the Sorvall SS-34 rotor, and stored in 1 ml aliquots at -80°C . Different bleedings from the same mice were not pooled.

Goat antiserum against purified Rauscher leukemia virus gp70 was kindly provided by Dr. R. Wilsnack, Huntington Research Laboratories, Brooklandville, Md.

Cytotoxicity Assay. The method used was modified from that of Sanderson (1965). Target mouse lymphocytes were purified from spleen cell suspensions by discontinuous density gradient centrifugation (Boyum 1968) on lymphocyte separation medium (Litton Bionetics). $1-2 \times 10^7$ cells were suspended in 0.5 ml minimal essential medium (Microbiological Associates, Bethesda, Md.) containing 5% fetal calf serum (MEM-FCS) and incubated with 0.2-0.3 mCi $\text{Na}^{51}\text{CrO}_4$ (New England Nuclear, Boston, Mass.) for one hour at 37°C . After incubation, the cells were washed twice with 10 ml HBSS and suspended in MEM-FCS at 10^7 cells/ml. 25 μl aliquots (2.5×10^5 cells) of this suspension were added to duplicate 25 μl aliquots of serial twofold dilutions of the antiserum in 12 x 75 mm plastic tubes. To each tube was added 50 μl of guinea pig complement (Gibco, Grand Island, N.Y.) that had been diluted fourfold with MEM-FCS and previously absorbed with mouse spleen cells.

The tubes were incubated at 25°C for two hours with occasional shaking. The assay was stopped by addition of 2 ml ice cold MEM to each tube. The tubes were centrifuged at 500g x 5 minutes and 1 ml of the supernatant was removed for radioactive counting. Tubes containing medium or normal mouse serum in place of alloantisera were included to determine spontaneous lysis.

Radiolabelling of Cells. Cell surface components were labeled with ^{125}I or ^{131}I by the lactoperoxidase procedure (Marchalonis et al. 1971). $1-2 \times 10^8$ spleen or lymphoma cells in 1 ml phosphate buffer saline (PBS) were labeled at 25°C by the addition of 2 mg lactoperoxidase (Sigma, St. Louis, Mo.), 1-2 mCi Na ^{125}I or Na ^{131}I and 0.1 ml 0.003% hydrogen peroxide. Two further 0.1 ml aliquots of hydrogen peroxide were added at five-minute intervals. Five minutes after the last addition, 10 mls of PBS containing 10 mM KI was added and the cells were washed twice in this buffer. The cell pellet was extracted for 30 minutes on ice with 1 ml of 0.5% Nonidet P-40 (NP40, Shell, N.Y.) in 10 mM Tris-HCl (pH 7.4) 1.5 mM MgCl_2 , 0.15 M NaCl. Nuclei were removed by centrifugation at 400g x 5 minutes and the supernatant was centrifuged at 100,000g for 60 minutes in the Beckman type 65 rotor. The supernatant from this centrifugation was used for immunoprecipitation. Alternatively, cells were labeled by incubation (8×10^6 cells/ml) in minimal essential medium (Gibco, Grand Island, N.Y.) supplemented with 1% BSA and containing 100 $\mu\text{Ci/ml}$ of ^3H , ^{14}C or ^{35}S -labeled amino acids (New England Nuclear, Boston, Mass.) for 4-6 hours at 37°C in a 5% CO_2 atmosphere. In preliminary experiments, 4-6 hours was determined to be the optimum labeling time. After washing, the internally labeled cells were extracted in 0.5% NP40 at a concentration of 10^7 cells/ml and processed as above.

Immunoprecipitation. 100 μl aliquots of radiolabeled cell extracts were incubated with 20 μl of alloantisera in microfuge tubes (Beckman) for 30 minutes at 4°C. Goat antiserum against mouse immunoglobulin (Miles, Elkhart, Ind.) was then added and the tubes incubated for three hours at 4°C. The volume of goat antiserum had been previously determined to be at equivalence with mouse alloantisera. The precipitates were collected by centrifugation in the microfuge (Beckman) for three minutes

and washed three times by resuspension in 10 mM Tris-HCl, 0.15 M NaCl pH 7.4. On the third wash the precipitates were transferred to a clean tube. The final precipitates were counted for radioactivity and stored at -20°C .

In later experiments formalin fixed Staphylococcus aureus bacteria (Cullen and Schwartz 1976), generously provided by Dr. Emil Gotschlich (The Rockefeller University, New York, N.Y.), or protein A-Sepharose (Miles, Elkhart, Ind.) were used as the precipitating agent in place of goat anti-mouse immunoglobulin. For immunoprecipitation of 20 μl of H-2 alloantiserum, 100 μl of a 10% w/v suspension of S. aureus bacteria or 50 μl of a 50% v/v suspension of protein A-Sepharose were used. Labeled material was eluted from the immunoprecipitates by boiling in SDS-sample buffer; the bacteria or protein A-Sepharose were removed by centrifugation for five minutes in the microfuge. The use of these agents had no effect on the precipitation of H-2 antigen and had the advantage of producing less protein in the final samples for gel electrophoresis.

Labeled immunoglobulin was removed from spleen cell extracts by precipitation with goat antiserum to mouse Ig (Miles Laboratories, Elkhart, Ind.). A donkey antiserum to goat Ig (Miles Laboratories) was used to precipitate the goat antiserum to gp70.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Immuno-precipitates were dissolved in 2% SDS in 62.5 mM Tris-HCl (pH 7) containing 5% 2-mercaptoethanol, heated at 100°C for five minutes and subjected to SDS-polyacrylamide gel electrophoresis using the method of Laemmli (1970). Tubes of 6 mm internal diameter were used. The gels were sliced using a Gilson Autogel Divider (Ranin Instruments, Fort Lee, N.J.) and analyzed for radioactivity. Molecular weights (MW) of labeled components in immunoprecipitates were estimated (Weber and Osborn 1969) from the positions of ^{131}I -labeled internal standards on SDS-PAGE: MOPC 104E μ -chain, MW 73,000 (Robinson et al. 1973); MOPC 21 γ -chain, MW 50,000 (Milstein et al. 1972); and MOPC 21 κ -chain, MW 23,000 (Svasti and Milstein 1972). These proteins were obtained from Litton Bionetics and labeled with ^{131}I by the chloramine-T technique (Greenwood et al. 1963).

Virus. Rauscher leukemia virus (Litton Bionetics, Inc., Kensington, Md.) was labeled with ^{125}I (New England Nuclear, Boston, Mass.) by the lactoperoxidase technique and extracted in 10 mM Tris-HCl (pH 7.4), 1.5 mM MgCl_2 , 0.15 M NaCl containing 0.5% Nonidet-P40. The supernatant from a 100,000g x 90 minute centrifugation of this extract was used for immunoprecipitation.

Table VI
Assays of Anti-H-2 Alloantisera

Alloantiserum	H-2 specificity	Target cells (H-2 type)					
		B10 (K ^b D ^b)	DBA/2 (K ^d D ^d)	C3H (K ^k D ^k)	C3H.OH (K ^d D ^k)	B10.A (K ^k D ^d)	B10.A(4R) (K ^k D ^b)
anti-H-2 ^k	K ^k D ^k	-		64	8	64	
anti-H-2 ^d	K ^d D ^d	-	128	-	16	32	
anti-H-2 ^b	K ^b D ^b	128				16	16
anti-H-2D ^b	D ^b	8				-	8
anti-H-2K ^b	K ^b	128	-		-	-	-
anti-H-2D ^d	D ^d	-	16-32			32-64	-
anti-H-2K ^d	K ^d	-			32-64	-	-
anti-H-2K ^k	K ^k	-			-	64	32

The values shown are the reciprocal of the highest dilution of the antiserum giving 50% cytotoxicity in the assay. A dash indicates that this value was less than 4; a blank indicates that the experiment was not done.

Pretreatment

Precipitating Alloantiserum

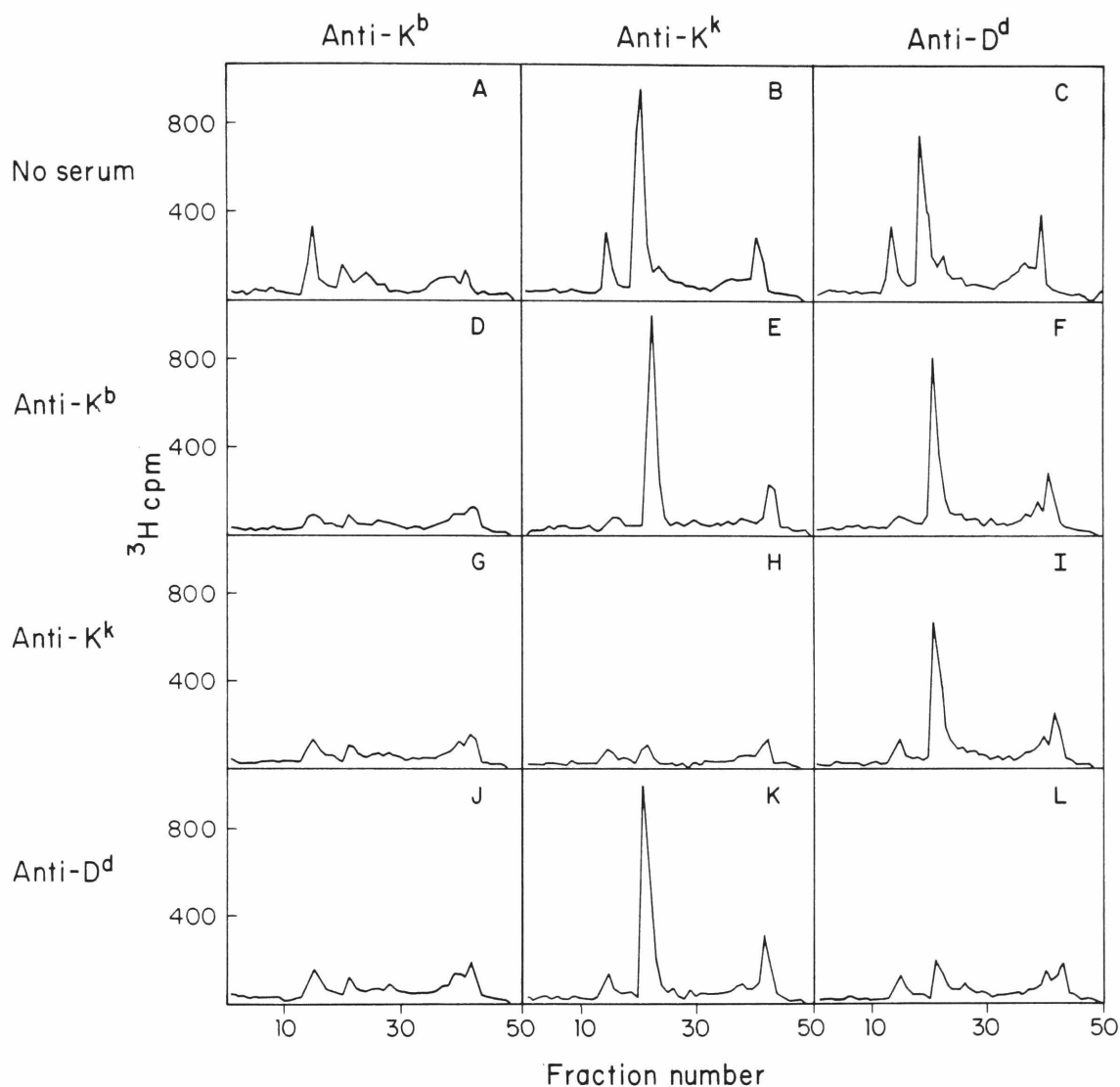


Fig. 4. Independent immunoprecipitation of H-2D and H-2K antigens. Immunoprecipitates were made from an NP40 extract of ASL1 leukemia cells (K^kD^d) labelled with ³H-leucine and run on 10% SDS gels in the presence of 2-mercaptoethanol. The extracts received no pretreatment (panels A,B,C), pretreatment with anti-H-2K^b antiserum (panels D,E,F), pretreatment with anti-H-2K^k antiserum (panels G,H,I) or pretreatment with anti-H-2D^d antiserum (panels J,K,L). After removal of immune complexes with *S. aureus* bacteria, immunoprecipitates were made from the extracts using anti-H-2K^b antiserum (panels A,D,G,J), anti-H-2K^k antiserum (panels B,E,H,K) or anti-H-2D^d antiserum (panels C,F,I,L).

precipitation of labeled H-2 antigens by an alloantiserum of a second specificity. Thus, pretreatment of the extract with anti-D^d alloantiserum removes all the labeled D^d antigens available for a second precipitation with anti-D^d but does not affect subsequent precipitation of K^k antigens. The results indicate, firstly, that the monospecific H-2 alloantisera do not cross-react in the immunoprecipitation assay and, secondly, that H-2D and H-2K antigens can be precipitated independently. This conclusion has also been demonstrated by Cullen et al. (1972) and is consistent with studies showing independent capping of H-2D and H-2K antigens (Neauport-Sautes 1973). Similar results have also been obtained using the other monospecific alloantisera.

Anti-MuLV Antibodies in H-2 Alloantisera. Immunoprecipitates were made from NP40 extracts of ¹²⁵I-labeled spleen cells from a variety of mouse strains using H-2 alloantisera and analyzed by SDS-PAGE. In many cases, labeled species of molecular weight 70,000-80,000 were observed in addition to the characteristic peaks of H-2 heavy (MW 46,000) and light (MW 12,000) chains (Henning et al. 1976a). This additional material was found in immunoprecipitates made from extracts of lymphocytes or leukemia cells from mice of many different strains using a variety of H-2 alloantisera (Table VII). For example, immunoprecipitates from DBA/2(H-2^d) lymphocytes contained a component with a molecular weight of 70,000 (Fig. 5a). The DBA/2-derived lymphoma P388 showed large amounts of a similar material with a higher molecular weight (80,000 daltons, Fig. 5b). This material was absent, however, from immunoprecipitates made with extracts of C57BL/10(H-2^b) lymphocytes (Fig. 5c), and from extracts of the C57BL/6(H-2^b)-derived leukemia EL4 (Fig. 5d), except when the precipitates were made with the anti-H-2D^b alloantiserum (Table VII). Similar results were obtained using different bleedings of the alloantisera.

Precipitation of this additional material was generally independent of the H-2 specificity of the antiserum used; for example, it was found in immunoprecipitates made from extracts of P388(H-2^d) cells using either anti-H-2D^d or anti-H-2K^b alloantisera (Table VII), although, as expected, the amount of H-2 heavy chain precipitated by the anti-H-2K^b alloantiserum was considerably decreased. The 70,000-80,000 dalton component was

Table VII

Detection of 70,000-80,000 dalton Components on Mouse Lymphocytes
and Leukemia Cells by H-2 Alloantisera

Strain and Cell Type	H-2 haplo- type	Alloantisera*					
		Anti- H-2Dd	Anti- H-2Kd	Anti- H-2Db	Anti- H-2Kb	Anti- H-2Kk	Anti- H-2b
DBA/2	lymphocyte	+	+		+	+	
DBA/2	lymphoma (P388)	+			+	+	
C57BL/10	lymphocyte	-	-	+	-	-	-
C57BL/6	lymphocyte			+	-		
C57BL/6	leukemia (EL4)	-		+	-		
(C57BL/10xDBA/2)F1	lymphocyte	+					
B10.A	lymphocyte	+			+		
B10.A(2R)	lymphocyte	+			+		
B10.A(4R)	lymphocyte					+	+
B10.A(5R)	lymphocyte	+		+			+
B10.BR	lymphocyte				+	+	
B10.D2	lymphocyte	+	+				
BALB/c	lymphocyte	+					
BALB/c	lymphoma (S49.1)	+			+		
C3H	lymphocyte					+	
C3H.OH	lymphocyte		+				
129	lymphocyte					+	
AKR	lymphocyte					+	
C58	lymphocyte	+				+	
RF	lymphocyte	+				+	

* Immunoprecipitates were made from ¹²⁵I-labelled NP40 cell extracts using various H-2 alloantisera; the presence of 70,000-80,000 dalton components was determined from SDS-PAGE of the immunoprecipitates. "+" = presence of 70,000-80,000 dalton components; "-" = their absence. Mouse strain combinations used to produce alloantisera are given in Table V.

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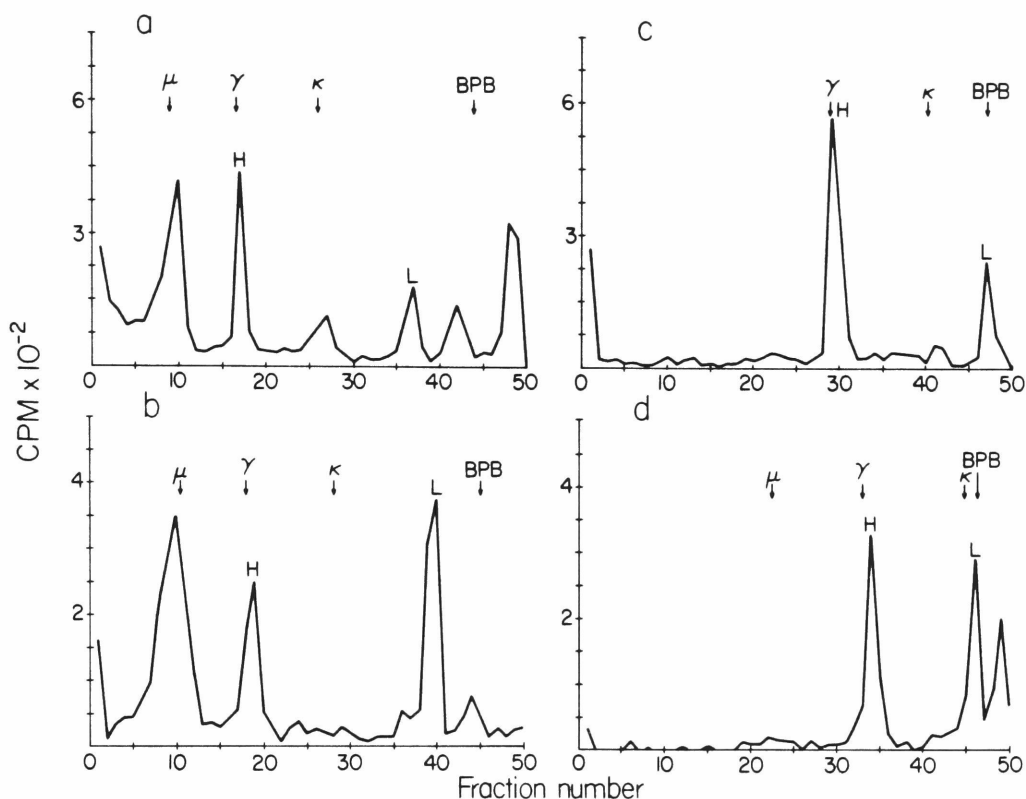


Fig. 5. Detection of cell surface components of murine spleen and leukemia cells by H-2 alloantisera. Immunoprecipitates were made from NP40 extracts of ¹²⁵I-labelled cells using H-2 alloantisera and run on SDS-polyacrylamide gels in the presence of 5% mercaptoethanol: (a) anti-H-2D^d with DBA/2 spleen cell extract, 12% gel; (b) anti-H-2D^d with P388 cell extract, 12% gel; (c) anti-H-2K^b with C57BL/10 spleen cell extract, 7.5% gel; (d) anti-H-2K^b with EL4 cell extract, 8.5% gel. Molecular weight markers are indicated by arrows; μ, MOPC 104E μ-chain (MW 73,000); γ, MOPC 21 γ-chain (MW 50,000); κ, MOPC 21 κ-chain (MW 23,000); BPB, bromophenol blue dye marker. H-2 heavy (H) and light (L) chains are also indicated. Reproduced with permission from Milner et al. (1976).

absent from precipitates made with sera from unimmunized mice, indicating that it is not trapped during the precipitation reaction. These observations suggest that the alloantisera contain antibodies which have an affinity for a cell surface antigen other than the H-2 antigens of mouse cells.

The identity of the antigen was suggested by the reaction of H-2 alloantisera with NP40 extracts of ^{125}I -labelled Rauscher leukemia virus. The predominant labelled component precipitated by the alloantisera was the viral envelope glycoprotein gp70 (Fig. 6a). In a reciprocal experiment, a goat antiserum against the gp70 of Rauscher leukemia virus precipitated labelled material from extracts of P388 cells which co-electrophoresed on SDS-PAGE with the additional component obtained with H-2 alloantisera (Fig. 6b). Precipitation of this material from P388 extracts by the goat antiserum against gp70 decreased significantly the amount of the 80,000 dalton component in subsequent immunoprecipitates made with anti-H-2^d alloantisera. The goat antiserum to gp70, however, also precipitated a 70,000 dalton component from extracts of EL4 cells (Fig. 6c), which did not occur in immunoprecipitates made with most H-2 alloantisera. These results suggest that the additional cell surface antigen precipitated by H-2 alloantisera is closely related to the viral glycoprotein gp70.

Origin of Anti-MuLV Antibodies in H-2 Alloantisera. Several possible mechanisms may account for the presence of anti-MuLV antibodies in H-2 alloantisera. It is conceivable, for example, that mice immunized with allogeneic cells might respond either to foreign viral antigens on the immunizing cells, or to endogenous MuLV or viral antigens induced by allogeneic stimulation. Alternatively, the levels of anti-MuLV antibodies may increase with age or as a consequence of a general stimulation of the immune system. To distinguish between these groups of possibilities, mice were given several injections of either allogeneic spleen cells or complete Freund's adjuvant. The mice were bled before the first injection ("pre-immune serum"), and after the series of injections. Serum was also obtained from unimmunized mice of the same age. The anti-MuLV antibody in serial two-fold dilutions of these sera was assayed by indirect immunoprecipitation using an NP40 extract

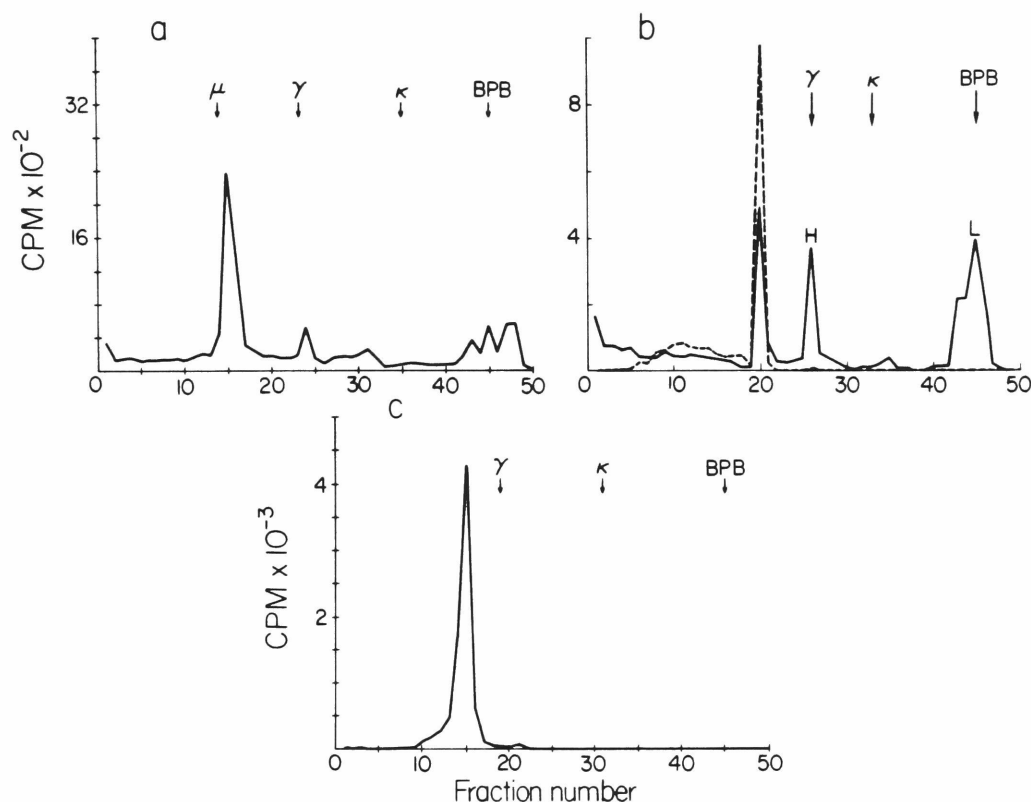


Fig. 6. Detection of gp70 molecules from Rauscher leukemia virus and murine leukemia cells. Immunoprecipitates were made and run on SDS-polyacrylamide gels in the presence of 5% mercaptoethanol as follows: (a) immunoprecipitate made from an NP40 extract of ^{125}I -labelled Rauscher leukemia virus using anti-H-2D^d alloantiserum; 10% gel; (b) co-electrophoresis of an immunoprecipitate made from an NP40 extract of ^{131}I -labelled P388 cells using anti-H-2D^d alloantiserum (—), and an immunoprecipitate to gp70 (---), 10% gel; (c) immunoprecipitate made from an NP40 extract of ^{125}I -labelled EL4 cells using a goat antiserum to gp70, 10% gel. Molecular weight markers are described in Fig. 5. Reproduced with permission from Milner et al. (1976).

of ^{125}I -labelled Rauscher leukemia virus. The results obtained are shown in Figure 7.

Serum from C57BL/10 mice, which were negative for the gp70 antigen detected by most H-2 alloantisera, showed a considerable increase in anti-MuLV antibody levels after injection of viral-antigen-positive B10.A spleen cells (Fig. 7a). On the other hand, immunization of (C57BL/10 x DBA/2) F_1 mice with B10.BR spleen cells, where both donor and recipient mice are positive for the viral antigen detected by H-2 alloantisera, produced no increase in serum anti-MuLV antibody levels compared to unimmunized mice of the same age (Fig. 7b). Aging of mice appeared to cause a slight increase in anti-MuLV antibody levels in (C57BL/10 x DBA/2) F_1 mice but not in C57BL/10 mice. In both cases, however, stimulation of the immune system by injection of adjuvant resulted in an increase in the amounts of these antibodies.

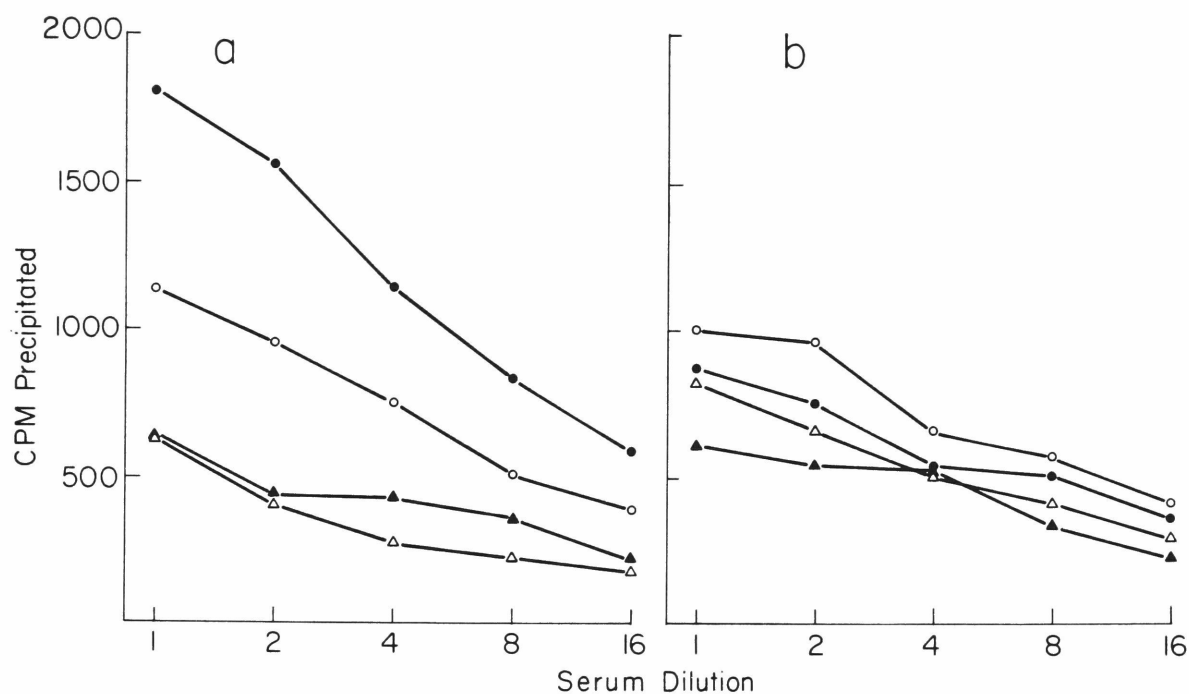


Fig. 7. Titration of antibodies to leukemia virus components in immune and nonimmune sera from (a) C57BL/10 and (b) (C57BL/10 x DBA/2) F1 mice. Each serum was pooled from five female mice. Immuno-precipitates were made from NP40 extracts of ^{125}I -labelled Rauscher leukemia virus using two-fold serial dilutions of the sera and a constant volume of goat antiserum to mouse immunoglobulin. Each precipitate was washed three times in ice-cold 10mM Tris-HCl, 0.15M NaCl (pH 7.4), and the radioactivity remaining in the final precipitate was measured. ●-●, serum from mice given multiple intraperitoneal injections of either B10.A (Fig. 7a) or B10.BR (Fig. 7b) spleen cells; ○-○, serum from mice given multiple intraperitoneal injections of complete Freund's adjuvant (Difco, Detroit, Mich.); △-△, serum from unimmunized age-matched mice; ▲-▲, serum from mice before injection with spleen cells or adjuvant. Reproduced with permission from Milner et al. (1976).

Discussion: The Origin of Anti-MuLV Antibodies in H-2 Alloantisera

Murine leukemia viruses (MuLV) are endogenous in the mouse, and multiple copies of the leukemia virus genome can be detected by DNA hybridization in mouse nuclear DNA (Lowy et al. 1974). In high leukemic mouse strains such as AKR, infectious virus is produced throughout life, whereas in low leukemic strains (e.g. BALB/c, C57BL/6), this occurs only with increasing age. In low leukemic strains, however, the production of infectious virus can be induced by many stimuli, including chemical agents and mitogens in vitro or by allogeneic stimulation during mixed lymphocyte or graft-versus-host reactions (Hirsch and Black 1974). The expression of viral genes is not limited to the production of infectious virus, for viral proteins such as the envelope glycoprotein (gp70) can be detected in normal unstimulated mouse cells (Strand et al. 1974, DelVillano et al. 1975). For example, the G_{IX} antigen has been shown to be present on gp70 molecules at the thymocyte cell surface (Tung et al. 1975a,b).

Despite the prevalence of MuLV and viral proteins, mice are generally not tolerant to viral antigens, and antibodies to MuLV components can be found in most nonimmune mouse sera (Ihle et al. 1974, Nowinski and Kaehler 1974). In particular, anti-MuLV antibodies have been detected in H-2 alloantisera and have been shown to be responsible for anomalous cytotoxicity reactions of these sera on mouse tumour cells (Klein 1975, Nowinski and Klein 1975). Although the sensitivity of the tumour cells to lysis by H-2 alloantisera was correlated with their production of MuLV (Nowinski and Klein 1975), the cell surface molecules detected by the anti-MuLV antibodies were not directly identified on the cells. Our studies using the immunoprecipitation technique indicate that these molecules are related to or identical to gp70 which occurs on many types of mouse cells.

The H-2 alloantisera did not detect all cell surface gp70 molecules indiscriminately. For example, goat antiserum to gp70 precipitated a 70,000 dalton molecule from extracts of EL4 cells, but a similar component was not detected by most H-2 alloantisera. The anti-MuLV antibodies in these alloantisera appear to recognize antigenic determinants on a subpopulation of gp70 cell surface molecules. This is

similar to the finding that the G_{IX} antigen is present on gp70 molecules of thymocytes of only certain mouse strains (Tung et al. 1975b). The detection of gp70 by H-2 alloantisera in strains congenic with C57BL/10 except for the H-2 region but not in C57BL/10 (except by the anti-D^b alloantiserum) suggests that the expression of this protein may be linked to the H-2 region. Alternatively, the congenic strains may have become infected with MuLV since their original production; for example, B10.A mice have recently been shown to be heavily infected with MuLV whereas congenic C57BL/10 mice are comparatively virus-free (Melief et al. 1975).

Cell surface gp70 molecules from different lymphoid tissues have been previously reported (Del Villano et al 1975) to show a variation in molecular weight similar to that described here for the components from DBA/2 splenic lymphocytes and P388 lymphoma cells. These observations suggest that P388 cells may have originated from a cell type not found in DBA/2 spleen. In addition, some 70,000-80,000 dalton components detected by H-2 alloantisera may not be gp70 molecules; for example, it has been shown that the cell surface antigen induced by Moloney leukemia virus is not carried by any known component of the virion (Fenyo and Klein 1976).

The alloantisera were also found to contain antibodies against Sendai virus (unpublished observations). This may have been due to an infection of our mouse colony by this virus, as has been reported for other colonies (Parker et al. 1964). There was no cross-reactivity between the two types of virus, however, as shown by immunoprecipitation using rabbit antisera to Sendai and Rauscher viruses and ¹²⁵I-labelled extracts of both viruses. In addition, purified Sendai virus did not inhibit the anti-H-2 activity of the alloantisera in a cytotoxicity assay, indicating the absence of cross-reaction with H-2 antigens.

Our results suggest that several factors may be involved in the production of anti-MuLV antibodies in H-2 alloantisera, and that the contribution of each in any particular immunization will depend on the nature of the mouse strains used. When donor and recipient mice differ in their expression of endogenous viral antigens, then a direct response to foreign viral antigens on the immunizing cells may be possible. This

probably occurs, for example, in the immunization of C57BL/10 mice with B10.A spleen cells. Recipients such as (C57BL/10 x DBA/2) F_1 mice, which express the viral antigen detected by H-2 alloantisera, do not respond readily to viral antigen-positive B10.BR spleen cells, indicating that these mice may be tolerant to this viral antigen. Alternatively, the levels of circulating anti-MuLV antibodies could be decreased by binding to endogenous viral antigens. General stimulation of the immune system, as shown by the increase in anti-MuLV antibody after injection of Freund's adjuvant, may contribute to the effect. The increased expression of MuLV and viral antigens with age (Hirsch and Black 1974) may also affect the levels of anti-MuLV antibodies. In addition, anti-gp70 antibody has been detected in a rabbit antiserum made against concanavalin A-stimulated spleen cells (G.R. Gunther, J.L. Wang and G.M. Edelman, unpublished observations). This suggests that these stimulated spleen cells may also express MuLV antigens and that antigen expression may be related to mitogenic stimulation.

The production of H-2 alloantisera involves mouse strains and cells which differ genetically in the H-2 region. Allogeneic stimulation by cells of a different H-2 type may induce MuLV in the recipient (Hirsch and Black 1974) and produce an immune response to endogenous viral antigens. Alternatively, other genes in this region influence the susceptibility of the mouse to infection by leukemia virus (Lilly and Pincus 1973) and hence may indirectly control the expression of viral antigens. Despite the detailed genetic and serological definition of the H-2 region, it is possible that even the most specific H-2 alloantisera will contain unforeseen activities against viral antigens, or the products of as yet undefined genes.

IV. THE STRUCTURE OF H-2 ANTIGENS

The genetics and serology of the H-2 gene complex and other loci on the 17th chromosome raise a number of questions about the products of these genes. For example: What is the basis of the polymorphism in the H-2 system and the nature of the public and private antigenic specificities? Are H-2K gene products evolutionarily related to H-2D gene products and to the products of other loci on the 17th chromosome? Are any of the molecules coded for by these genes related to antibodies? The answers to these questions and an understanding of the function of H-2 antigens depend upon knowledge of the structure of H-2 antigens in solution and on the cell surface.

To provide a basis for detailed analysis of the structure of these cell-surface glycoproteins, I have attempted to define precisely the overall structure of the molecule in terms of the type and number of its polypeptide chains, the nature of the interactions between chains, and the organization of the chains. The basic properties of the molecule were determined in solution and, where possible, compared with H-2 antigens on the cell surface. On the basis of these and other studies, a model is proposed for the H-2 antigens which includes the arrangement of the polypeptide chains, the location of interactions between the chains, and the orientation of these proteins on the cell surface.

Materials and Methods

Radiolabelling and Solubilization of H-2 Antigens. Splenic lymphocytes from inbred mouse strains and lymphoma cells were labelled with ¹²⁵I by the lactoperoxidase technique and extracted with 0.5% NP40 as described in the previous section.

Spleen cell membrane fractions were obtained as a pellet by centrifugation (105,000 x g, 60 min) of the supernatant from labelled cells that had been repeatedly frozen and thawed in phosphate-buffered saline and centrifuged at 400 x g for 5 min. Isolated membranes were extracted with 0.5% NP40 or 0.5% sodium desoxycholate (DOC), (Sigma) in 0.01 M Tris-HCl buffer, pH 8.0, 0.15 M NaCl, and centrifuged for 60 minutes at 100,000 x g. The clear supernatant was used for gel chroma-

tography and sucrose density gradient centrifugation.

Water-soluble antigens were prepared by papain treatment of cells or detergent extracts using 2.0 mg of papain (Sigma) for 2×10^8 cells in 1 ml of phosphate-buffered saline or for 1 ml of detergent extract in the presence of 2mM cysteine for one hour at 37°. Proteolysis was terminated by addition of iodoacetamide to a final concentration of 5mM. Alkylation of cells or membrane fractions was performed using 0.01M iodoacetamide in 0.01M Tris-HCl buffer, pH 8.0, 0.15M NaCl for 30 minutes at 25°C.

Molecular Weight Determinations. Immunoprecipitates of ^{125}I labelled H-2 antigens were dissolved in 2% sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis, as described in the last section. Gels were sliced and the fractions analyzed for radioactivity. Molecular weights of labelled components were determined by comparison with marker proteins labelled with ^{131}I run on the same gel (Weber and Osborn 1969). The following proteins, labelled with ^{131}I using chloramine T (Greenwood et al. 1963) were used as standards: MOPC 104 E IgM (Bionetics), MW of μ chain = 73,000 (Robinson et al. 1973); MOPC 21 IgG (Bionetics), MW of γ chain = 50,000 (Milstein et al. 1972), MW of light chain = 23,000 (Svasti and Milstein 1972).

Gel exclusion chromatography was performed on Sephadex G-200 (1 x 75cm columns in 0.01M Tris-HCl (pH 8.0), 0.15M NaCl, 0.5% DOC); spleen cell membranes solubilized in either 0.5% NP40 or 0.5% DOC were loaded in a volume of 1 ml, and 1 ml fractions were collected. Fractions were assayed for H-2 antigenic activity by inhibition of cytotoxicity (see below). Chromatography of papain solubilized extracts was performed in the absence of detergent. The columns were calibrated using the following proteins labelled with ^{131}I or ^{125}I by the chloramine T technique (Greenwood et al. 1963): MOPC 21 immunoglobulin (Litton Bionetics), MW 146,000; bovine serum albumin (Sigma), MW 67,000; chymotrypsinogen (Worthington), MW 25,000; and cytochrome C (Sigma), MW 12,400. The values for molecular weight, diffusion coefficient and Stoke's radius for these proteins were obtained from tables in Andrews (1970). Dextran blue (Pharmacia) and phenol red were used to indicate the void volume (V_0) and the total bed volume (V_t) respectively of the

columns. The partition coefficient, K_D , for H-2 antigens and marker proteins was determined from the volume, V_e , at which each eluted, using the relation,

$$K_D = \frac{V_e - V_o}{V_t - V_o} \quad (\text{Akers, 1970}).$$

Diffusion coefficients ($D_{20,w}$) and Stokes' radii for H-2 antigens were determined from plots of the inverse error function complement of the partition coefficient ($\text{erfc}^{-1} K_D$) against reciprocal diffusion coefficients and Stokes' radius respectively, as described by Akers (1970) (Figs. 12, 13).

Sucrose density gradient centrifugation was performed on 4-20% sucrose gradients in 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl, 0.5% DOC centrifuged for 25 hr at 35,000 rpm (5°) in an SW 39 Beckman rotor. Fractionation was by tube puncture and drop counting, 6 drops/fraction. Each fraction was assayed for H-2 antigenic activity by inhibition of cytotoxicity (see below). The sedimentation coefficients ($S_{20,w}$) for H-2 antigens were determined from the sucrose concentrations at the peak of antigenic activity by use of the tables of time integrals in McEwen (1967). A particle density of 1.4 g cm^{-3} (equivalent to a partial specific volume of $0.714 \text{ cm}^3 \text{ g}^{-1}$) was assumed for the H-2 antigens.

The molecular weights of H-2 antigens under nondissociating conditions were determined from the diffusion and sedimentation coefficients by the Svedberg equation (Svedberg and Pederson 1940) assuming a partial specific volume of $0.725 \text{ cm}^3/\text{g}$ for the H-2 antigens.

Assay of H-2 Antigenicity. H-2 antigens were assayed by inhibition of antibody mediated cytotoxicity. 25 μl aliquots of the samples to be assayed were incubated with 25 μl of H-2 alloantiserum in 12 x 75 mm plastic tubes (Falcon) for 30 minutes at 25°C . The dilution of H-2 alloantiserum used was the maximum dilution giving 100% cytolysis. For the assay of extracts containing detergent, 25 μl 35% w/v bovine serum albumin (Miles) was added to the aliquot of extract ten minutes before addition of alloantiserum to prevent subsequent lysis of the target cells by detergent (Dawson et al. 1973). The activity of the H-2 alloantiserum was then assayed by addition of ^{51}Cr -labeled target cells and complement as described in the last section. The inhibition of

cytolysis by the extract was calculated according to the formula: %

$$\text{inhibition} = \frac{100 \times (\text{cpm released by serum alone}) - (\text{cpm released by serum \& extract})}{(\text{cpm released by serum alone})}$$

Cross-linking Experiments. Spleen or lymphoma cells labeled with ^{125}I were treated with the cleavable cross-linking reagent dimethyl-3,3'-dithiobispropionimidate (Pierce) for 20 minutes at 25°C in 0.106 M phosphate buffer (pH 8.0) using 2 mg/ml of the reagent at a cell concentration of 5×10^7 cells/ml (Wang and Richards 1974). An excess of ice cold phosphate buffer containing 50 mM glycine was added to stop the reaction. The cells were centrifuged and extracted with 0.5% NP40 in the presence of 10 mM iodoacetamide or N-ethylmaleimide to alkylate free sulfhydryl groups.

Aliquots of the extracts or immunoprecipitates made from the extracts were analyzed by two-dimensional polyacrylamide gel electrophoresis. The technique used was modified from that of Wang and Richards (1974). The first dimension was run in the absence of reducing agent in 2.5 mm (i.d.) x 120 mm glass tubes at 0.5 mA/tube constant current until the bromophenol blue dye marker was 0.5 cm from the end of the tubes. The gels were removed and soaked for two hours at 25°C in 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS and 10% 2-mercaptoethanol. The second dimension gel system was modified from that of O'Farrell (1975) and consisted of three horizontal layers: the main gel was cast to 3 cm from the top of the plates, above this was a 2 cm layer of stacker gel and on this was cast a 1 cm layer of agarose in 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and 5% mercaptoethanol. The first dimension gel was laid horizontally on this layer and fixed in place with the same agarose mixture. The second dimension gels were run at a constant current of 20 mA/gel until the bromophenol blue dye from the first dimension had reached 0.5 cm from the bottom of the gel. The gel was removed, fixed for 15-30 minutes in 10% trichloroacetic acid and stained for 4 hours in 25% methanol, 7% acetic acid containing 0.1% Coomassie brilliant blue (Schwarz/Mann). Gels were destained first in 25% methanol, 7% acetic acid, then in 7% acetic acid and dried on 3 M filter paper (Whatman). Autoradiographs were made using Cronex X-ray film (DuPont).

Results

H-2 Antigens in Solution. H-2 antigens are tightly bound to the cell surface and must be solubilized by either detergent extraction or papain treatment (Nathenson and Cullen 1974). Comparison of the products obtained by each method provides some details about the H-2 structure. The size of the polypeptide chains and the interactions between them were assessed by SDS-polyacrylamide gel electrophoresis of H-2K^b antigens prepared by immunoprecipitation from either NP40 detergent extracts or papain-treated detergent extracts of ¹²⁵I-labelled C57BL/10J spleen cells using the anti-H-2K^b alloantiserum. In the presence of reducing agents SDS gel electrophoresis of immunoprecipitates of detergent solubilized H-2 antigens showed two species: a heavy chain (MW 47,000) and a light chain (MW 12,000) (Figs. 8C,9). The smaller component has been previously identified as β_2 -microglobulin (Silver and Hood 1974, Appella et al. 1976). On the other hand, SDS gel electrophoresis of H-2 antigens obtained by papain treatment of detergent extracts showed a heavy chain component of molecular weight 39,000 and an intact light chain (Figs. 8D,9). Similar results were obtained for H-2 antigens solubilized by papain treatment of intact, labelled cells. We have designated the papain fragment of the heavy chain F_H (Henning et al. 1976a).

In the absence of any reducing agents, the detergent-solubilized protein produced a major component at a position corresponding to twice the molecular weight of the fully reduced heavy chain (Figs. 8A,9). Elution of this material from the gel and re-electrophoresis under reducing conditions gave only the 47,000 dalton component (Fig. 8B). This observation suggests that the H-2 heavy chains in the detergent-solubilized molecule are linked by at least one disulfide bond to form a dimer. The light chains, however, do not appear to be covalently linked either to the heavy chain or to each other.

The gel profile (Fig. 8D) of the papain-solubilized molecule was similar both in the presence and absence of reducing agents, indicating that this molecule contains no disulfide bonds between the heavy chains. Because the papain treatment is performed in the presence of 2mM cysteine which might have reduced any disulfide bonds between the heavy chains,

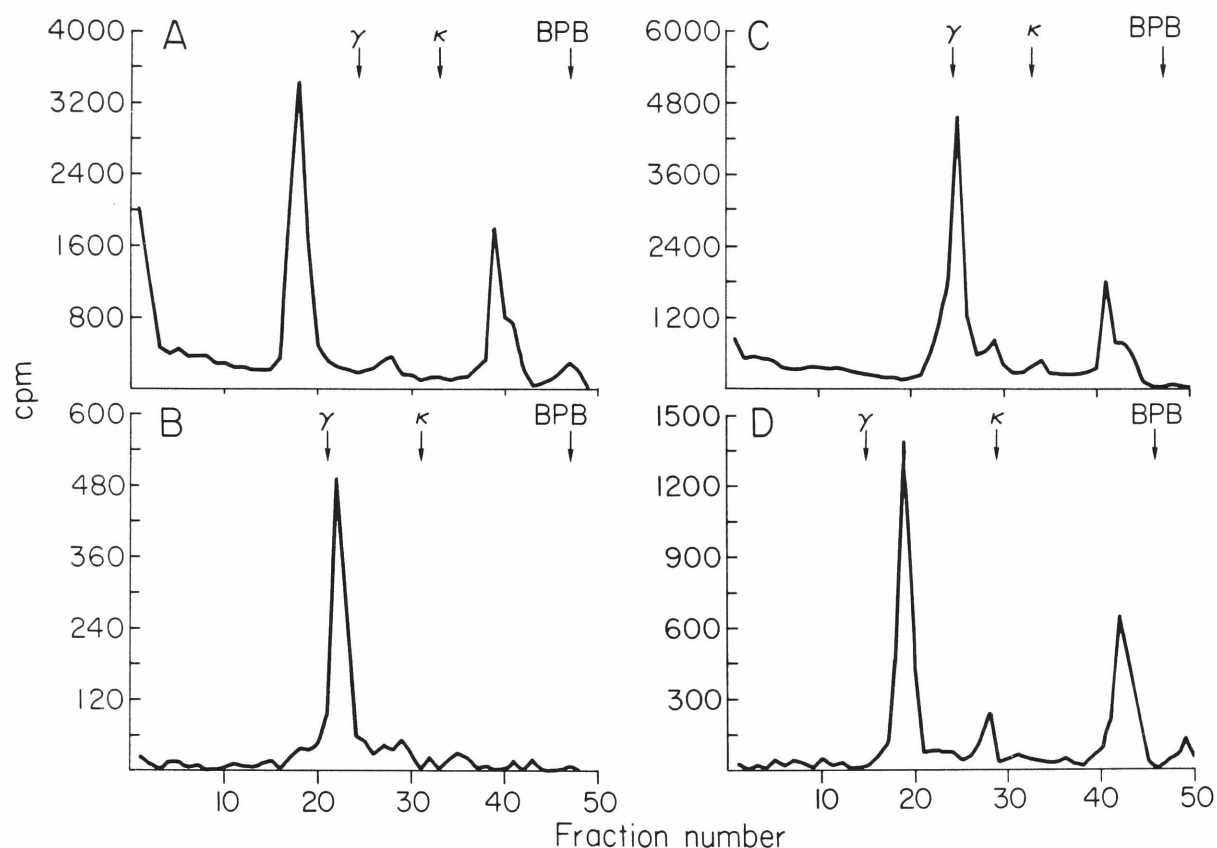


Fig. 8. SDS-polyacrylamide gel electrophoresis of immunoprecipitates of NP40 solubilized H-2K^b antigens from ¹²⁵I-labelled C57BL/10 spleen cells: (A) unreduced immunoprecipitate (10% polyacrylamide gel); (B) re-electrophoresis of material eluted from fractions 18-20 of the gel shown in panel A after reduction with 2-mercaptoethanol (10% gel); (C) immunoprecipitate after reduction with 2-mercaptoethanol (10% gel); (D) immunoprecipitate from papain-treated NP40 extract after reduction with 2-mercaptoethanol (12% gel). γ, MOPC 21 heavy chain (MW 50,000); κ, MOPC 21 light chain (MW 23,000); BPB, bromophenol blue. From Henning et al. (1976a).

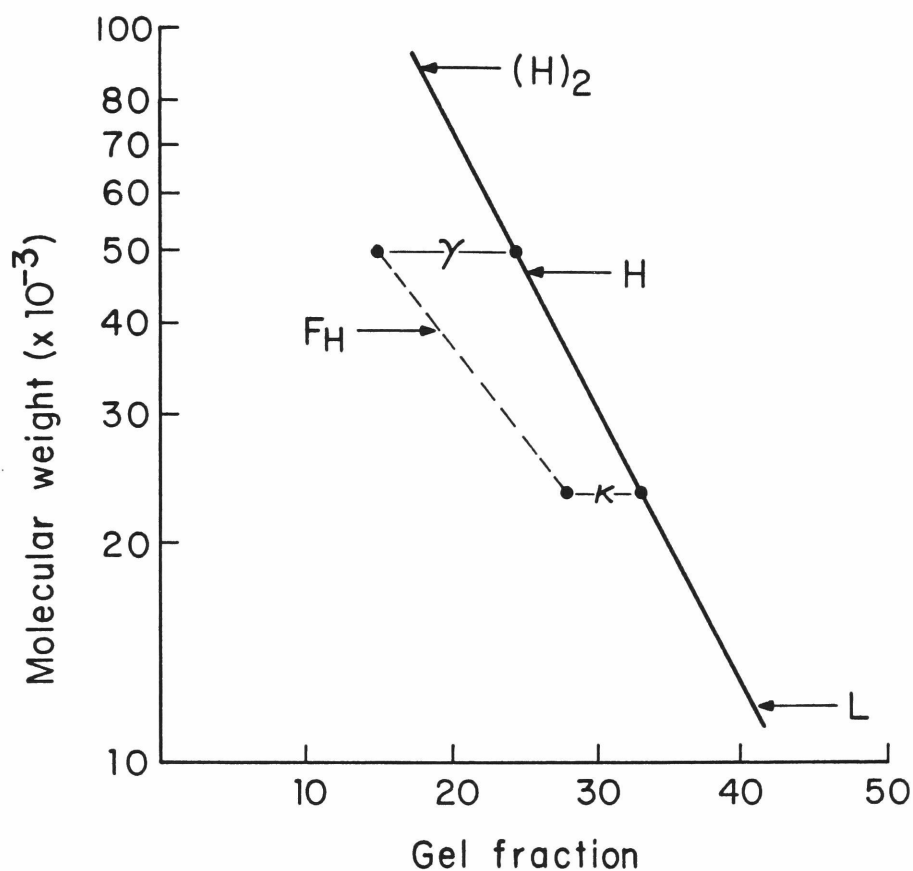


Fig. 9. Determination of the molecular weight of H-2 heavy and light chains. The gels shown in Fig. 8 were calibrated with internal standards. The data shown are for Figs. 8A and 8C (—), and for Fig. 8D (---). Molecular weight standards are γ , MOPC 21 heavy chain (MW 50,000) and κ , MOPC 21 light chain (MW 23,000). The positions of H-2 heavy chain monomer, H, heavy chain dimer, (H)₂, papain fragment, F_H, and light chain, L, are indicated.

this experiment was also performed using papain which was activated with 2mM cysteine and dialysed overnight against PBS to remove residual cysteine. Again no difference was observed between the molecular weights of the papain-solubilized H-2 antigens in the presence or absence of reducing agents.

The molecular weights of the detergent and papain-solubilized molecules were also compared in the absence of SDS. Gel exclusion chromatography of NP40 or DOC-solubilized spleen or lymphoma cell membrane material on Sephadex G-200 gave a major component with H-2 antigenic activity that eluted between the IgG and BSA markers with an approximate molecular weight of 120,000 (Fig. 10). Lower molecular weight material retaining antigenic activity was also observed frequently. Omission of DOC from the elution buffer or the use of NP40 instead of DOC increased the apparent MW considerably, indicating the formation of aggregates. Under nondissociating conditions and in the absence of detergent, gel chromatography of papain-solubilized H-2 antigenic material indicated an MW of approximately 50,000 (Fig. 11). The partition coefficients for detergent and papain-solubilized H-2 antigens were used to determine their apparent diffusion coefficients and Stokes' radii (Figs. 12, 13 and Table VIII) by the method of Akers (1970).

Ultracentrifugation of detergent-solubilized H-2 antigens on sucrose density gradients in 0.5% DOC gave a peak of antigenic activity at 5.9S with some minor components at lower S values (Fig. 14). In the absence of DOC most of the H-2 antigenic material was found at the bottom of the centrifuge tube, indicating aggregation of the antigen under these conditions. The same sedimentation coefficient was also obtained for H-2K^b antigens. In sucrose density gradient centrifugation experiments, papain-treated detergent extracts showed a major peak of activity at 3.7S (Fig. 14).

The results of these experiments are summarized in Table VIII. Calculation of molecular weights by the Svedberg equation using the determined values of the diffusion and sedimentation coefficients gave values of 120,000 for the intact molecule in detergent solution and 50,000 for the major fragment produced by papain cleavage (Table VIII). These results are consistent with the hypothesis that intact H-2 antigens

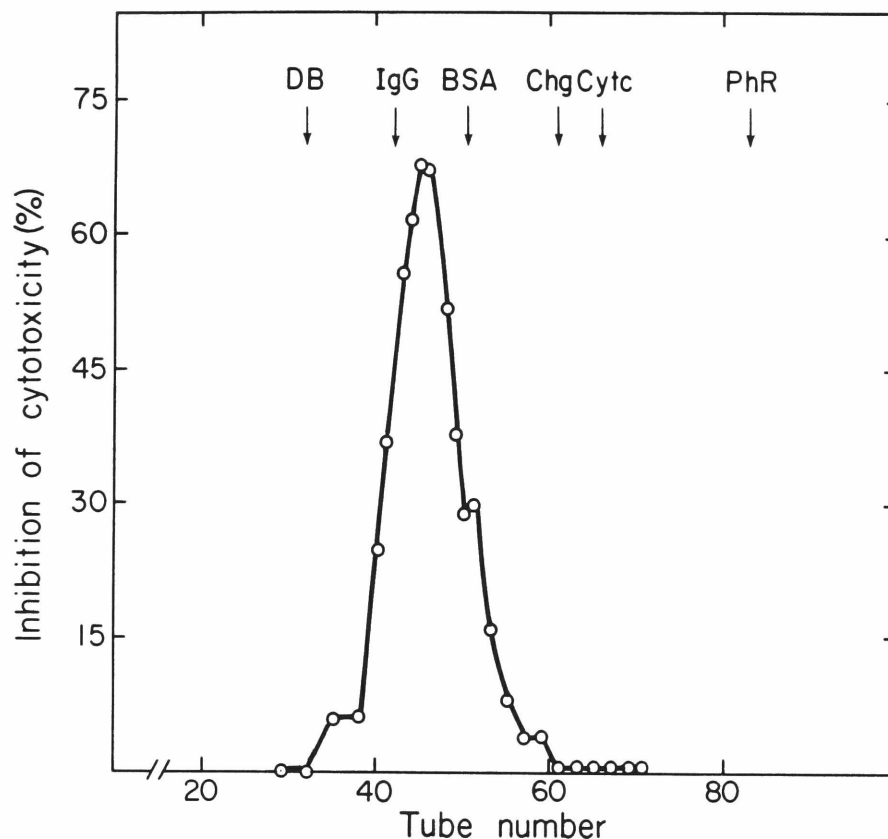


Fig. 10 Gel exclusion chromatography of detergent solubilized H-2 antigens on Sephadex G-200. 1 ml of 0.5% DOC extract of a membrane fraction of C57BL/10J spleens was loaded on a calibrated Sephadex G-200 column (1.2 x 75cm). Eluant: 0.01M Tris-HCl buffer, pH 8.0, 0.15M NaCl, 0.5% DOC, 5ml/hr. Assay: anti-H-2K^b on ⁵¹Cr-loaded C57BL/10J spleen lymphocytes. DB, dextran blue; IgG, MOPC 21 immunoglobulin; BSA, bovine serum albumin; Chg, chymotrypsinogen; CytC, cytochrome C; PhR, phenol red. Reproduced with permission from Henning et al. (1976c).

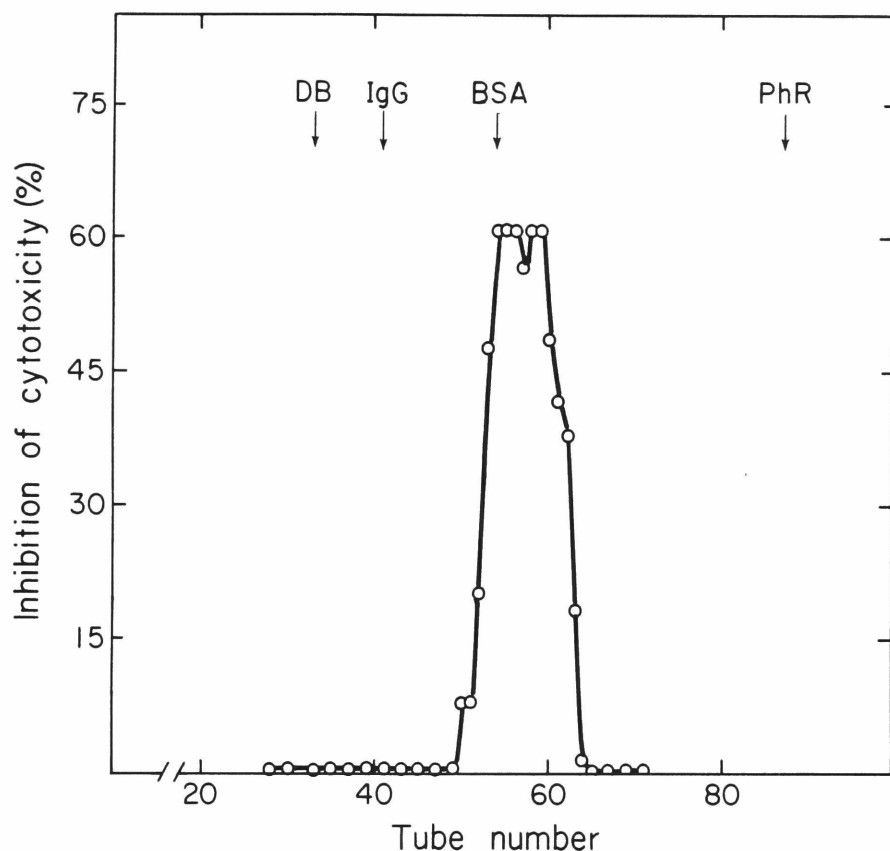


Fig. 11 Gel exclusion chromatography of papain solubilized H-2 antigens on Sephadex G-200. 1 ml of a 0.5% NP40 extract of a membrane fraction of 10 BALB/c spleens was digested with papain and loaded on a calibrated Sephadex G-200 column (1.2 x 75cm). Eluant: 0.01M Tris-HCl, pH 8.0, 4 ml/hr. Assay: anti-H-2D^d on ⁵¹Cr-loaded BALB/c spleen lymphocytes. DB, dextran blue; IgG MOPC 21 immunoglobulin; BSA, bovine serum albumin; PhR, phenol red. Reproduced with permission from Henning et al. (1976c).

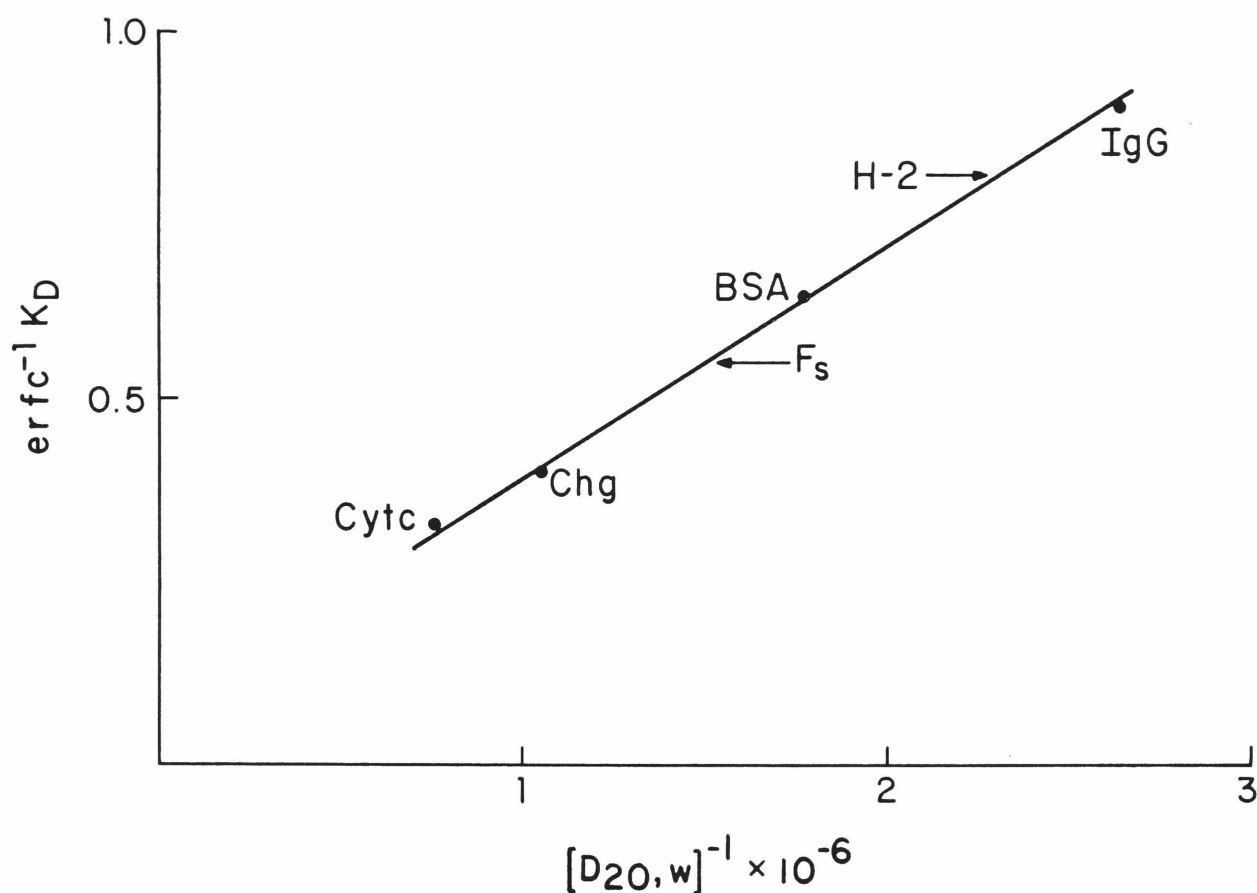


Fig. 12 Determination of the diffusion coefficient of H-2 antigens. The inverse error function complement of the partition coefficient ($\text{erfc}^{-1} K_D$) is plotted against the reciprocal diffusion constant for known standard proteins. The values for detergent solubilized H-2 antigens (H-2) and papain solubilized antigens (F_s) are determined by interpolation. The standard proteins used were: IgG, MOPC21 immunoglobulin; BSA, bovine serum albumin; Chg, chymotrypsinogen; Cytc, cytochrome.

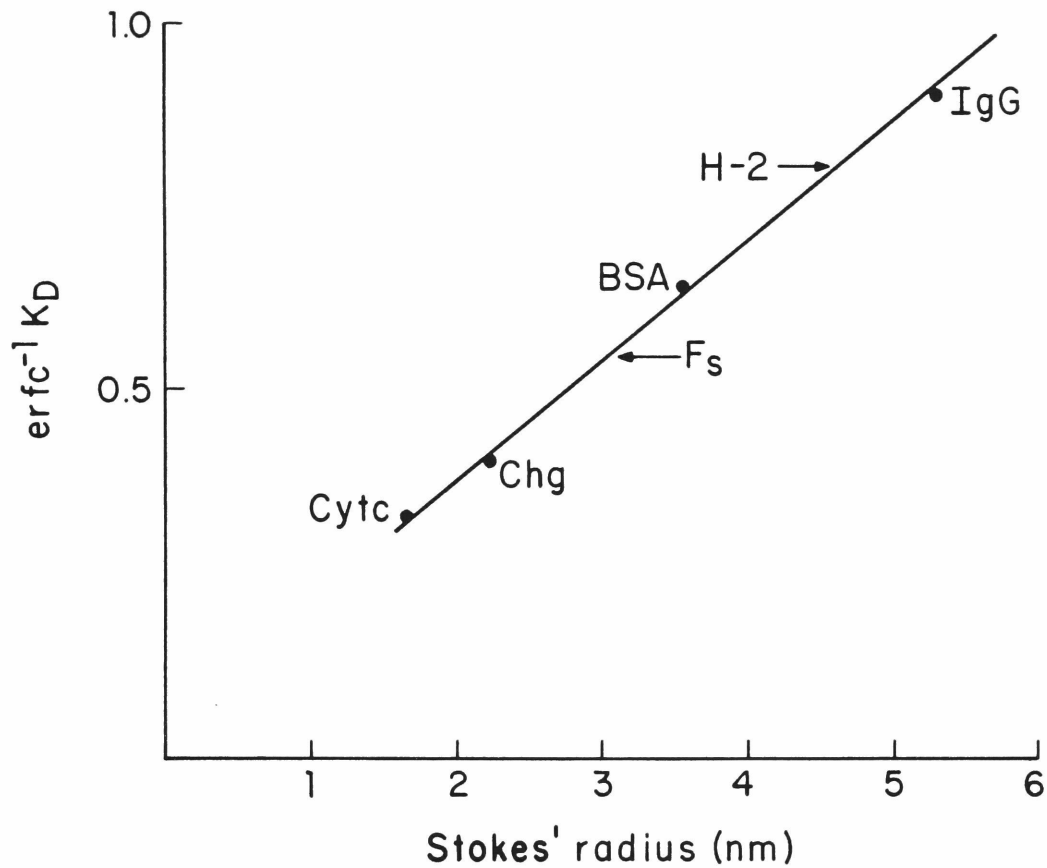


Fig. 13 Determination of the Stokes' radius of H-2 antigens. The inverse error function complement of the partition coefficient ($\text{erfc}^{-1} K_D$) is plotted against the Stokes' radius for known standard proteins. The values for detergent-solubilized H-2 antigens (H-2) and papain-solubilized antigens (F_s) are determined by interpolation. The standard proteins used were: IgG, MOPC21 immunoglobulin; BSA, bovine serum albumin; Chg, chymotrypsinogen; Cytc, cytochrome C.

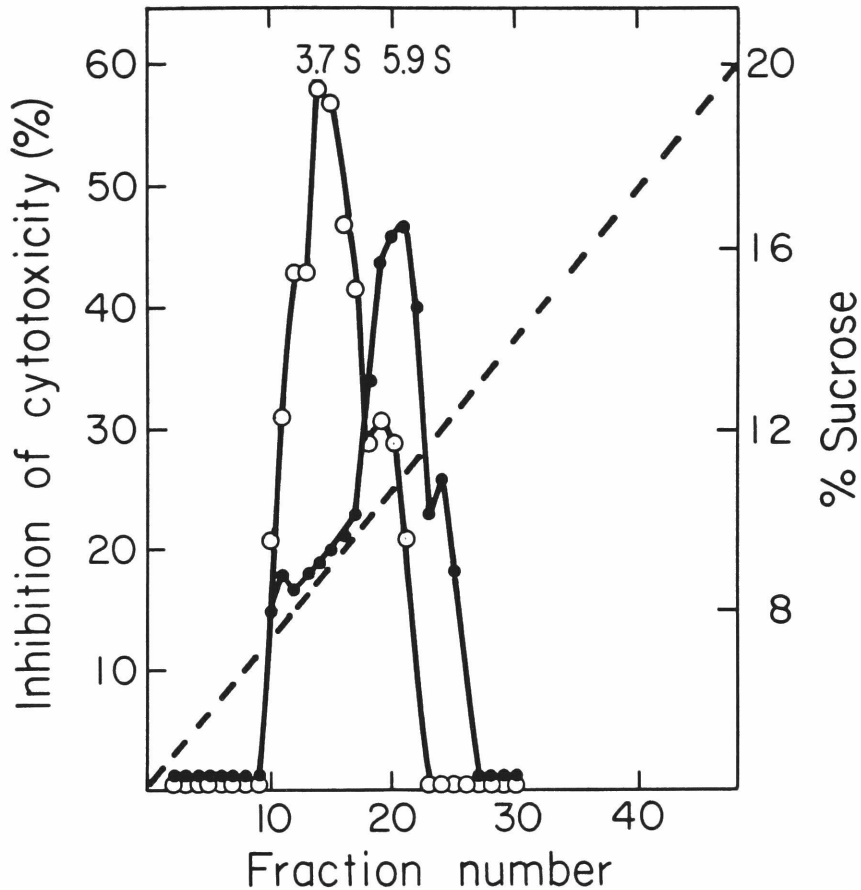


Fig. 14 Sucrose density gradient centrifugation of detergent and papain solubilized H-2 antigens. A 0.5% NP40 extract (75 μ l) from membranes of DBA/2J spleens (10 spleens per ml) was treated with papain, mixed with 75 μ l of an untreated extract from C57BL/10J spleen cell membranes, loaded onto a 4-20% sucrose gradient in 0.01M Tris-HCl, pH 8.0, 0.15M NaCl 0.5% DOC, and centrifuged for 25 hr at 35,000 rpm (5°) in an SW39 Beckman rotor. Fractionation was by tube puncture and drop counting, 6 drops/fraction. Each fraction was assayed for H-2K^d and H-2K^b by inhibition of cytotoxicity of anti-H-2K^d and anti-H-2K^b on DBA/2J and C57BL/10J targets, respectively.
 (o) H-2K^d: detergent-solubilized and papain treated.
 (●) H-2K^b: detergent-solubilized. (---) % sucrose.
 Reproduced with permission from Cunningham et al. (1976b) and Henning et al. (1976c).

Table VIII

Physicochemical Properties of H-2 Antigens

	<u>Molecular Weight</u>	<u>Stokes' radius</u>	<u>Diffusion Coefficient^a</u>	<u>Sedimentation Coefficient</u>
H-2 dimer (in DOC)	120,000 ^b	4.6 nm	4.4×10^{-7}	5.9 S
H chain	47,000 ^c	---	---	---
L chain (β_2)	12,000 ^c	---	---	---
Papain fragment (F _S)	50,000 ^b	3.1 nm	6.7×10^{-7}	3.7 S
H chain (F _H)	39,000 ^c	---	---	---
L chain (β_2)	12,000 ^c	---	---	---

The data shown are for the H-2K^b gene product from C57BL/10 spleen cells.
^acm²sec⁻¹

^bCalculated using the Svedberg equation (Ackers 1970).

^cSDS-PAGE (Weber and Osborn 1969).

in detergent solution are composed of two heavy chains of 47,000 daltons each and two noncovalently associated β_2 -microglobulin molecules. In contrast, the papain-solubilized molecule, which we have designated F_s for soluble fragment (Henning et al. 1976), contains only one F_H fragment and one light chain.

H-2 Antigens on the Cell Surface. The physiological function of the H-2 antigens is probably mediated directly at the cell surface. It was therefore necessary to relate the structure of the H-2 antigens in solution to their structure on the cell surface, in particular to determine their orientation with respect to the plasma membrane.

Polyacrylamide gel electrophoresis of detergent-solubilized molecules in the absence of reducing agents indicated that the molecules on the cell surface might differ from those in solution. Although the pattern shown in Fig. 8A was usually obtained, immunoprecipitates from detergent extracts occasionally showed the simultaneous presence of H-2 heavy-chain monomers and dimers on SDS-polyacrylamide gels in the absence of reducing agents (Fig. 15). Similar observations have been reported by other laboratories for both H-2 and HLA antigens (Schwartz et al. 1973, Cresswell and Dawson 1975). These results raised the possibility that the H-2 heavy chain may not exist as a covalently linked dimer on the cell surface, and that the disulfide bridge might be formed after extraction from the membrane. In accord with this hypothesis, treatment of cells or membrane fractions with iodoacetamide prior to detergent extraction significantly increased the amount of H-2 heavy-chain monomer found on SDS-polyacrylamide gels in the absence of reducing agents (Fig. 15).

These results indicate that the majority of heavy chains on the cell surface are not linked by disulfide bonds and, therefore, that they may exist either as monomers or as noncovalently associated dimers. Preliminary experiments indicated that some dimers may exist. For example, oxidation of sulfhydryl groups with o-phenanthroline and copper sulfate (Steck 1972) prior to alkylation of cells slightly increased the amount of heavy-chain dimer, suggesting that some H-2 molecules may be closely associated with each other as noncovalently bonded dimers.

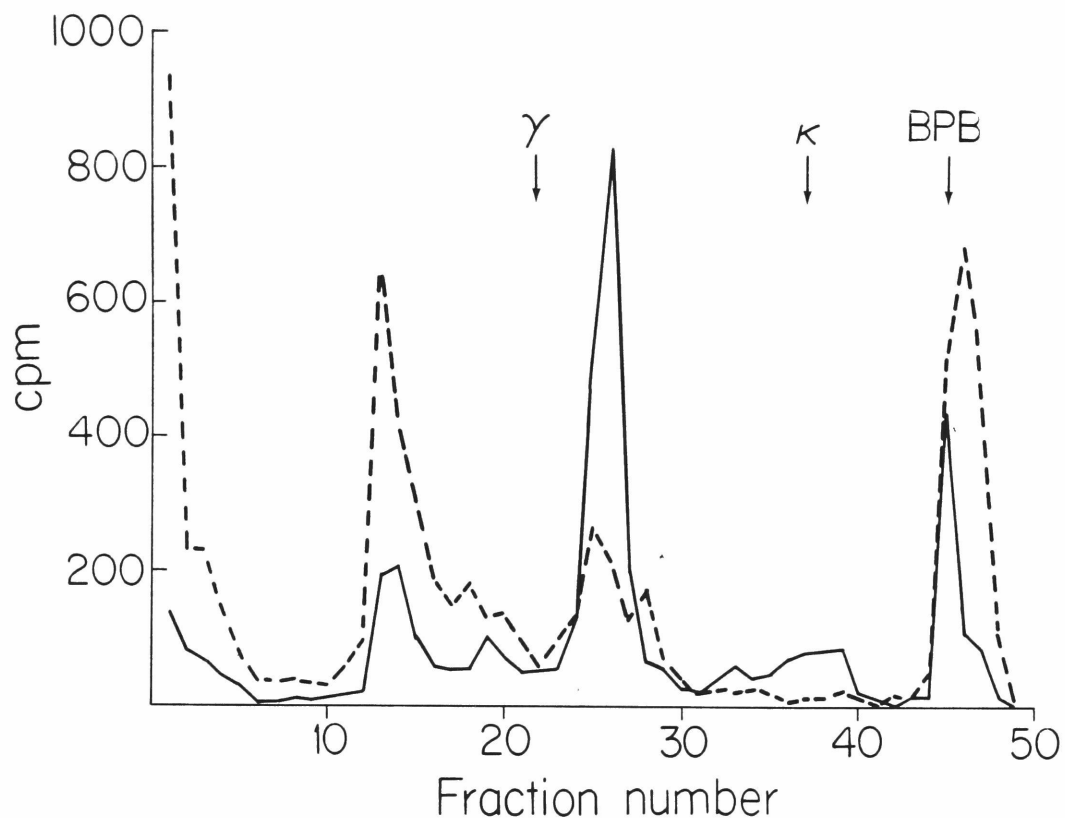


Fig. 15. Alkylation of H-2 antigens on the cell surface. SDS-PAGE of immune precipitates of NP40-solubilized H-2K^b antigens from ¹²⁵I-labelled EL-4 cells. (—) Cells treated with 5mM iodoacetamide before detergent extraction; (---) cells not treated before extraction. Ten percent polyacrylamide gels run in the absence of reducing agents. Molecular weight markers are as described in the legend to Figure 8. Reproduced with permission from Cunningham et al. (1976a).

Cross-linking of H-2 Heavy and Light Chains. The association between the small and large subunits of H-2 antigens was also demonstrated by cross-linking experiments using the reagent dimethyl-3,3'-dithiobispropionimidate which contains a disulfide bond cleavable by reduction (Wang and Richards 1974). H-2K^b antigens were isolated by immunoprecipitation from NP40 extracts of cross-linked ¹²⁵I-labelled RBL tumour cells and analyzed by SDS-polyacrylamide gel electrophoresis. In the absence of reducing agents the immunoprecipitate made from a cross-linked extract displayed an extra component with a molecular weight slightly higher than immunoglobulin heavy chain that was not found in the immunoprecipitate made from an untreated extract (Fig. 16A slots 5,6). In the presence of reducing agents, however, immunoprecipitates from both extracts showed the same heavy and light chain components (Fig. 16B, slots 5,6).

In the absence of reducing agents the H-2 antigens from both extracts show two heavy chain components with slightly different mobilities. This heterogeneity is not seen in the presence of reducing agent suggesting that the two components may differ in intra-chain disulfide bonding. The specificity of the immunoprecipitation of the H-2 antigens is shown by control precipitates made using anti-K^k alloantiserum (Fig 16A and 16B, slots 1 and 2). The higher molecular weight component found in the control and anti-K^b immunoprecipitates is probably the viral glycoprotein gp70 as indicated by specific immunoprecipitates made with goat antiserum against gp70 (Figs. 16A and 16B, Slots 3 and 4). After cross-linking most of the gp70 appears to migrate with a higher molecular weight, probably due to dimer formation.

The cross-linking is also shown by two-dimensional SDS-polyacrylamide gel electrophoresis in which the first dimension is run in the absence of reducing agent and the second in the presence of reducing agent (Wang and Richards 1974). Components which have the same apparent molecular weights in the presence and absence of reducing agents will run on the diagonal of the second dimension gel. Components whose electrophoretic behaviour is different in the presence and absence of reducing agent, such as those cross-linked by the disulfide containing reagent, will run off the diagonal in the second dimension.

Immunoprecipitates of K^b antigens made from both cross-linked and untreated NP40 extracts of ^{125}I -labelled RBL cells show major heavy and light chain components which lie on the diagonal of the two-dimensional gel (Fig. 17). The higher molecular weight component in both gels is probably gp70. The immunoprecipitate of H-2K^b antigens made from the extract of cells that had been treated with the cross-linking reagent shows two additional components which have the same molecular weight as H-2 heavy chains in the second dimension but which lie off the diagonal. Vertically below one of these components (marked with an arrow in Fig. 17) is a component with the same molecular weight in the second dimension as β_2 -microglobulin (also indicated by an arrow in Fig. 17). These results suggest that H-2 heavy and light chains may be cross-linked to form a species with an approximate molecular weight of 55,000. In addition, this experiment reveals a larger aggregate of H-2 antigen seen as a smear in Fig. 17B to the left of the H-2 heavy chain spot. This component is probably H-2 heavy chain dimer, suggesting that at least some heavy chains may be closely associated on the cell surface. The fact that not all heavy chains and light chains are cross-linked probably reflects the inefficiency of the cross-linking reaction. Similar results, with similar cross-linking efficiencies, have been reported for HLA antigens (Springer et al. 1977). As a result of the inefficiency of the cross-linking reagent, a quantitative estimate of the amount of H-2 heavy chain monomer and dimer cannot be made.

The Orientation of H-2 Antigens on the Cell Surface. β_2 -microglobulin is not attached to the cell membrane and its orientation is difficult to ascertain. The heavy chain, however, appears to be firmly embedded in the membrane and is released as an intact molecule by detergent or as the F_H fragment by treatment with papain. Comparison of the amino acid sequences of the F_H fragment with those of the H-2 heavy chain from the detergent-solubilized molecule should therefore indicate which region of the chain is closely associated with the cell membrane. To determine the orientation of the H-2K^b heavy chain, experiments were carried out using antigens and their papain fragments internally labelled with tritiated tyrosine, histidine, or arginine. The positions of the labelled residues in the purified heavy chains and F_H fragments were determined for each sample both manually and in

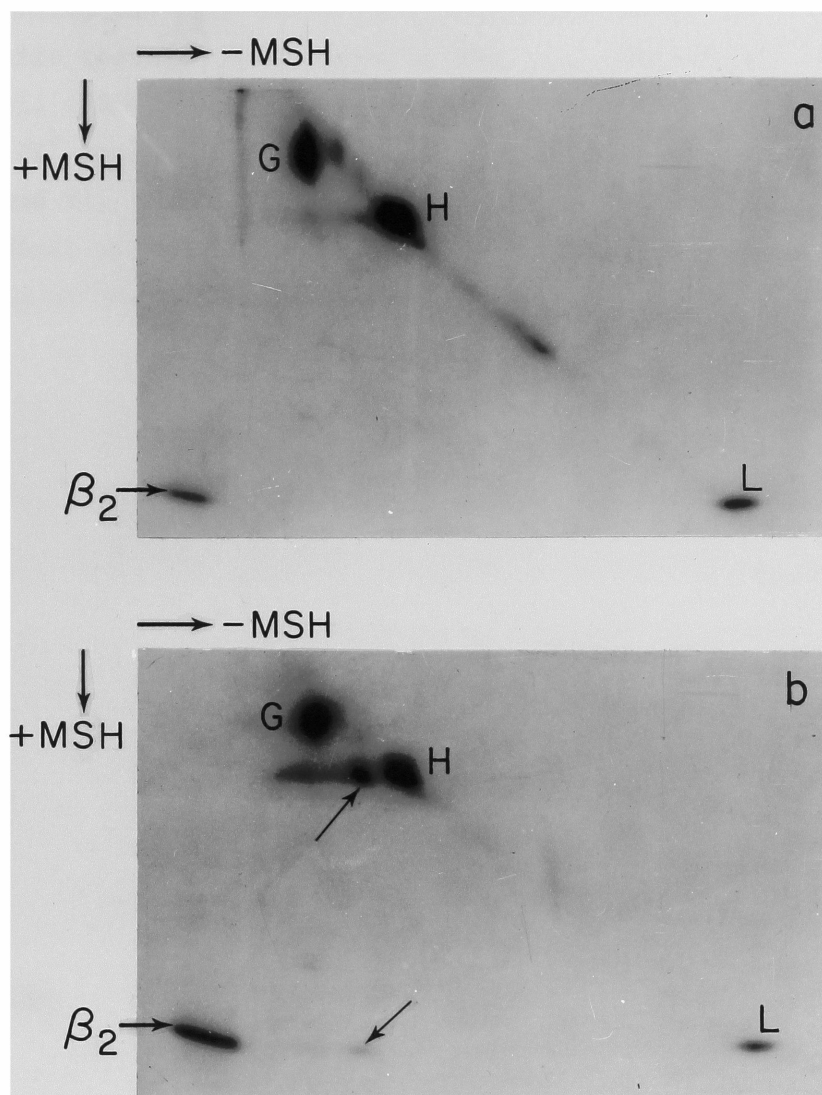


Fig. 17. Analysis of H-2K^b antigens by the two dimensional gel system of Wang and Richards (1975). The samples are run first in the horizontal dimension in the absence of 2-mercaptoethanol (-MSH) and in the vertical direction in the presence of 2-mercaptoethanol (+MSH). Immunoprecipitates were made with anti-H-2K^b anti-serum from NP40 extracts of radioiodinated RBL cells that were untreated (a) or treated with dimethyl-3,3'-dithiobispropionimidate before extraction (b). β_2 indicates the position of radioiodinated β_2 -microglobulin standard run in the second dimension. The positions of spots due to H-2 heavy chain (H), H-2 light chain (L) and gp70 (G) are indicated. Arrows indicate spots due to cross-linked components. The polyacrylamide concentration in both dimensions was 12%. Gels were exposed to film for 15 days.

the automatic sequencer. The results (Table IX and Fig.27) show no differences between the F_H fragment and the $H-2K^b$ heavy chain for the three amino acids tested. These results have also been shown by Ewenstein et al. (1976), who showed that the leucinyl and arginyl residues in $H-2K^b$ and its papain fragment F_H are identical in positions 5, 6, 14, 17 and 21. These data suggest that the F_H fragment contains the amino-terminal part of the H-2 heavy chain, and that the carboxyl-terminal region of the molecule is closely associated with the cell membrane.

Table IX

Amino-Terminal Sequences of H-2K^b Heavy Chain
and the H-2K^b F_H Fragment

			1	2	3	4	5	6	7
H-2K ^b	H	H ₂ N	-	-	His	-	-	Arg	Tyr
H-2K ^b	F _H	H ₂ N	-	-	His	-	-	Arg	Tyr

Sequences were determined by manual and automatic methods.

Discussion: A Model for the Structure of H-2 Antigens

From the data described here and other studies a model has been proposed for the subunit structure of H-2 antigens on the cell surface and in solution (Henning et al. 1976a; Fig. 18).

The present data indicate that the detergent solubilized H-2 molecule in solution is composed of two disulfide-linked heavy chains and two noncovalently associated light chains (β_2 -microglobulin). Data from other laboratories have suggested that H-2 and HLA molecules consist of two disulfide-linked heavy chains and two noncovalently associated β_2 -microglobulin chains (Strominger et al. 1974, Peterson et al. 1975). The amount and nature of higher and lower molecular weight species in both systems are not well defined. Higher polymers of HLA antigens (Strominger et al. 1974) and H-2 antigens (Schwartz et al. 1973) are seen in nonionic detergents and probably result from the formation of large micelles by these detergents. Both 120,000- and 60,000-dalton HLA species have been observed in DOC (Cresswell and Dawson 1975), and the simultaneous presence of H-2 heavy chain dimers and monomers has been seen in the absence of reducing agents (Schwartz et al. 1973). In addition, a 50,000-dalton component of HLA antigens was observed under dissociating conditions in the absence of reducing agents (Peterson et al. 1975).

More recent data on HLA antigens, however, have indicated that the formation of heavy chain dimers in detergent solution is an artefact of the extraction procedure (Snary et al. 1975) and can be prevented by alkylation of the cells prior to extraction (Springer et al. 1977) as I have described for H-2 antigens.

These findings suggest that the H-2 heavy chain may not exist predominantly as a covalently bonded dimer on the cell surface. It may therefore exist as either a monomer or a non-covalently associated dimer (Fig. 19). The efficient formation of disulfide bonds between the heavy chains once these are removed from the cell membrane may indicate that the heavy chains already exist as non-covalently associated dimers in the cell membrane. Furthermore, if the association between heavy chains occurs only after they are extracted from the membrane

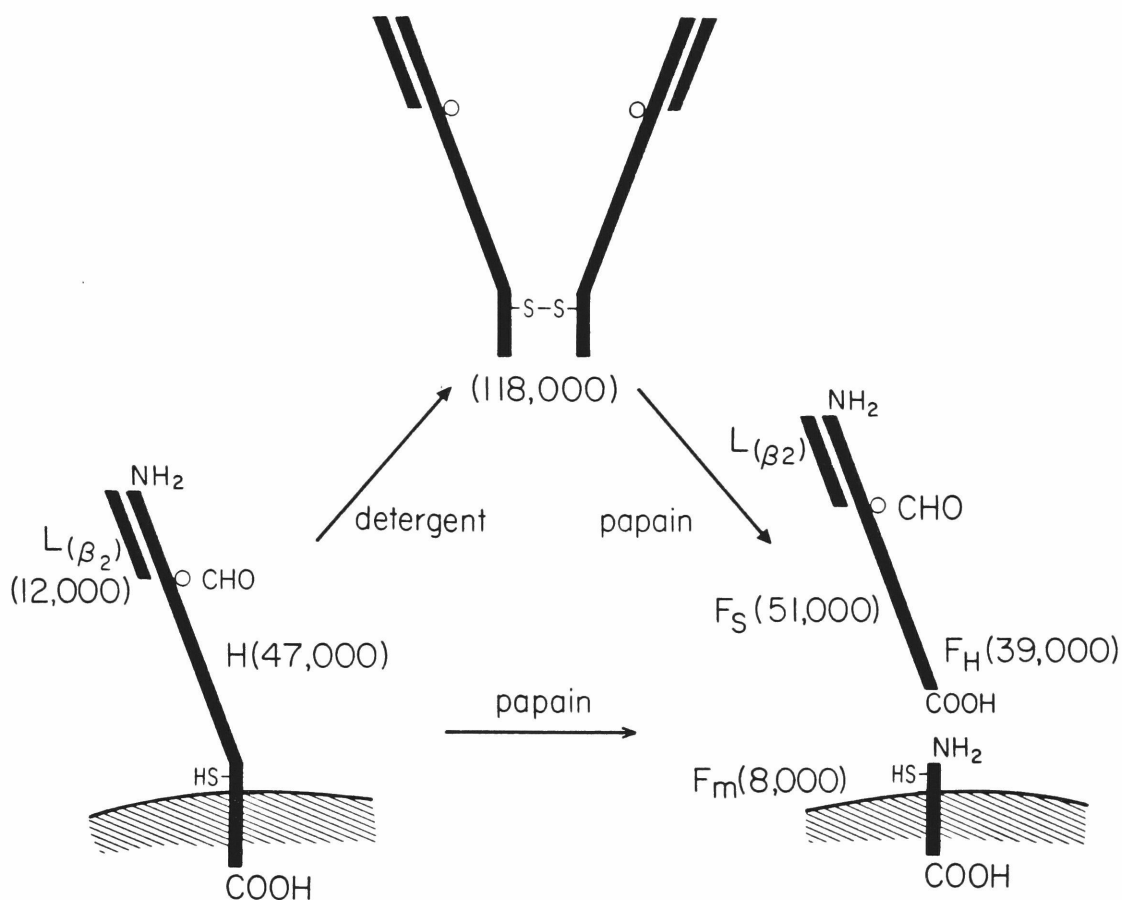


Fig. 18. Subunit structure and orientation of H-2 antigens in solution and on the cell surface. Detergent-solubilized H-2 antigens contain two heavy and two light chains with at least one disulfide bond linking the two heavy chains. Papain treatment of intact cells or detergent extracts generates H-2 heavy-chain fragments of identical size (the data given here are for the H-2K^b molecule). H = H-2 heavy chain; L(β₂) = H-2 light chain (β₂-microglobulin); F_S = water-soluble fragment (F_H plus L) obtained after papain treatment of cell surfaces or detergent extracts; F_H = fragment of the H-2 heavy chain obtained after papain treatment; F_m = portion of the H-2 heavy chain cleaved from the bulk of the molecule by papain and apparently associated with the cell membrane. Molecular weights are given in parentheses.

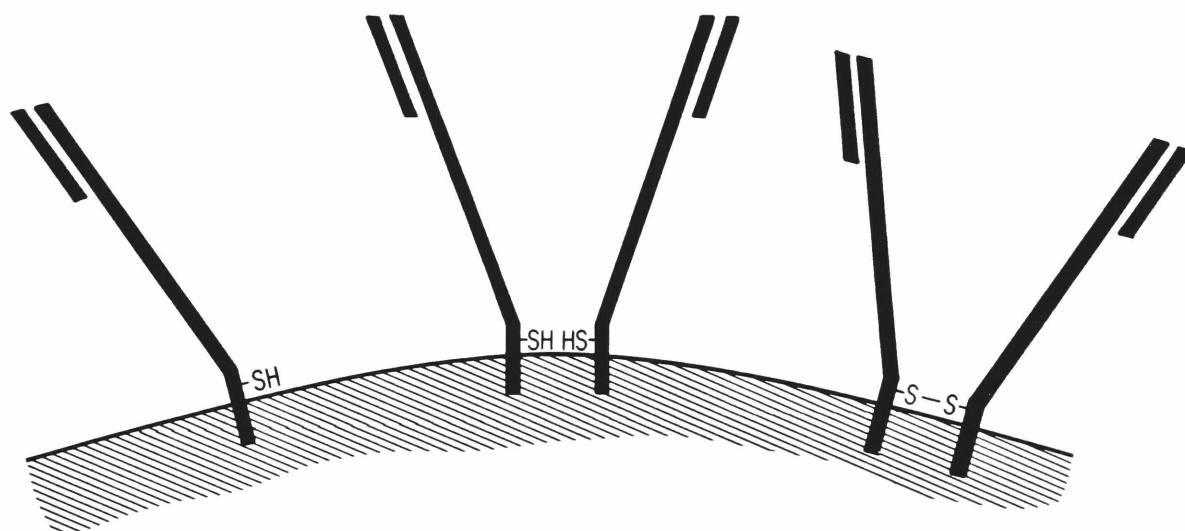


Fig. 19. Possible forms of H-2 antigens on the cell surface.

then formation of H-2K-H-2D heterodimers might be expected. The independent immunoprecipitation of H-2K and H-2D antigens (Fig. 4), however, indicates that formation of heterodimers on detergent extraction does not occur to any great extent, if at all. In addition, several reports (Neauport-Sautes et al. 1973, Hauptfeld et al. 1975) have demonstrated that the H-2D and H-2K antigens are not associated on the cell surface and are able, for example, to be capped independently.

One intriguing possibility is that there is an equilibrium between the dimeric and monomeric forms of the H-2 antigens on the cell surface. Alteration of this equilibrium may influence the function of H-2 antigens. For example, the monomer form of the antigen on the cell surface might have sites available for interaction with other molecules such as viral components.

Papain treatment of H-2 antigens generates a large water-soluble fragment, F_s , which retains the private antigenic determinants. Other studies have shown that the heavy chain fragment, F_H , also carries the same carbohydrate side chain as the intact heavy chain (Schwartz et al. 1973). The remaining fragment of the heavy chain, which has been termed F_m for membrane fragment (Fig. 18, Henning et al. 1976a), is probably responsible for anchorage of the heavy chain in the cell membrane. This putative fragment, which would have a molecular weight of approximately 8,000, has not yet been isolated and characterized. Treatment of detergent-solubilized HLA antigens with papain, however, results in the complete degradation of the F_m region of the HLA heavy chain suggesting that the F_m fragment of the H-2 heavy chain may not exist after papain treatment (Strominger et al. 1976). Other studies of the HLA antigens indicate that the heavy chains of the HLA antigens span the cell membrane and have access to both the external and cytoplasmic spaces (Walsh and Crumpton 1977). Consistent with this finding is the report that the region of the HLA heavy chain homologous to the F_m fragment contains a region of hydrophobic amino acids, presumably in contact with the lipid bilayer of the cell membrane, and a region of hydrophilic amino acids, presumable in contact with the cytoplasm (Strominger et al. 1976).

The F_H fragment of the heavy chain, in contrast to the detergent-solubilized molecule, does not appear to form covalently bonded heavy chain dimers, indicating that the F_H fragment does not contain the cysteine residues responsible for interchain disulfide bonding between intact heavy chains. These residues are probably located in the F_m fragment (Fig. 18). Furthermore, the F_s fragments of H-2 antigens do not form non-covalently associated dimers, suggesting that the residues responsible for these interactions may also reside in the F_m fragment. This is in contrast to papain solubilized HLA antigens, which have been reported to exist as non-covalently associated dimers (Strominger et al. 1974).

The solubilization of H-2 antigens by papain has allowed us to determine the orientation of these molecules with respect to the cell surface. The papain- and detergent-solubilized heavy chains possess the same amino-terminal amino acid sequences, indicating that the amino-terminal of the heavy chain extends away from the cell surface while the carboxyl-terminal is associated with the cell membrane. This orientation resembles that of other membrane glycoproteins, such as glycophorin (Bretscher 1971, Segrest et al. 1973) and immunoglobulin (Fu and Kunkel 1974), and it may be a general feature of all membrane proteins. The F_m region of H-2 antigens should include this carboxyl-terminal region and may have many features in common with similar portions of other membrane proteins, including similarities in amino acid composition or sequence.

V. STRUCTURAL COMPARISONS OF H-2D AND H-2K GENE PRODUCTS

The genetics and serology of the H-2 antigens suggest that these molecules may have unusual properties. For one thing, the H-2 genes are extremely polymorphic with many variants in the population. Each H-2 gene product is defined by a unique private antigenic determinant, as well as by public antigenic determinants that are common to several H-2 gene products. The presence of shared and unique antigenic determinants on different H-2 molecules suggests that they will have some structural features that are in common and some that are specific for a given H-2 molecule. Moreover, from their genetic and serological analysis of the H-2 system, Klein and Schreffler (1971) suggested that the H-2K and H-2D loci arose from a single precursor gene.

In this section I will describe the properties of H-2 antigens determined by the H-2K and H-2D genes of the haplotypes b, d and k. These gene products are compared by molecular weight, isoelectric point and amino-terminal amino acid sequence. Comparison of the structural properties of these molecules allows certain conclusions to be made about the origin and diversity of histocompatibility antigens.

Materials and Methods

Details of radiolabeling, immunoprecipitation and SDS-PAGE techniques are described in the preceding sections.

Comparison of H-2D and H-2K gene products on SDS-PAGE. For the comparison of the molecular weights of H-2 heavy chains of different haplotypes, the heavy chains were purified on SDS-polyacrylamide gels. The labeled heavy chains were eluted from gel fractions into 2% SDS by shaking overnight at 37°C. Gel particles were removed by centrifugation and the supernatant was lyophilized. Excess SDS was removed by extraction with methanol. The samples were lyophilized, resuspended in SDS sample buffer and run on SDS-polyacrylamide gels in the presence of molecular weight standards or H-2 heavy chains of a different genotype labeled with a different isotope.

Two-dimensional gel electrophoresis. For analysis by two-dimensional gel electrophoresis (O'Farrell 1975), cells labeled with ^{125}I by the lactoperoxidase technique were extracted in 10 mM Tris-HCl (pH 7.0)

containing 0.5% NP40, 50 μ g/ml ribonuclease (Worthington), 50 μ g/ml deoxyribonuclease (Schwartz-Mann), 200 units/ml trasylol (Sigma) and 10 mM iodoacetamide and centrifuged at 100,000 x g for 60 min. Aliquots of the supernatant were mixed with equal volumes of isoelectric focusing (IEF) sample buffer (0.5 M urea, 2% (v/v) NP40, 2% Ampholines (LKB) (a mixture of 1.6% pH range 5-7 and 0.4% pH range 3.5-10), and 5% 2-mercaptoethanol) and concentrated to the original volume (Speed Vac Concentrator, Savant Instruments, Hicksville, N.Y.). Immunoprecipitates were prepared for two-dimensional electrophoresis by extraction with IEF sample buffer.

Samples of labeled cell extracts or immunoprecipitates were isoelectrofocussed in thin glass tubes (130 mm x 2.5 mm inside diameter), by using a gel mixture consisting of 9 M urea, 2% (v/v) NP40, 2% Ampholines (1.6% pH 5-7 and 0.4% pH 3.5-10), and 4% acrylamide. The IEF gels were extruded from the tubes and equilibrated for 2 hours at 25°C in 5 ml SDS sample buffer (2.3% (w/v) SDS, 10% (w/v) sucrose, 5% 2-mercaptoethanol, and 0.0625 M Tris-HCl, pH 6.8). The equilibrated gels were stored frozen in SDS sample buffer at -70°C before the second electrophoresis.

The second dimension electrophoresis was done as described by O'Farrell (1975). The equilibrated IEF tube gel was placed on top of the stacking gel, and anchored on with 1 ml of melted agarose. The slab gels were run at 20 mA constant current per gel. The gels were fixed and stained as described in the preceding section.

For neuraminidase treatment radioiodinated EL4 cells were incubated in PBS at 5×10^7 cells/ml with 50 U/ml *Vibrio cholerae* neuraminidase (Behring Diagnostics, American Hoechst Corp., Summerville, N.J.) for one hour at 37°C. The cells were centrifuged and the cell pellet extracted as described above.

Preparative Immunoprecipitation of H-2 Antigens. Mouse leukemia cells were radioactively labeled by incubation at 10^7 cells/ml with 0.1 mCi/ml ^3H or ^{35}S -amino acids (New England Nuclear, Boston, Mass.) in minimal essential medium lacking the labeled amino acid (Gibco, Grand Island, N.Y.). The cells were extracted with 0.5% NP40 in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl at 10^8 cells/ml and centrifuged at 100,000 x g for 60 minutes. The supernatant was used for immunoprecipitation:

usually 0.1 ml anti-H-2 alloantiserum was used for 1 ml cell extract. The immune complexes were precipitated with 0.5 ml goat antiserum against mouse IgG (Miles, Elkhart, Ind.) and the precipitates were washed three times in 2 ml 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.25% NP40. Immuno-precipitates were dissolved in 2 ml 6 M guanidine-HCl (pH 8.5), 1 M Tris, 1.25 mg/ml EDTA. 1.5 mg dithiothreitol (Sigma, St. Louis, Mo.) was added and the mixture incubated at 37°C for two hours under nitrogen. 3.5 mg iodoacetamide (recrystallized, ICN Pharmaceuticals, Inc., Plainview, N.Y.) was added and the mixture incubated for one hour at 25°C. The immunoprecipitate was dialysed against water overnight, lyophilized and redissolved in 0.5 ml SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol).

Preparative SDS-PAGE. The immunoprecipitate dissolved in sample buffer was loaded on a 2 x 20 cm preparative SDS-polyacrylamide gel (7.5% acrylamide concentration) using the Laemmli (1970) system. The gel was run at 5 mA for 12 hours at 4°C. The position of H-2 heavy chain component was determined by use of a marker protein labeled with 5-dimethylamino-1-naphthalene sulphonyl chloride which co-electrophoresed with H-2 heavy chains. The fluorescent band was cut from the gel and the gel slice was placed on a 2 x 15 cm column of Biogel P2 (Bio Rad) which was equilibrated with electrode buffer. Acrylamide gel was polymerized over the gel slice and labeled H-2 heavy chains were eluted from the slice into the Biogel P2 by electrophoresis at 250-300 V for 30-60 minutes. Labeled heavy chains were eluted from the Biogel P2 with water, counted for radioactivity and lyophilized.

Amino Acid Sequence Determination. The amino acid sequences of H-2 heavy chains were determined by manual and automatic methods. All sequence analysis was performed using an internal radioactively labeled standard, MOPC21 light chain. This was labeled with a single radioactive amino acid and prepared from MOPC21 myeloma cells (obtained from the Salk Institute, La Jolla, Cal.) by the same method as the H-2 heavy chains using immunoprecipitation with goat antiserum against mouse immunoglobulin. Usually the light chain was labeled with ³⁵S-methionine, except when this label was used in the H-2 heavy chain; the MOPC21 light chain was then labeled with ¹⁴C-valine. The amino-terminal amino acid sequence of MOPC21 light chain is shown in Table IX.

Table IX

Amino Terminal Amino Acid Sequence of the Light Chain
of MOPC21 Immunoglobulin

	5	10
Asn-Ile-Val-Met-Thr-Gln-Ser-Pro-Lys-Ser		
<u> </u> <u> </u>		
	15	20
Met-Ser-Met-Ser-Val-Gly-Glu-Arg-Val-Thr		
<u> </u> <u> </u> <u> </u> <u> </u>		
	25	30
Leu-Thr-Cys-Lys-Ala-Ser-Glu-Asn-Val-Val		
		<u> </u> <u> </u>

Data from Svasti and Milstein (1972).

The manual sequence procedure was a modification of the method of Wiener et al. (1972) to obtain phenylthiohydantoin (PTH) amino acids. The lyophilized sample was dissolved in 0.4 ml 0.2 M NaHCO_3 (pH 9.8) in a conical glass tube and made 1% in SDS. The H-2 heavy chain sample usually contained 50,000 to 200,000 cpm radioactivity. Approximately 10,000 cpm MOPC21 light chain and 0.6 mg succinylated concanavalin A (Gunther et al. 1973) were added. The sample was heated at 50°C for 5 minutes under N_2 . On cooling, the sample was sonicated for 10 to 30 seconds and 25 - 50 μl phenylisothiocyanate added under N_2 . The sample was mixed well and incubated under N_2 for 30 minutes at 50°C . After cooling, the sample was extracted twice with 4.5 mls acetone and the residue dried in vacuo at 60°C . After cooling, 0.4 ml heptafluorobutyric acid was added under N_2 and the mixture incubated at 50°C for five minutes. (At this point samples which were to be sequenced automatically were put in the sequencer cup.) The tube was flushed with N_2 and dried at 60°C in vacuo. The residue was extracted twice with 0.5 ml butyl chloride containing 0.1% ethanethiol (Eastman). The extracts were pooled and set aside: these contained the thiazolinone amino acid from the first residue. The remainder of the sample was dried under N_2 and the procedure restarted.

For automatic sequence determination, samples were treated according to the manual procedure up to the point indicated above. The sample was put into the sequencer cup (Beckman model 890C sequencer) and the Quadrol double cleavage program (Beckman Instruments) was started at step 47.

The thiazolinone amino acids in the butyl chloride extracts from both automatic and manual procedures were dried under N_2 at 45°C . For conversion to the PTH amino acids, 0.2 ml 1 N HCl (containing 0.1% ethanethiol) was added under N_2 . The tube was stoppered tightly and incubated for ten minutes in an oil bath at 80°C . The samples were extracted twice with 0.7 ml ethyl acetate and the pooled organic phases and the aqueous phase dried under N_2 . The phases were then dissolved in 10 ml Aquasol (New England Nuclear) and counted directly for radioactivity.

Alternatively, the organic phase was analysed by thin layer chromatography (Summers et al. 1973). The dried organic phase was dissolved in 10-20 μl ethyl acetate and spotted with a mixture of

standard PTH-amino acids on 5 x 5 cm polyamide thin layer sheets (Schleicher and Schuell, Keene, N.H.). Appropriate spots were cut out and counted for radioactivity in Aquasol.

Sequencer reagents (heptafluorobutyric acid, butyl chloride, and phenylisothiocyanate) were obtained from Beckman Instruments (Palo Alto, Cal.). Glass distilled organic solvents were obtained from Burdick and Jackson (Muskegon, MI.).

TL Alloantiserum. An alloantiserum against TL antigens 1, 2 and 3 was produced by multiple injections of (C57BL/6 x A.Tla^b)F₁ mice (generously provided by Dr. E. A. Boyse, Sloan-Kettering Institute for Cancer Research, New York, N.Y.) with ASL1 leukemia cells (also provided by Dr. E. A. Boyse). The first injection of 10^6 ASL1 cells was given subcutaneously. Palpable tumours appeared at the site of injection 9-10 days later. The mice were given two intraperitoneal injections of 4 mg asparaginase six hours apart to prevent further growth of the tumour cells. Two further injections of 5×10^6 ASL1 cells were given subcutaneously ten and twenty days later. All subsequent injections were of $1-3 \times 10^7$ cells given intraperitoneally at ten day intervals. The mice were first bled after the sixth injection. The ASL1 tumour was maintained by passage in A/J mice.

Results

Relative Sizes of H-2 Heavy Chains and F_H Fragments. The model for the structure of the H-2 antigens (Fig. 18) is based on data obtained for one H-2 antigen, H-2K^b. Further studies indicated that all H-2 antigens have the same general structure. The experiments described in this section were designed to test for a possible association between the molecular weights of the H-2 heavy chains and the H-2K or H-2D gene coding for them.

Radiolabeled H-2K and H-2D antigens of haplotypes b, d and k were solubilized by NP40 and isolated by immunoprecipitation. The heavy chains were purified from the immunoprecipitates by SDS-PAGE in order to prevent distortion of the running behaviour of the heavy chain by the unlabeled immunoglobulin in the immunoprecipitate. The molecular weights of the H-2 heavy chains were then estimated by SDS-PAGE (Table X). For each haplotype, there is a small but consistent difference in the mobilities of the gene products from the two loci, corresponding to a difference of 2,000-3,000 daltons in the apparent molecular weights. Although the results are expressed as differences in molecular weights, other factors, such as carbohydrate content or the extent of SDS binding, could be responsible for small differences in mobility on the gels.

Because these differences in mobility are close to the limit of resolution of the gels, a different type of comparison was carried out. Products of the K and D loci for each of the three haplotypes were labeled with different isotopes (¹²⁵I or ¹³¹I) and isolated on polyacrylamide gels. The purified H-2K and H-2D gene products for each haplotype were then combined and the mixture run on polyacrylamide gels (Fig. 20). By this method, the differences indicated in Table X were verified.

These results confirm previous observations (Nathenson and Cullen (1974) and indicate consistent and distinct differences that appear to be characteristic of each gene product of the K and D loci. The products of alleles at one locus, however, are not consistently larger in apparent molecular weight than those at another. For example, D^b heavy chain is larger than K^b, but the K^d heavy chain is larger than D^d.

Table X

Molecular Weights of H-2 Heavy Chains
Solubilized by NP-40 and by Papain Digestion

<u>Haplotype</u>	<u>Locus</u>	<u>Molecular weight of heavy chains solubilized by</u>	
		<u>detergent</u>	<u>papain</u>
b	K	47,000	39,000
	D	50,000	43,000; 20,000
d	K	50,000	43,000; 20,000
	D	48,000	39,000
k	K	N.D.	39,000
	D	N.D.	N.D.

H-2 heavy chains were prepared by immunoprecipitation from detergent- or papain-treated extracts of ^{125}I -labelled spleen cells or thymocytes of C57BL/10 mice (H-2^b), DBA/2 and B10.D2 mice (H-2^d) and AKR and B10.BR mice (H-2^k). Detergent-solubilized heavy chains were prepurified on SDS-polyacrylamide gels. Molecular weights were estimated on SDS-polyacrylamide gels using internally ^{131}I -labelled-marker proteins. (N.D. = not determined).

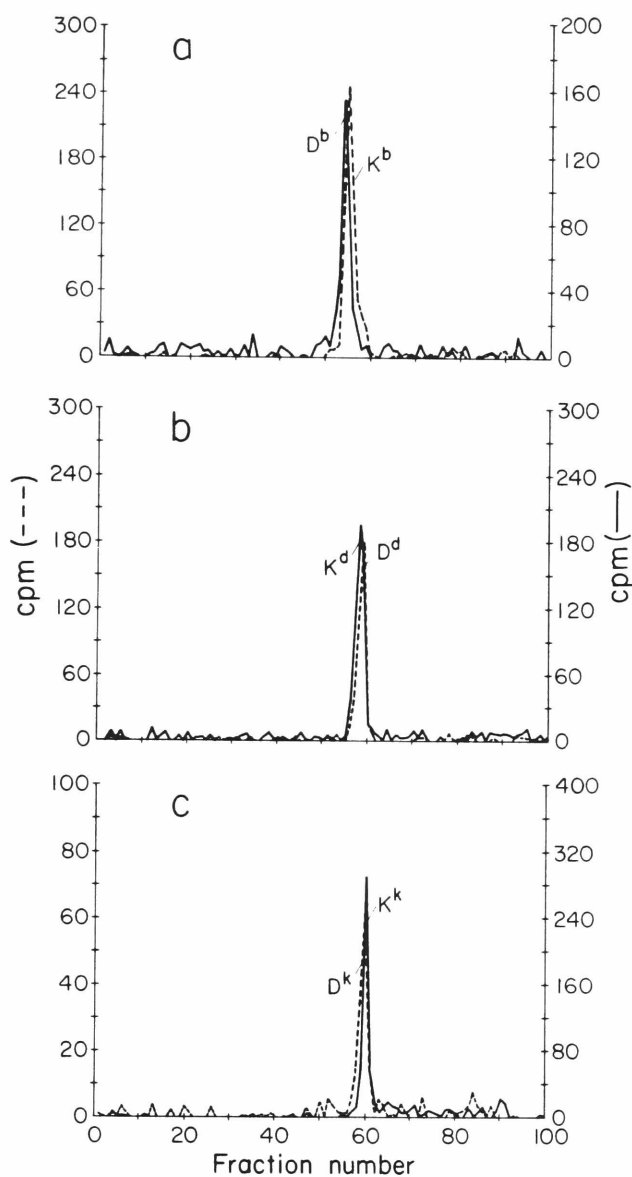


Fig. 20. Comparison of detergent-solubilized H-2 heavy chains of D and K loci on SDS-polyacrylamide gels. (a) ^{125}I -labelled D^b and ^{131}I -labelled K^b heavy chains; (b) ^{125}I -labelled K^d and ^{131}I -labelled D^d heavy chains; (c) ^{125}I -labelled K^k and ^{131}I -labelled D^k heavy chains. All gels were 8.5% polyacrylamide. (—) ^{125}I cpm; (---) ^{131}I cpm. Samples for these gels were pre-purified by elution of heavy-chain peak fractions from SDS-polyacrylamide gels of appropriate immunoprecipitates. Reproduced with permission from Cunningham et al. (1976a).

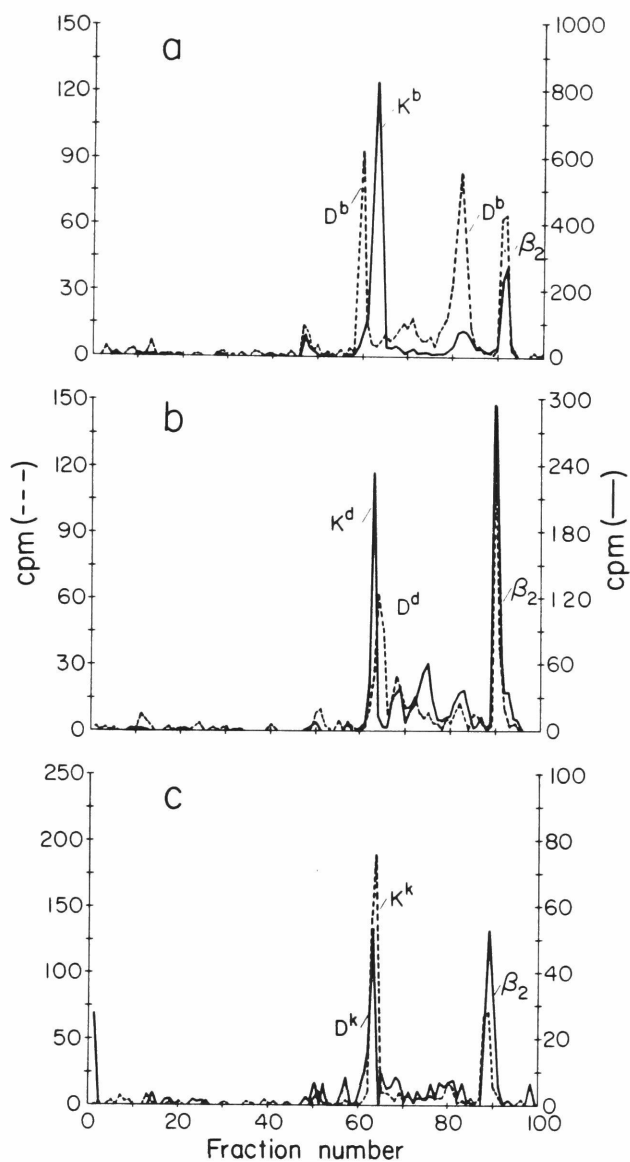


Fig. 21. Comparison of papain-solubilized H-2 heavy chains of D and K loci on SDS-polyacrylamide gels. (a) ^{125}I -labelled D^b and ^{131}I -labelled K^b heavy chains; (b) ^{125}I -labelled K^d and ^{131}I -labelled D^d heavy chains; (c) ^{125}I -labelled K^k and ^{131}I -labelled D^k heavy chains. (—) ^{125}I cpm; (---) ^{131}I cpm. Samples for these gels were immunoprecipitates made from papain digests of NP40 extracts of labelled cells. All gels were 8.5% polyacrylamide and were run in the presence of reducing agents. Reproduced with permission from Cunningham et al. (1976a).

Differences in fragmentation with papain have also been observed for H-2 heavy chains (Nathenson and Cullen 1974). Similar analyses were therefore performed for the fragments of H-2 heavy chains obtained by papain digestion of NP40 extracts (Table X and Fig. 21). Each H-2 heavy chain produces a large fragment (F_H) of molecular weight 39,000-43,000, some 7,000-9,000 daltons smaller than the intact, detergent-solubilized molecule. There is a striking parallel between the sizes of the F_H fragments from different gene products and the sizes of the intact molecules; that is, the F_H fragment of the D^b heavy chain is larger than that of K^b , but the F_H fragment of K^d is larger than that of D^d . Furthermore, the larger gene products in these two haplotypes also produce additional papain fragments of approximately 20,000 daltons (Fig. 21).

Comparison of H-2 Antigens by Two-Dimensional Gel Electrophoresis

The two-dimensional (2D) gel system of O'Farrell (1975) separates protein mixtures by isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension. This technique enables the majority of cellular proteins to be resolved on a single gel. The proteins are characterized by two parameters, their isoelectric point and molecular weight. Analysis of H-2 antigens by this technique, therefore, provides further information about the similarities and differences between different gene products.

Spleen cells from C57BL/10 mice were radioactively labeled by incubation for four hours with ^{35}S -methionine, extracted with 0.5% NP40 and analysed by 2D-electrophoresis. The autoradiograph of this gel (Fig. 22a) displays several hundred spots which probably represent a major fraction of the total cell proteins. The pH gradient in the IEF gels was determined as described by O'Farrell (1975) and found to be between pH 5.0 and pH 7.0 as indicated in Fig. 22. The most intense spot in this gel (indicated by an arrow in Fig. 22a) is probably actin (Jones 1977). In contrast, C57BL/10 spleen cells labeled with ^{125}I by the lactoperoxidase technique produce a quite different 2D gel pattern (Fig. 22b). The lactoperoxidase technique labels only cellular proteins that are exposed to the enzyme. The absence of any spot in this gel in a position corresponding to that of actin indicates that mainly

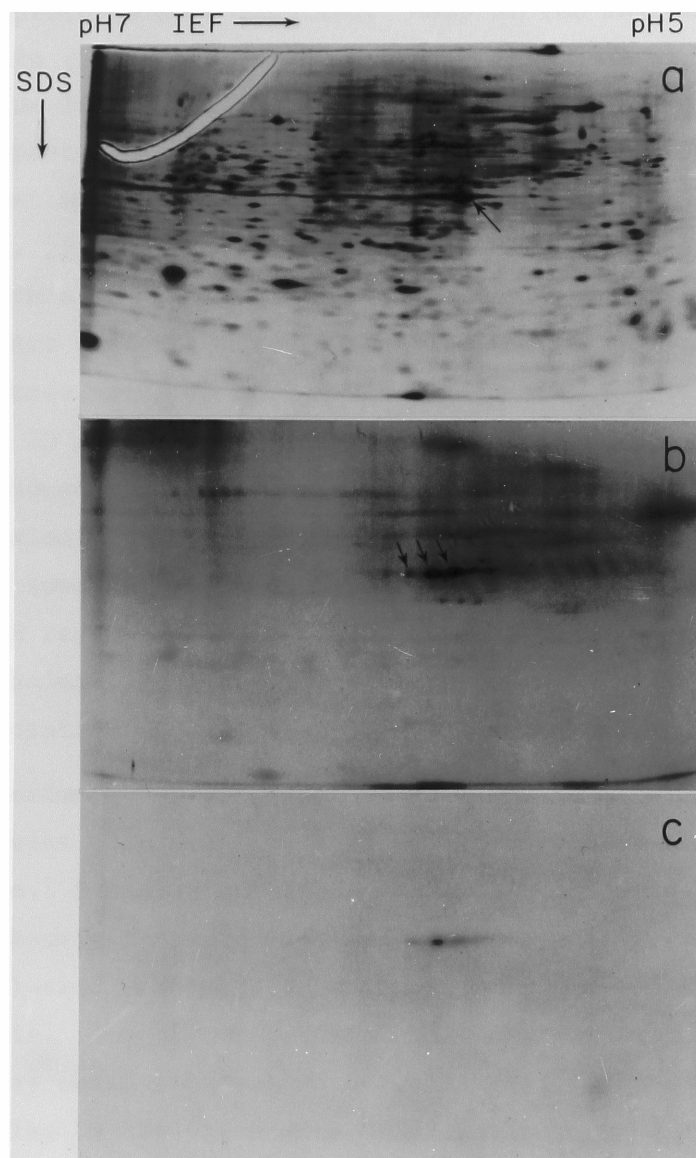


Fig. 22. Autoradiographs of 2D gels of spleen cells and H-2 antigens: (a) NP40 extract of C57BL/10 spleen cells labelled with ^{35}S -methionine, the arrow indicates the spot assumed to be actin; (b) NP40 extract of C57BL/10 spleen cells labelled with ^{125}I , the arrows indicate the positions of spots identified as H-2K^b heavy chains; (c) immunoprecipitate made from an aliquot of the extract shown in (b) using an anti-H-2K^b alloantiserum. The pH gradient of the IEF gels is as indicated at the top; the polyacrylamide concentration in the second dimension was 10%. The gels were exposed to film for 15 days (a,b) and 11 days (c).

cell surface proteins were labelled. Many of the components in this gel are streaked in the horizontal direction. This is probably not an artefact but is due instead to the high carbohydrate content of many cell surface glycoproteins. On the other hand, some components in this gel formed discrete spots. The most intense of these spots (indicated by arrows in Fig. 22b) were identified as the H-2K^b heavy chain by comparison with a 2D gel (Fig. 22c) of an immunoprecipitate made from the same extract using an anti-H-2K^b alloantiserum. Control immunoprecipitates made using an anti-H-2K^k alloantiserum gave no radioactive components on 2D gels. One-dimensional SDS-PAGE of the H-2K^b immunoprecipitate showed that it contained only components with molecular weights equivalent to H-2 heavy and light chains. The H-2 light chain was not seen in the 2D gel: this may have an isoelectric point outside the pH range of the IEF gel. These results indicate that H-2K^b heavy chains form a cluster of spots with slightly different isoelectric points.

An experiment was performed to test whether the differences in isoelectric point were due to heavy chains containing different amounts of sialic acid. Radioiodinated EL4 leukemia cells were incubated at 37°C for one hour with neuraminidase. Immunoprecipitates using anti-H-2K^b alloantiserum were made from extracts of these cells and from untreated cells. The 2D gel patterns of these extracts are shown in Fig. 23. The H-2K^b heavy chain from untreated cells (Fig. 23a) forms a cluster of spots similar to that seen with H-2K^b antigens from spleen cells (Fig. 22c). The smear of radioactivity to the left of the spots is an artefact and did not appear reproducibly. The H-2K^b antigens from neuraminidase-treated cells (Fig. 23b) produce spots with a higher isoelectric point than the untreated antigens. This result indicates that at least part of the microheterogeneity of the heavy chains is due to differences in their sialic acid content. The fact that the heavy chains were not reduced to a single spot is probably due to incomplete removal of all sialic acid residues. It is also possible, however, that part of this heterogeneity could be due to variations in the amino acid sequence of the heavy chain.

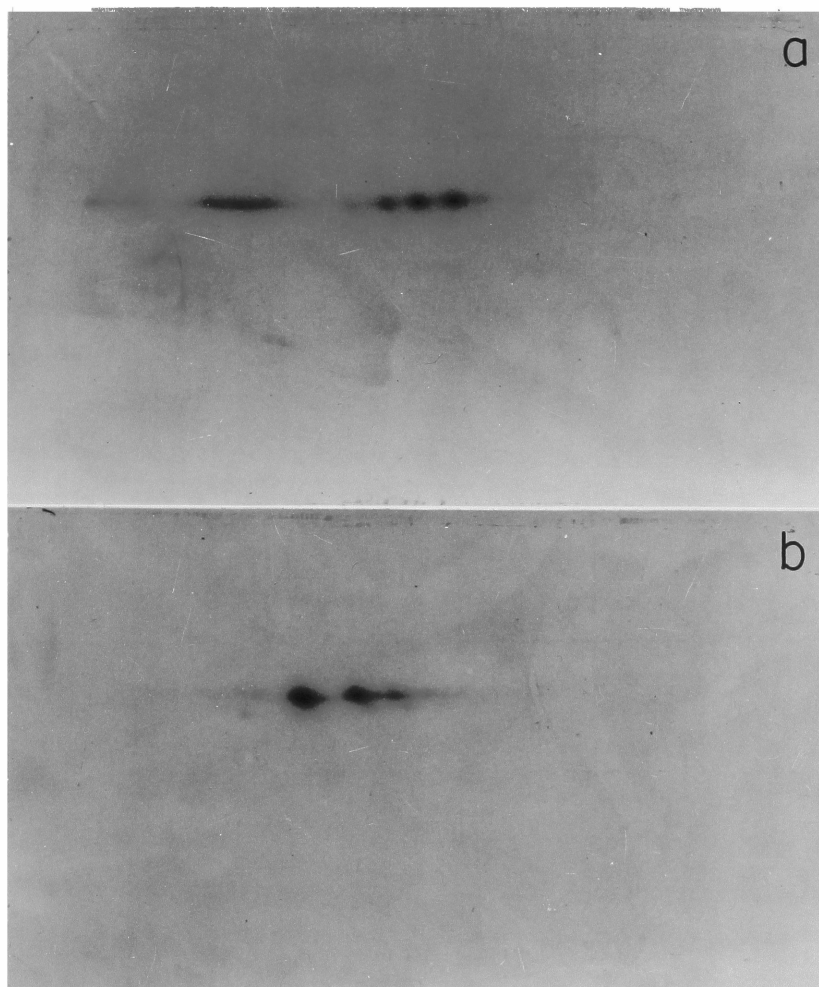


Fig. 23. Effect of neuraminidase treatment on the 2D gel patterns of H-2K^b heavy chains. H-2K^b antigens were immunoprecipitated from NP40 extracts of ¹²⁵I-labelled EL4 cells: (a) untreated EL4 cells; (b) neuraminidase treated EL4 cells. The polyacrylamide concentration in the second dimension was 10%. Gels were exposed to film for 20 days.

Despite the fact that immunoprecipitates of H-2 heavy chains gave a number of spots on 2D gel electrophoresis, each H-2 heavy chain gave characteristically different patterns. Spleen cells from B10.D2 and B10.A(4R) mice were labeled with ^{125}I and extracted with NP40. Immunoprecipitates of H-2K^d, D^d, K^k and D^b antigens were prepared from these extracts using specific alloantisera. The 2D gel patterns of these immunoprecipitates are compared with the patterns of the whole extracts in Figures 24 and 25. The heavy chain spots are identified in the gels of the NP40 extracts by arrows. Control immunoprecipitates from the NP40 extracts did not give any radioactive spots on 2D gels. Each H-2 heavy chain produces a cluster of spots having the same apparent molecular weight but different isoelectric points. The number of spots produced and their isoelectric points are characteristic for each heavy chain. For example, the K^k heavy chain produces a family of four or five spots with isoelectric points of approximately 6.0 to 6.5, whereas the D^b antigen produces two spots with an approximate isoelectric point of 5.8 (Fig. 24). The D_b antigen labels weakly with ^{125}I : two spots are visible in the gel of the immunoprecipitates (Fig. 24c) but only one spot can be identified in the gel of the extract (Fig. 24a). The K^d and D^d heavy chains have similar isoelectric points but slightly different molecular weights (Fig. 25a). The K^d heavy chain has a higher apparent molecular weight than D^d, as was shown in Fig. 20. The K^d immunoprecipitate also produced an intense spot with a lower molecular weight (Fig. 25b). This is probably an Ia antigen coded by a gene in the I region and precipitated by anti-Ia antibodies in the anti-K^d alloantiserum. Similar patterns to those described here have been reported for the H-2K^d, D^d and K^k antigens (Jones 1977).

The finding that each heavy chain produces a cluster of spots with slightly different isoelectric points is probably due to variations in sialic acid content between different molecules of the same H-2 heavy chain, as was shown for the H-2K^b heavy chain. The differences in the patterns of spots obtained for heavy chains of different H-2 type, however, are probably a reflection of the polymorphism of the H-2 antigens. The differences between the patterns produced by different H-2 heavy chains may also be due in part to variations in sialic acid

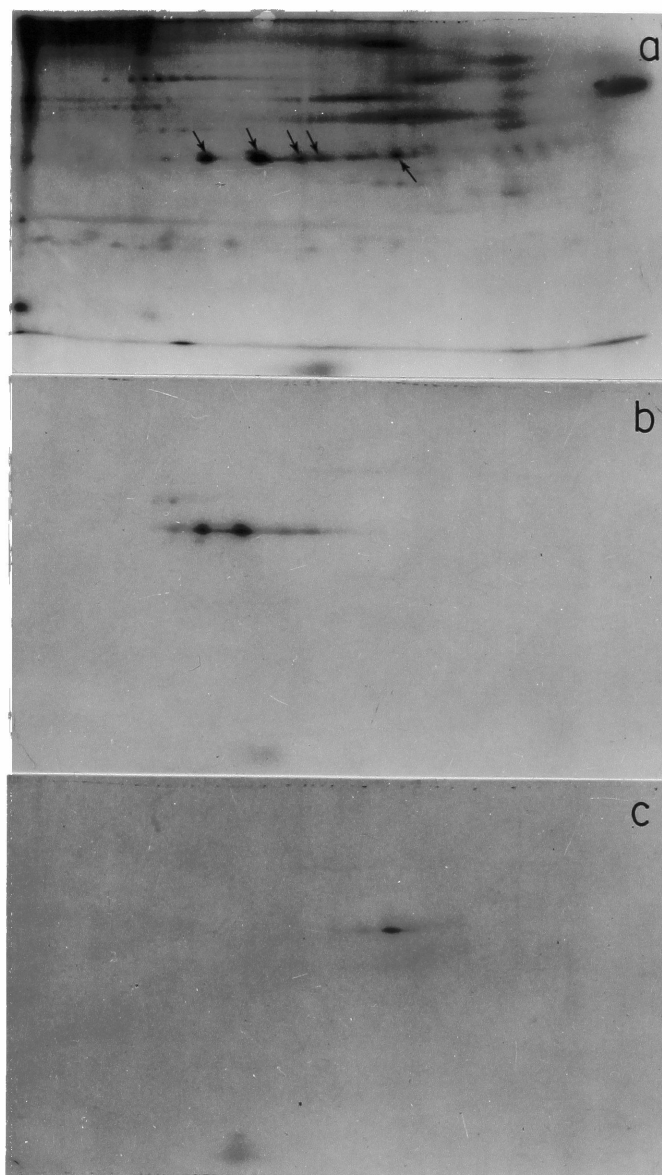


Fig. 24. Autoradiographs of 2D gels of H-2K^k and H-2D^b antigens: (a) NP40 extract of ¹²⁵I-labelled B10.A(4R) spleen cells, the downward pointing arrows indicate the positions of spots identified as H-2K^k heavy chains, the upward pointing arrow indicates the positions of a spot identified as H-2D^b; (b) immunoprecipitate of H-2K^k antigen prepared from B10.A(4R) extract; (c) immunoprecipitate of H-2D^b antigen from B10.A(4R) extract. The polyacrylamide concentration in the second dimension was 10%. Gels were exposed to film for 15 days (a) and 11 days (b,c).

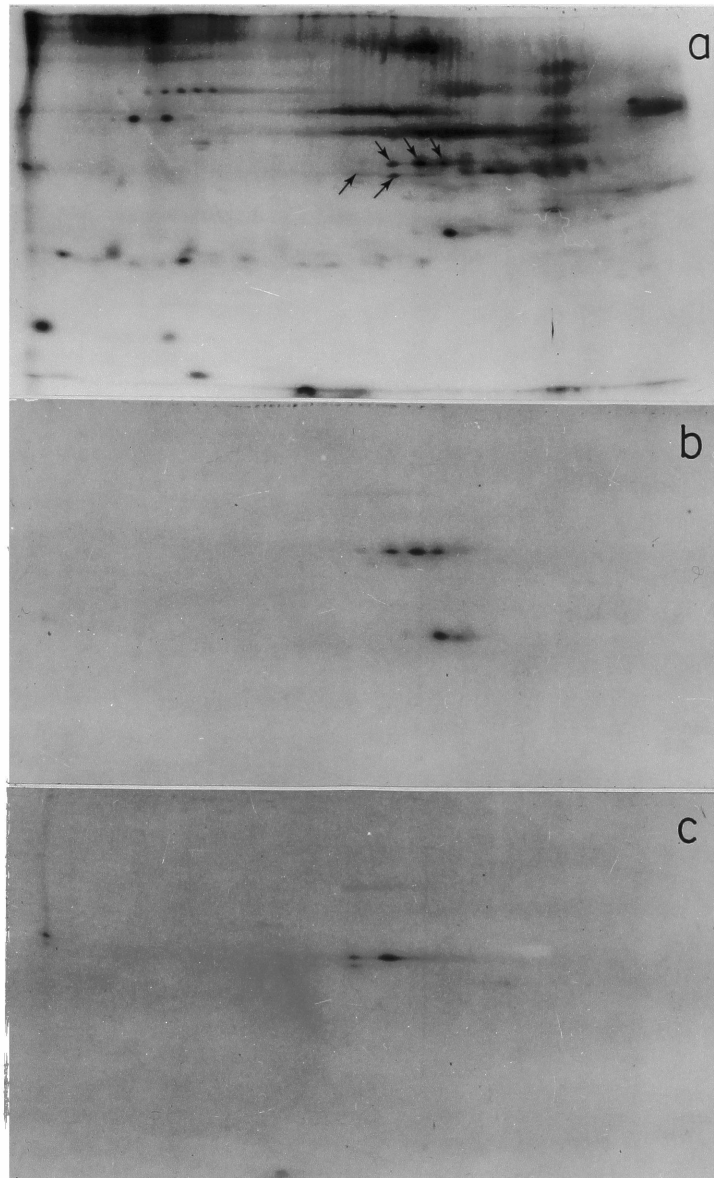


Fig. 25. Autoradiographs of 2D gels of H-2K^d and H-2D^d antigens: (a) NP40 extract of ¹²⁵I-labelled B10.D2 spleen cells, the downward pointing arrows indicate the positions of spots identified as H-2K^d heavy chains, the upward pointing arrows indicate the positions of spots identified as H-2D^d heavy chains; (b) immunoprecipitate of H-2K^d antigen prepared from B10.D2 extract; (c) immunoprecipitate of H-2D^d antigen from B10.D2 extract. The polyacrylamide concentration in the second dimension was 10%. Gels were exposed to film for 15 days (a) and 11 days (b,c).

content. For example, the pattern produced by H-2K^k heavy chains (Fig. 24b) is similar to that obtained for H-2K^b heavy chains after neuraminidase treatment (Fig. 23b), suggesting that the H-2K^k heavy chain has a lower sialic acid content.

The H-2 heavy chains are identified on 2D gels by virtue of their molecular weights and reactivities with specific anti-H-2 antisera. Further information concerning the identity of these molecules can be obtained by comparison of 2D gels of NP40 extracts from spleen cells of mice possessing different H-2 genotypes. Spleen cells from mice of the strains B10.BR, B10.A(2R), B10.A and B10.A(5R) were radioiodinated, extracted with NP40 and analyzed by 2D electrophoresis. The patterns obtained are shown in Fig. 26. These strains are congenic resistant mice which differ in H-2 genotype but have the same C57BL/10 genetic background. The alleles carried by each strain for regions of the H-2 complex are shown in Fig. 26. All of the patterns obtained are remarkably similar but there are differences in the positions of some spots.

The strains B10.BR and B10.A(2R) differ in genes in the C, S, G and D regions. 2D gels from these strains show two major differences. A spot with the molecular weight of H-2 heavy chain in the gel of B10.BR (indicated by an arrow in Fig. 26a) is absent from the gel of B10.A(2R). In this gel, however, there is a spot with a similar molecular weight (indicated by the upper arrow in Fig. 26b) that is absent from the gel of B10.BR. These spots are probably H-2D^k and H-2D^b heavy chains. In addition, there is a spot in the gel of B10.A(2R) cells (indicated by the lower arrow in Fig. 26b) that is absent from B10.BR. This could be the product of a gene in any of the C, S, G or D regions, possibly Ia antigens.

The strains B10.A and B10.A(5R) differ in genes in the K, A and B regions. The 2D gels from these strains also show prominent differences. The most intense group of spots in each gel (indicated by downward pointing arrows in Fig. 26c, d) have similar molecular weights but different isoelectric points. These spots can be identified as H-2K^k and H-2K^b heavy chains by comparison with the patterns of immuno-

<u>Strain</u>	<u>K</u>	<u>A</u>	<u>B</u>	<u>J</u>	<u>E</u>	<u>C</u>	<u>S</u>	<u>G</u>	<u>D</u>
a. B10.BR	k	k	k	k	k	k	k	k	k
b. B10.A(2R)	k	k	k	k	k	d	d	d	b
c. B10.A	k	k	k	k	k	d	d	d	d
d. B10.A(5R)	b	b	b	k	k	d	d	d	d

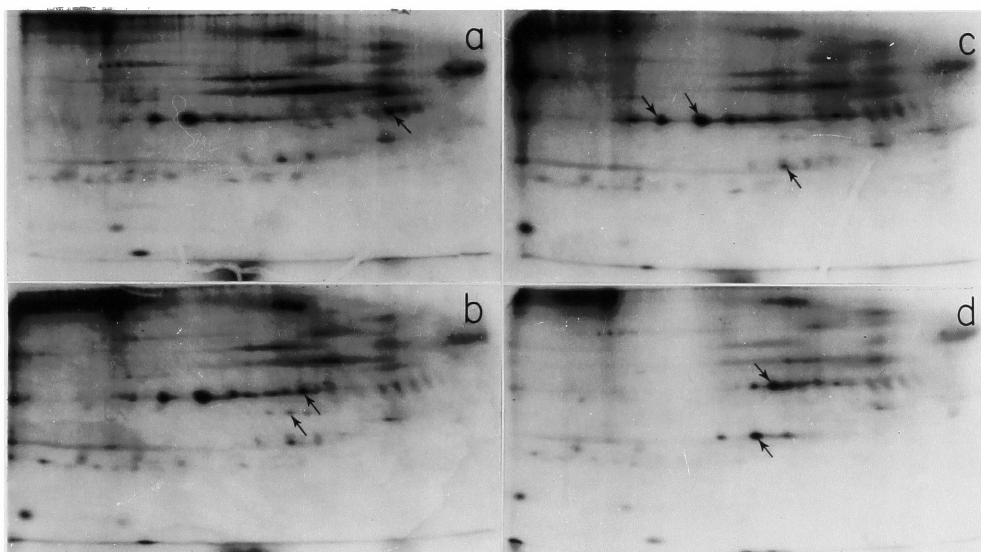


Fig. 26. Comparison of 2D gel patterns of different mouse strains. The samples were NP40 extracts of radioiodinated spleen cells from female mice of the following strains: (a) B10.BR; (b) B10.A(2R); (c) B10.A; (d) B10.A(5R). The alleles carried by each strain in regions of the H-2 gene complex are indicated at the top. The arrows indicate differences between the gel patterns as discussed in the text. The polyacrylamide concentration in the second dimension was 10%. Gels were exposed to film for 15 days.

precipitated heavy chains (Figs. 22b and 24b). There are also differences in spots with a lower apparent molecular weight (indicated by upward pointing arrows in Fig. 26c,d). These spots could be the products of genes in the K, A or B regions, probably Ia antigens coded by A region genes.

The comparisons of 2D gels of cells from different mouse strains support the identification of the spots due to H-2 antigens. This experiment also allows the tentative identification of spots due to other products of genes in the H-2 region. This technique may be particularly useful in the identification of H-2 gene products that are not detected by antisera. This approach is limited, however, by the possibility that not all components may be resolved on 2D gels. For example, components with isoelectric points outside the range of the IEF gels or components which are not labelled by ^{125}I or which are not solubilized by NP40 will not be visualized in this gel system. Thus, components of some Ia antigens are too basic or acidic to be resolved by 2D electrophoresis (Jones 1977). The 25,000 dalton component of Ia antigens also labels poorly with ^{125}I (Schwartz et al. 1978).

Attempts have also been made to analyze thymus leukemia (TL) antigens by 2D electrophoresis. These molecules are structurally similar to H-2 antigens and are also associated with β_2 -microglobulin (Vitetta et al. 1976b). Immunoprecipitates were made from NP40 extracts of radioiodinated B10.A thymocytes, which express the antigens Tla.1, 2 and 3, and radioiodinated B10.D2 thymocytes, which express the antigen Tla.2, using an antiserum against Tla.1,2 and 3 antigens. Electrophoresis of aliquots of these immunoprecipitates on one-dimensional SDS gels showed that they contained radioactive components with approximate molecular weights of 45,000 and 12,000 that were absent from control immunoprecipitates. The 2D gels of the TL antigen immunoprecipitates, however, showed no radioactive components. This suggests that TL antigens have an isoelectric point outside the range of the IEF gels or that they are not solubilized in the NP40-8M urea IEF sample buffer.

Amino Acid Sequence Analysis of H-2 Heavy Chains. The available evidence indicates that the polymorphism of the H-2 antigens is restricted to the H-2 heavy chain. The key questions regarding the polymorphism and evolution of the histocompatibility antigens can therefore only be resolved by analysis of the amino acid sequences of the heavy chains. For a variety of reasons, the amino acid sequences of H-2 heavy chains cannot be readily determined by conventional techniques. The approach to this problem has been to use new radiochemical sequencing techniques (Jacobs et al. 1974) coupled with the use of specific immunoprecipitation.

Mouse leukemia cells (EL4, P388) were labeled by incubation with one ^3H or ^{35}S -amino acid for four hours. The cells were extracted with NP40, and the radioactively labeled H-2 antigens isolated by immunoprecipitation and preparative gel electrophoresis. After removal of SDS, the heavy chains were sequenced in the automatic sequencer. As an internal standard, MOPC21 light chain (Svasti and Milstein 1972), labeled in a single amino acid but with a different isotope, was sequenced simultaneously with the H-2 heavy chain. The thiazolinone amino acids obtained from the sequencer were converted to the phenylthiohydantoin (PTH) amino acids. These were either counted directly for radioactivity or the residue was identified by thin layer chromatography.

The data obtained for H-2K^b heavy chains are shown in Fig. 27. A peak of radioactivity indicates a labeled residue at that position in the polypeptide chain. The detergent-solubilized H-2K^b heavy chain, therefore, has a histidine residue at position 3 (Fig. 27a) and a tyrosine residue at position 7 (Fig. 27b). The same amino acids were found in these positions for the H-2K^b heavy chain prepared by papain treatment (Fig. 27a and b). As described previously, this result indicates that the papain and detergent-solubilized heavy chains have the same amino terminus. The ^{35}S -methionine labeled MOPC21 light chain sequenced simultaneously with the H-2K^b heavy chain produced a peak of radioactivity in residue 4 (Fig. 27a and b) as expected from the known sequence of this protein (Table IX). In this experiment, the identities of the PTH-amino acids were confirmed by analysis by thin layer chromatography. The same results were also obtained by manual sequencing methods. Although the papain fragment of the heavy chain

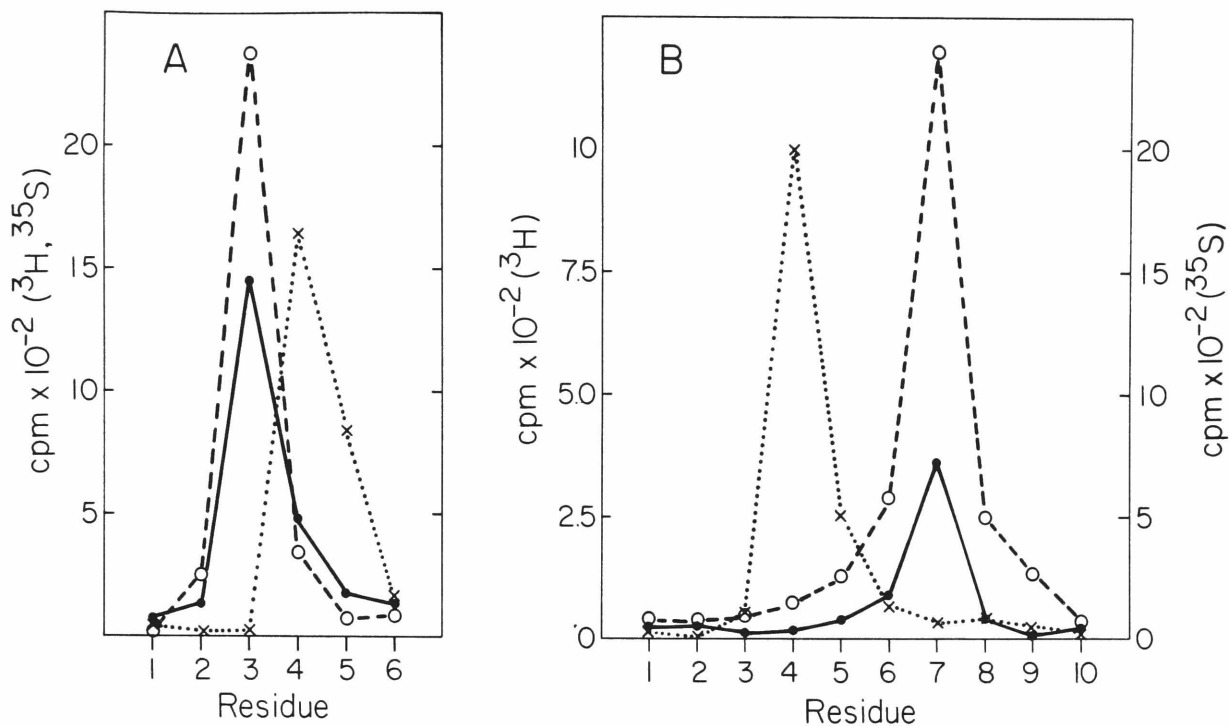


Fig. 27. Amino acid sequence analysis of H-2K^b heavy chains solubilized by detergent and by papain. H-2K^b heavy chains labelled with (A) ^3H -histidine or (B) ^3H -tyrosine were sequenced automatically in the presence of ^{35}S -methionine labelled MOPC21 light chain. The radioactivity detected in the PTH-amino acid is plotted for each residue: o---o, detergent solubilized H-2K^b heavy chain; ●—●, papain solubilized H-2 heavy chain; x.....x, ^{35}S Met-MOPC21 light chain.

was isolated with a lower yield, it routinely gave better efficiencies in sequencing. This increased efficiency is probably due to the better solubility of the papain fragment in aqueous solution. This factor could be particularly important in later steps of the sequencer run when most of the SDS would be expected to have been removed from the sample.

Further data for detergent-solubilized heavy chains are shown in Fig. 28. In addition to the tyrosine residue at position 7, H-2K^b heavy chain has tyrosine at positions 22 and 27. The ³⁵S-methionine labeled MOPC21 light chain has methionine residues at portions 4, 11 and 13 as expected from the known sequence (Table IX). The H-2K^b heavy chain also has leucine residues at positions 5 and 17, and a phenylalanine residue at position 8. In contrast, the H-2D^d heavy chain has phenylalanine residues at positions 8 and 17. These results therefore indicate that there is a positive difference between the sequences of the H-2K^b and H-2D^d heavy chains at position 17.

Amino acids are assigned to particular positions if there is a significant increase in the radioactivity of the PTH-amino acid at that position. In some cases, this may be confused by two phenomena: "preview," an increase in radioactivity in the residue before the peak, and "carry-over," a significant amount of radioactivity in the residue immediately after the peak. Both of these features can be seen particularly in the data for ³H-phenylalanine labeled H-2D^d heavy chains (bottom panel, Fig. 28). These phenomena are due to molecules being sequenced out of phase and probably occur because the sample is not completely soluble. Another feature of the data is that the background radioactivity in each residue tends to increase at higher residue numbers. This tends to obscure the smaller peaks. These artifacts vary from sample to sample. All of the assignments reported here appeared reproducibly in sequence determinations of several different sample preparations.

Several factors may generate artifacts in the sequence results. Detergent extracts labeled with ¹²⁵I or internally with ³H- or ³⁵S-

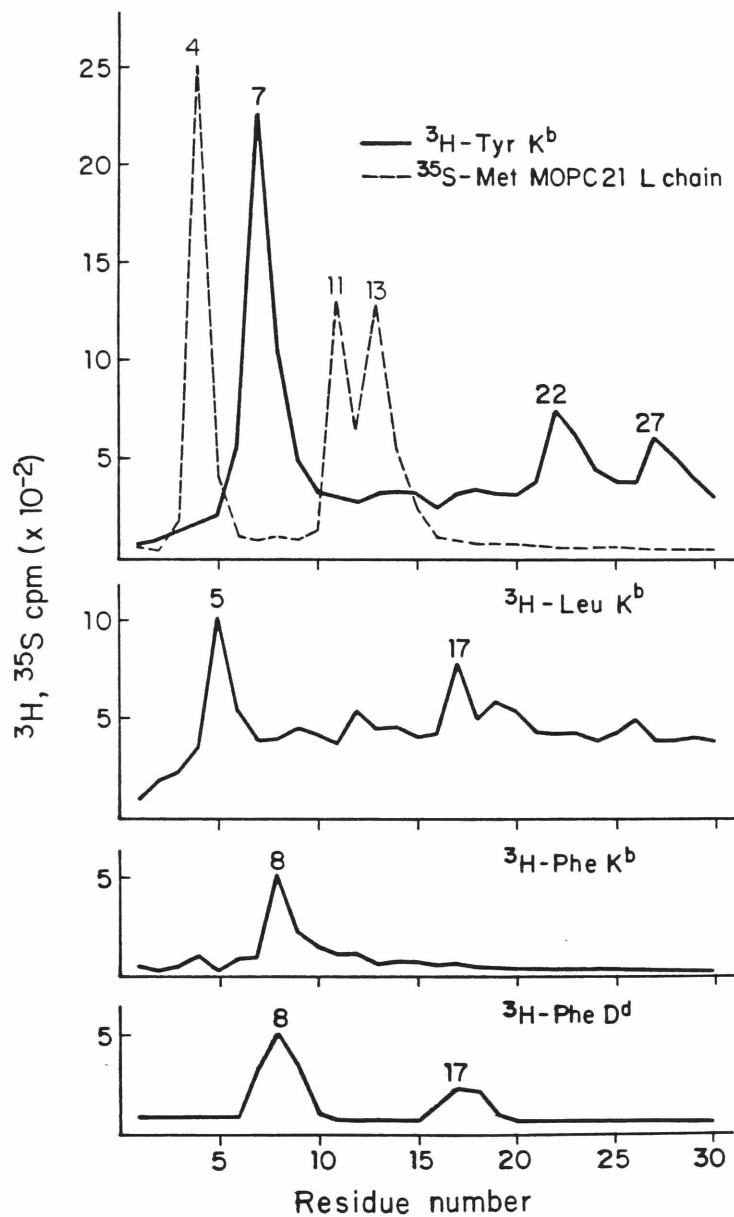


Fig. 28. Amino acid sequence analysis of detergent-solubilized H-2 heavy chains. The radioactivity detected in the PTH-amino acid is plotted for each residue. The numbers over the peaks indicate the positions of assigned residues.

amino acids contain a labelled component that migrates on SDS-polyacrylamide gels in the region of H-2 heavy chains (Fig. 29a). This material also appears in immunoprecipitates but is generally removed by the careful and extensive washing of these precipitates routinely carried out before solubilization. This material, however, could contribute to some sequence variations. This material has been labelled with ^3H -tyrosine, isolated by SDS-PAGE and sequenced in the automatic sequencer. The results indicate that the material may contain some H-2 antigens, but the material does not account for the sequence results obtained for H-2 heavy chains.

A second potential problem arises from viral components on some of the tumour cells used in sequence studies. Immunoprecipitates of extracts of EL4 cells with antibodies to the viral protein gp70 (Fig. 29b) give the expected component in the region of 70,000 daltons but they also give a component of 45,000 daltons (gp45). The H-2 alloantisera used in the sequence studies also contain antibodies against gp70, and therefore the putative H-2 heavy chains isolated with these antisera may contain some gp45. Some of the antisera do not detect viral components, and yet the same sequences for H-2 antigens are obtained, suggesting that such contamination does not significantly affect the results.

These potential difficulties suggest that detailed genetic interpretations derived from the present data must be viewed with some caution. Nevertheless, the fact that different gene products from the same cell show differences in sequence (Table XI), the identity of the amino-terminal sequences of the detergent- and papain-solubilized heavy chains (Fig. 27), the striking similarities between the sequences of H-2 antigens determined in different laboratories (Table XII) and the similarities between the sequences of H-2 and HLA antigens (Table XII) indicate that the general and most important conclusions from the sequence data are valid.

The data obtained for 5 different H-2 gene products are summarized in Table XI. Some of the residues shown are tentative assignments usually because the residue is difficult to detect. For example,

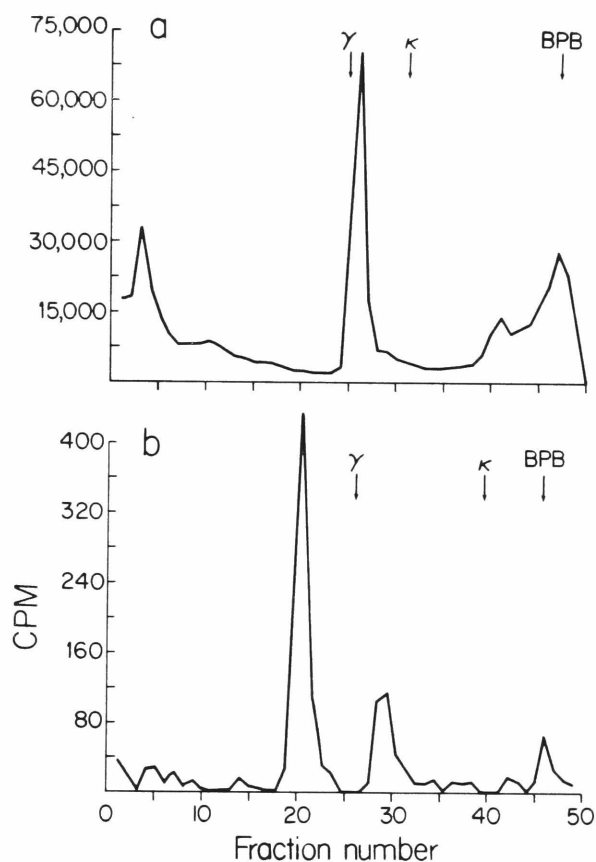


Fig. 29. SDS-polyacrylamide gels illustrating components other than H-2 heavy chains that may be present in immunoprecipitates. (a) NP40 extract of ^3H -tyrosine-labelled EL4 cells on 10% polyacrylamide gel; (b) Immunoprecipitate made from an NP40 extract of ^{125}I -labelled EL4 cells using a goat antiserum against Moloney virus gp70 (8.5% gel). The gel shows gp70 and gp45 components. Both gels were run in the presence of 2-mercaptoethanol. Molecular weight markers are indicated: γ , MOPC21 heavy chain (MW 50,000); κ , MOPC21 light chain (MW 23,000). BPB indicates the position of bromophenol blue dye marker.

the threonine residues at positions 10 and 13 are tentative assignments. The cells do not incorporate ^3H -threonine as readily as ^3H -tyrosine and the data are not as convincing as those for the tyrosine at position 7. Nevertheless small amounts of radioactivity do appear reproducibly at these positions in ^3H -threonine labelled heavy chains. Some support for the assignment at position 10 is provided by the fact that position 10 in some HLA antigens is also a threonine (Terhorst et al. 1976, Bridgen et al. 1976). In the same way, the proline residues at positions 15 and 20 of H-2K^{d} and position 20 of H-2K^{b} , the phenylalanine at position 22 of H-2K^{d} , and the leucine at position 26 of H-2K^{b} are also tentative assignments.

The most striking conclusion from these results is that there is considerable similarity between the sequences of different H-2 heavy chains (Table XI). The residues at positions 3, 6, 7, and 27 appear to be the most highly conserved. This applies both to the products of alleles at one locus, for example, K^{b} and K^{d} , and to the products of the K and D loci of the same haplotype, for example, K^{d} and D^{d} . These observations strongly support the hypothesis (Klein and Shreffler 1971) that the K and D loci arose by gene duplication.

There are also differences between the sequences of different H-2 heavy chains. Most important are the two positive differences at position 17 in D^{d} and at position 22 in K^{d} . Although the phenylalanine at position 22 is tentative, it has been observed in HLA antigens (Terhorst et al. 1976, Bridgen et al. 1976). Other positive differences are observed on comparison with the K^{k} sequences of Vitetta et al. (1976a). In addition, there are several negative differences, that is, the absence in some gene products of residues that are present in others. The absence of methionine at position 1 in K^{b} , and proline, valine, and valine at positions 2, 9 and 12, respectively, in K^{d} are examples of these negative differences.

In some of these positions, however, a small amount of the expected residue is frequently seen. For example, a very weak proline has been seen at position 2 in D^{d} and a weak phenylalanine at position 8 in K^{d} . In some cases, such as the phenylalanine at position 8 in K^{d} , this could be due to contamination by the H-2D gene product. In the H-2K^{d} heavy chain, two residues, leucine and valine, were detected

Table XI

Amino Acid Sequences of H-2 Heavy Chains

	1				5					10				15
H-2K ^b	NH ₂	X	Pro	His	-	Leu	Arg	Tyr	Phe	Val(Thr)	-	Val	-	Arg Pro
K ^d	NH ₂	Met	X	His	-	(<u>Leu</u> Val)	Arg	Tyr	(X)	X (Thr)	-	X	-	Arg(Pro)
D ^d	NH ₂	Met	(X)	His	-	Leu	Arg	Tyr	Phe	Val(Thr)	-	Val(Thr)	Arg	-
D ^b	NH ₂	-	-	-	-	-	(Arg)	Tyr	-	-	-	-	-	-
K ^k	NH ₂	-	-	His	-	-	Arg	Tyr	-	-	-	-	-	-
	16					20				25				
H-2K ^b	-	Leu	-	-	(Pro)	Arg	Tyr	-	-	-	(Leu)	Tyr		
K ^d	-	(<u>Leu</u> Val)	-	-	(Pro)	Arg	(Phe)	-	-	-	-	Tyr		
D ^d	-	Phe	-	-	-	Arg	Tyr	-	-	-	-	Tyr		
D ^b	-	-	-	-	-	-	Tyr	-	-	-	-	Tyr		

(-) Denotes the absence of all other amino acids listed for this gene product; (X) denotes the absence of residues found in other heavy chains at this position. Residues in parentheses are preliminary results.

weakly but reproducibly at positions 5 and 17. While the presence of the leucine residue could reflect contamination by the H-2D^d heavy chain, this would not apply to the valine residue. This result may reflect heterogeneity of the K^d heavy chain similar to that seen in the two-dimensional gel patterns.

All of these observations indicate that there are clear differences between the products of the various alleles of the K and D loci. These differences undoubtedly reflect the genetic polymorphism but the data are too limited at present to relate any differences in sequence to particular antigenic determinants. In no case, however, is there a feature that allows these proteins to be identified as the products of the K or the D loci. In fact some K and D gene products appear to resemble each other more closely than products of alleles at the same locus. For example, K^b and D^d heavy chains have valine residues at positions 9 and 12 but these are absent from the K^d heavy chain.

The heavy chains of H-2 antigens have been sequenced by the same technique in other laboratories (Silver and Hood 1976, Ewenstein et al. 1976, Capra et al. 1976, Vitetta et al. 1976b). Essentially similar results were obtained to those described here. The general conclusions from these sequences and from comparisons of the sequences of the H-2 heavy chains with the heavy chains of other species will be discussed in the next section.

Extensive comparisons have been made between these sequences and those of immunoglobulin light and heavy chains. No convincing homology was found. Furthermore, no half-cystinyl residues, characteristic of immunoglobulin sequences were found in the first 30 residues of H-2 antigens. Nevertheless, the possibility of homology in other portions of the H-2 molecules with some regions of the immunoglobulins can by no means be excluded. For example, a modest homology has been reported between the sequence of an internal fragment of HLA heavy chains and the sequences of immunoglobulin variable regions (Strominger et al. 1978).

In addition, attempts were made to sequence the 45,000 dalton heavy chain of TL antigens by the radiochemical technique. Labelled

TL antigen was isolated by immunoprecipitation with an anti-TL 1,2,3 antiserum from extracts of ASL1 leukemia cells or from thymocytes. Eight preparations of TL antigens were tested, each labelled with a different amino acid. In no case was a residue convincingly seen at any position. This observation suggests that the amino-terminal residue of the TL heavy chain may be blocked or that the solubility properties of the TL heavy chain are sufficiently different from those of the H-2 heavy chains to prevent sequence analysis of these molecules using the same procedure.

Discussion: The Polymorphism of the H-2 Antigens

The genetics and serology of the H-2 system suggest that different H-2 gene products may have detailed features that are unique for each antigen while sharing a common overall structure. The experiments described in this section were designed to test for possible similarities and differences in the structures of the heavy chain products of several H-2K and H-2D genes. The main conclusions of these experiments can be summarized as follows:

1. Different H-2 antigens show similarities in the apparent molecular weight of the detergent solubilized heavy chain and in the site of papain cleavage of the heavy chain. The heavy chains also show homologies in their amino terminal amino acid sequences.
2. Differences in the H-2 antigens are revealed by detailed comparisons of the molecular weights of the detergent- and papain-solubilized heavy chains, differences in the isoelectric points of the heavy chains, and variations in the amino-terminal amino acid sequences of the heavy chains.

These conclusions strongly support the hypothesis of Klein and Shreffler (1971) that the H-2D and H-2K loci evolved by gene duplication. At the same time, these results indicate that the polymorphism of the H-2 system is reflected by differences in the structure of the heavy chains.

More detailed conclusions concerning the nature of the polymorphism and its genetic implications can be derived from comparisons of all the known sequences of the H-2 heavy chains with the sequences for the heavy chains of the major histocompatibility antigens of man and of the guinea pig (Table XII).

All of the H-2 heavy chain sequences determined independently in different laboratories (Cunningham et al. 1976b, Silver and Hood 1976, Vitetta et al. 1976b, Capra et al. 1976) are consistent with each other. The combined sequence data provides further support for the main conclusions reached on the basis of the data presented in this thesis. Thus, there is considerable homology in sequence between products of

Table XII

Amino Acid Sequences of Histocompatibility Antigen
Heavy Chains from Different Species

		1	5	10	
Mouse	K ^b	NH ₂	X Pro His —	Leu Arg Tyr Phe Val(Thr)Ala Val —	Arg
H-2	K ^d	NH ₂	Met X His —	X Arg Tyr X X (Thr) —	X — Arg
	K ^k	NH ₂	Met Pro His —	Leu Arg Tyr Phe <u>His</u> —	Ala Val — <u>Ile</u>
	D ^b	NH ₂	— Pro — —	— Arg Tyr — —	— Ala Val — Arg
	D ^d	NH ₂	Met X His —	Leu Arg Tyr Phe Val(Thr)Ala Val(Thr)	Arg
Human	A2	NH ₂	Gly Ser — Ser	Met Arg Tyr Phe Phe Thr Ser Val	Ser Arg
HLA	A1,2	NH ₂	— Ser — Ser	Met Arg Tyr Phe Phe Thr Ser Val	<u>Ala</u> Arg
	B7	NH ₂	Gly Ser His Ser	Met Arg Tyr Phe <u>Tyr</u> Thr Ser Val	Ser Arg
	B8,13	NH ₂	— Ser — Ser	Met Arg Tyr <u>Tyr</u> <u>Tyr</u> <u>Ser</u> <u>Ala</u>	Val Ser Arg
Guinea Pig B.1		NH ₂	— — His —	Leu Arg Tyr Phe Tyr —	Ala Val — —
GPLA					
		15	20	25	
Mouse	K ^b	Pro — Leu — —	(Pro)Arg Tyr Met — —	(Leu)Tyr	
H-2	K ^d	Pro — X — —	(Pro)Arg(<u>Phe</u>) — — —	Tyr	
	K ^k	Pro — Leu — Lys	Pro <u>Phe</u> <u>Ala</u> Met — — —	Tyr	
	D ^b	Pro — Leu — —	Pro Arg Tyr — — —	Tyr	
	D ^d	Pro — <u>Phe</u> — —	Pro Arg Tyr — — —	Tyr	
Human	A2	Pro Gly — Gly Glu — —	Phe Ile Ala Val — —		
HLA	A1,2	Pro Gly — — — —	— — — — —		
	B7	Pro Gly Arg Gly Glu	Pro Phe Phe Ile Ala Val Gly Tyr		
	B8,13	Pro Gly — — — —	— — — — —		
Guinea Pig B.1		Pro — — — — —	Phe Val — — —	Tyr	
GPLA					

The data for H-2 antigens are from Silver and Hood (1976), Ewenstein et al. (1976), Vitetta et al. (1976b), Capra et al. (1976) and Table XI of this thesis. The data for the HLA antigens are from Terhorst et al. (1976), Bridgen et al. (1976) and Strominger et al. (1978). The data for guinea pig GPLA antigens is from Schwartz et al. (1976). Residues which differ within a species are underlined. Residues in parentheses are preliminary results.

the H-2K locus and products of the H-2D locus, indicating the common evolutionary origin of these genes. There are also differences in the sequences: particularly prominent are the positive differences at positions 9, 14, 21 and 22 in the H-2K^k heavy chain reported by Vitetta et al.(1976a). In addition, there are a number of negative differences.

If the sequences of the H-2 heavy chains are compared in detail two further conclusions emerge. Firstly, the sequence of each heavy chain differs in multiple residues from the sequences of each other heavy chain. This applies even to the products of alleles at the same locus. For example, the sequences of K^b and K^d heavy chains differ in eight out of the 17 residues that can be compared. This is not a surprising finding, however, considering that the H-2K^b and K^d antigens differ in eight public antigenic determinants (Table II). It indicates that there has been a considerable evolutionary separation between the K^b and K^d genes. The two H-2D gene products differ in four out of the 14 residues that can be compared. Interestingly, differences between the H-2K^b and K^d antigens are also found at these positions (2, 5, 9 and 17), suggesting that they may be particularly variable residues. From this data, H-2 antigens can be classified as "complex allotypes" in that they are allelic gene products that differ by multiple amino acid substitutions (Gutman et al. 1975). This is in contrast to "simple allotypes" which differ by only one or a few amino acid substitutions.

The second conclusion from these sequences is that the H-2D gene products appear no more closely related to each other than to the H-2K gene products. In fact, some H-2K gene products appear to resemble H-2D gene products more closely than the products of other alleles of the H-2K locus. For example, the sequences of the K^b and D^b heavy chains differ only at positions 5 and 9. Furthermore, there are no features that distinguish products of the H-2K locus from products of the H-2D locus. This feature of the H-2 sequences has been termed a lack of "Kness" or "Dness" (Silver and Hood 1976) and may suggest an unusual genetic or evolutionary relationship between the H-2K and H-2D genes.

The A and B genes of the HLA system of man are believed to be homologous with the H-2K and H-2D genes of the mouse (Klein 1975a). The amino acid sequences of the HLA heavy chains are very similar (Table XII), indicating that the HLA-A and HLA-B genes also have a common evolutionary origin. In fact, the HLA heavy chains display fewer differences in amino acid sequence than the H-2 heavy chains. For example, the sequences of the A2 and B7 molecules differ only at residue 9. This difference appears to distinguish products of the A gene from products of the B gene. There are also multiple differences in the sequences of products of alleles at the same locus, indicating that the HLA heavy chains can also be described as complex allotypes.

The amino acid sequences of the heavy chains of the histocompatibility antigens of the mouse, human and guinea pig display significant homologies (Table XII). The amino acids at positions 3, 6, 7, 15 and 27 are the same in all heavy chains and there is considerable similarity at positions 8, 10, 12 and 14. There are also differences, particularly between the mouse and human sequences. The extent of the homology is less than that between the sequences of the respective β_2 -microglobulins (Fig. 2). There can be no doubt, however, that the heavy chains are related and that the major histocompatibility genes of these three species have a common evolutionary origin.

The H-2 heavy chains display common amino acid residues at some positions which distinguish these sequences from those of the HLA heavy chains. These have been termed species-specific residues (Silver et al. 1976) and occur at positions 1, 2, 5, 9 and 23 (Table XII). At each of these positions the H-2K and H-2D gene products have the same amino acid that is different from the amino acid in the same position in the HLA heavy chains. The surprising implication of this finding is that the H-2 genes in the mouse and the HLA genes in man were not duplicated until after the two species had diverged in evolution. An alternative explanation would require identical mutations in the duplicated genes.

I should, of course, stress that the present sequence data are very limited and describe only one tenth or less of the total sequences of the polypeptide chain. In addition, it is probable that the amino-terminal sequences are more variable than the sequences of the internal

regions of the chains. This variability at the amino terminus can be clearly seen, for example, in the sequences of β_2 -microglobulins from different species (Fig. 2). The serology of the H-2 antigens and the extensive differences in the structures of the heavy chains shown by peptide mapping techniques (Brown et al. 1974) suggest, however, that the variability extends throughout the heavy chain.

The radiochemical technique used for determining the H-2 sequences may also be less reliable than classical sequence methods. It is striking that there is much less variation between the HLA heavy chain sequences, which have been determined by non-radioactive methods. Some of the differences seen in the H-2 sequences may be artifacts due to contamination by molecules which have co-purified with the H-2 heavy chains.

It is also possible that the H-2K and H-2D and the HLA-A and HLA-B genes are not exactly homologous to each other. There are known to be more than two genes coding for major histocompatibility antigens in both the H-2 and HLA complexes. A third gene, HLA-C in the HLA complex codes for a product similar to those of the A and B genes (Rask et al. 1976). There is also a third gene in the H-2 complex, closely linked to H-2D, which determines a similar product to the H-2 antigens (Hansen et al. 1977b). Furthermore, the H-2 and HLA molecules that have been sequenced are only a fraction of the possible histocompatibility antigens that are present in the human and mouse populations. We do not know whether the present sequences are representative of all the possible H-2 and HLA antigens. More histocompatibility antigens must be sequenced before definite conclusions can be made.

An interesting picture has emerged, even from comparisons of the present limited data. The major histocompatibility complex appears to be an unusually dynamic genetic and evolutionary system. The polymorphism of the H-2 antigens is reflected by multiple amino acid substitutions in the sequences of the H-2 heavy chains. We can now ask what is the genetic and evolutionary origin of the polymorphism? However, any hypothesis concerning the polymorphism must be constrained by the observations that the H-2 heavy chains do not display features of Knese or Dnase and that the H-2 genes were probably not duplicated until after the human and mouse ancestors had diverged in evolution.

What are the factors that are involved in the generation of the polymorphism?

The most obvious factor is mutation. Since the diversification of the H-2 alleles must have occurred after the mouse and human ancestors diverged in evolution, a large number of mutations would have been required to generate the large number of differences seen between H-2 alleles. In fact, the H-2 system is known to have a very high mutation rate; for example, the H-2K^b gene has a spontaneous mutation rate of 5.5×10^{-4} per generation (Melvold and Kohn 1974). This is the highest mutation rate for a single gene in any mammal. In contrast, the rate of mutation for most genes in the mouse is usually below 10^{-5} per generation (Klein 1974). This may indicate that the H-2 genes are unusually unstable. Alternatively, the H-2 molecule may be more tolerant of structural alterations, particularly if it has no enzymatic function, or the skin grafting technique used to detect the H-2 mutants may be more sensitive than other methods of detecting mutations. Nevertheless, the mutation rate of the H-2 genes is probably sufficient to account for the observed differences in sequence.

The population structure of wild mice may influence the rate of fixation of new variants. In the wild, mice live in small isolated population groups (demes) which undergo cycles of contraction and expansion of their numbers with the season of the year. There may be migration of mice out of the deme but there is no migration into the deme (Klein 1974). New variants may be rapidly fixed in the small populations by random drift so that different demes are genetically diverse. Wild mice captured in the same areas have similar H-2 haplotypes as compared to mice from other localities (Klein 1972).

The population genetics of the H-2 system is also affected by the t complex, which is located on the 17th chromosome. Homozygous lethal t alleles of the t complex may be present in up to 50% of a wild mouse population. The high frequency of the alleles in the population is due to their property of distorting the male transmission ratio (Lewontin and Dunn 1960). In addition, t alleles suppress recombination over a large segment of the 17th chromosome. Studies on the H-2 types of wild mice carrying t alleles indicate that there is linkage

disequilibrium between the t complex and H-2 genes; that is, the H-2 types of the mice correlated with their t alleles (Hammerberg and Klein 1975). This observation suggests that suppression of recombination by the t alleles may preserve the entire T-H-2 region of the 17th chromosome as a single unit. Therefore any selection of a gene in this region of the chromosome will also select the H-2 genes with which it is linked. This might apply particularly to genes regulating the immune response.

The combination of high mutation rate, rapid fixation of new mutants and the influence of the t complex is probably sufficient to explain the extent of the polymorphism. These factors may also explain the multiple differences between H-2 alleles, even if the H-2 genes had not been duplicated when the human and mouse ancestors had diverged. But these factors alone do not explain the absence of Kness or Dness in the sequences of the H-2 heavy chains. One possible explanation would be that each heavy chain is not the product of a single gene but consists of a family of very similar molecules coded by several closely linked genes. Variations between the structures of the heavy chains in any one family might mask any properties of Kness or Dness. The heterogeneity seen in the two-dimensional gel patterns and the leucine/valine heterogeneity seen in the sequences of the H-2K^d heavy chain would be consistent with this hypothesis.

A more complex mechanism for the evolution of the H-2 genes suggests that the H-2 genes were extensively duplicated and mutated. Later, most of these genes were eliminated by unequal crossing over to leave only the H-2K and H-2D genes. This model would allow many more mutations to be fixed in the duplicated genes. But to account for the large number of different alleles, similar unequal crossing over events would have to occur independently.

An attractive alternative interpretation of the genetics of histocompatibility antigens has been proposed by Bodmer (1973). He proposed that the histocompatibility genes are not structural genes but regulatory genes that control the expression of the histocompatibility antigens rather than code for the antigens themselves. Each animal

would possess the structural genes for all the histocompatibility antigens in the species as a closely linked set of duplicated genes. These genes may be located on chromosome 17 but they could also map anywhere in the genome. The structural genes would exist as a single family of genes and would not be divided into K genes or D genes. It would therefore not be surprising that the sequences of the H-2 heavy chains do not display features of Kness or Dness. This model also suggests that mutation of H-2 genes may reflect a switch in the control mechanism resulting in the expression of a different structural gene. The mutant H-2 gene product would be another allele and might show considerable structural differences from the wild type gene product. One mutant, H-2K^{bd}, probably differs in two amino acid positions from the H-2K^b wild type heavy chain (Nathenson et al. 1978). Generally, H-2 mutants do not show marked structural differences from the wild type genes.

There is a precedent for this model in the genetics of the Tla gene which is linked to the H-2 complex. The TL antigens are expressed on the thymocytes of some strains of mice but not on the thymocytes of other strains (Boyse and Old 1969). Some TL antigens may be expressed, however, on leukemia cells from strains that do not show expression of TL antigens on their thymocytes. The structural genes for these antigens are possessed by all mice; the Tla gene determines which of the structural genes are expressed. As I have described, the TL antigens are structurally very similar to the H-2 antigens and a similar mechanism might also apply to the H-2 antigens.

A similar model has been proposed to account for the expression of immunoglobulin allotypes (Gutman et al. 1975). In rabbits, there is evidence that animals may express variable region allotypes that should not be present in the individual according to the genotype of its parents (Strosberg et al. 1974, Mudgett et al. 1975). The same phenomenon also occurs with constant region allotypes in mice, particularly when the animals are debilitated by the growth of a transplanted plasmacytoma (Bosma and Bosma 1974). These results suggest that, under certain circumstances, structural genes that are normally silent may be stimulated to express their gene product. Similarly, it has been reported

that mouse cells infected with vaccinia virus may express H-2 antigens not normally expressed by the uninfected cells (Garrido et al. 1976, 1977). Although this result may be due to contaminating antiviral antibodies in the H-2 antiserum, it would provide strong support for Bodmer's hypothesis.

It is possible to account for the polymorphism of the H-2 antigens without considering their function. On the other hand, it is likely that the polymorphism will be related to the function of the H-2 antigens. A frequent explanation of the maintenance of a polymorphism is the better fitness of heterozygotes over homozygotes. In the mouse there is evidence that animals heterozygous for the H-2 complex produce an enhanced immune response (Doherty and Zinkernagel 1975). In man, one population study indicates that HLA homozygotes may be at a selective disadvantage (Degos et al. 1974).

The experiments of Zinkernagel, Doherty and others have indicated that the H-2 antigens are important in the recognition of abnormal cells by the immune system (Doherty and Zinkernagel 1974). In addition, the H-2 antigens may interact with other components on the cell surface (Schrader et al. 1975). More recent data suggests that different H-2 antigens may vary in their ability to carry out these functions. For example, H-2D^b antigens but not H-2K^b antigens are incorporated into Friend virus particles (Bubbers et al. 1978). Similarly, the cytotoxic T cells specific for murine sarcoma virus infected cells recognize only the H-2D^b antigen on H-2^b cells and the H-2K^d antigen on H-2^d cells; the H-2K^b and H-2D^d antigens are irrelevant (Gomard et al. 1977). Some H-2 antigens may therefore be better adapted to interact with other components on the cell surface or to interact with receptors on the T cell. These H-2 antigens might therefore have a selective advantage in the wild population over H-2 antigens that function less efficiently in the immune surveillance system.

Possible functional differences between H-2 antigens of different types may be reflected by differences in their structures. For example, there is a curious resemblance between the structures of the H-2D^b and H-2K^d heavy chains: the detergent and papain solubilized H-2D^b and H-2K^d

heavy chains are both larger than the H-2K^b and H-2D^d heavy chains (Figs. 20, 21 and Table X). The H-2D^b and H-2K^d antigens but not the H-2K^b and H-2D^d antigens are also recognized by cytotoxic T cells directed against murine sarcoma virus infected cells (Gomard et al. 1977). Perhaps the structural differences in the H-2 heavy chains reflect their ability to associate with viral antigens on the cell surface or their ability to be recognized by antigen-binding receptors on the T cells. More detailed characterization of these differences may define the functional properties of the H-2 antigens.

Further characterization of the H-2 antigens may therefore provide clues as to the function of these molecules. It may be possible, for example, to define the regions of the H-2 heavy chain which interact with the light chain, with other cell surface components or with the antigen-binding receptors on the cytotoxic T cell. Extension of the analysis of the amino acid sequence of the heavy chain to internal regions of the molecule and to other H-2 antigens will provide more detailed information about the nature and origin of the genetic polymorphism of the H-2 antigens. The experiments described in this thesis form the basis for these further studies.

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