CHARACTERIZATION OF ANTIGENS OF THE DOG MAJOR HISTOCOMPATIBILITY COMPLEX

PROEFSCHRIFT

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ABBREVIATIONS

anti-HC	Anti-heavy chain
β_2m	β_2-microglobulin
BSA	Bovine serum albumin
2D	Two-dimensional
DEAE	Diethylaminoethyl
DLA	The dog MHC
DNA	Deoxyribonucleic acid
cDNA	copy DNA
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetate
ELISA	Enzyme-linked immunosorbent assay
E.R.	Endoplasmic reticulum
GA,GT	Copolymers of L-glutamic acid with L-alanine, copolymers of L-glutamic acid with L-tyrosine
H-2	The murine MHC
HLA	The human MHC
HPLC	High performance liquid chromatography
H-Y	Male-specific histocompatibility antigen
Ia	I-region associated
Ig, IgG	Immunoglobulin, immunoglobulin G
Ir	Immune response
LD	Lymphocyte defined
MHC	Major Histocompatibility Complex
MLR	Mixed lymphocyte reaction
MW	Molecular weight
NDS	Normal dog serum
NK cell	Natural killer cell
NRS	Normal rabbit serum
PBL	Peripheral blood lymphocyte(s)
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
pI	Isoelectric point
PMSF	Phenylmethylsulfonylfluoride
PTH	Phenylthiohydantoin
RhLA	The rhesus monkey MHC
RNA	Ribonucleic acid
mRNA	messenger RNA
R.R.	Relative risk
RT-1	The rat MHC
SD	Serologically defined
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLA	The (miniature) swine MHC
Staph A	Staphylococcus aureus (strain Cowan 1) protein A
TCA	Trichloroacetic acid
Tla	Thymus leukemia antigen
Tris	Tris(hydroxymethyl)aminomethane

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

I.1 Some historical notes

Many reports on scientific research end with suggestions for further investigations. In his thesis on the Major Histocompatibility Complex (MHC) of the dog (1973) H.M. Vriesendorp states: "As already mentioned, the true and final picture of the detailed structure of the SD part of a Major Histocompatibility System will not be available until after a detailed biochemical characterization of the antigens involved. Till that time the two loci concept will be a useful but still unverified model".
Since then MHC antigens of several species have been biochemically investigated (Finkelman et al., 1975 (the guinea pig), Giphart et al., 1978 (the rhesus monkey), Katagiri et al., 1975 (the rat), Kimball et al., 1979 (the rabbit), Lunney and Sachs, 1978 (the miniature swine), Parham and Ploegh, 1980 (the owl monkey), Phillips et al., 1978 (the Syrian hamster), Strominger et al., 1981 (man and mouse), Ziegler and Pink, 1975 (the chicken). In this thesis the isolation and biochemical characterization of dog MHC antigens is described.

Terminology has changed since 1973. The Major Histocompatibility System is now called Major Histocompatibility Complex (MHC), as it has been shown to contain several loci. It is a chromosomal region that codes for several molecules (MHC antigens) which serve important functions in the immune system. It has originally been discovered in the mouse because of its role in the rejection of transplantable tumours. Since its first discovery, MHC's have been detected in several species (Götze, 1977). Several histocompatibility systems exist, but the MHC presents the major transplantation barrier, hence its name. Genetic differences between recipient and donor have turned out to be crucial for transplant survival. When a recipient recognizes structures on donor tissue as non-self, an immune response will be mounted which leads to destruction of the transplant.
In his Nobel lecture in 1930, Landsteiner (1931) stressed the parallel between the problems of matching for blood transfusion and transplantation. He suggested that "serum reactions" might be useful to detect the elements which are important for the outcome of organ transplantation. Gorer started to work on this basis and discovered an antigen on mouse red blood cells. Matching for this antigen had a major effect on the survival of a transplantable tumour (Gorer, 1937). In co-operation with Snell he defined the first histocompatibility complex to be described, the H-2 complex in the mouse. A brief account of these early studies is given by Snell in his 1980 Nobel lecture (Snell, 1981).

The dog MHC antigens are named DLA antigens (*D*og *L*eucocyte *A*ntigen) (Vriesendorp et al., 1977). Two types of antigen have been defined in the MHC of the dog with possible relevance for transplantation. They were initially named according to the techniques with which they could be demonstrated; SD or *s*erologically *d*efined antigens and LD or *l*ymphocyte *d*efined antigens. They are described further below. A schematic view of the arrangement of the MHC loci on the chromosome is presented in Fig. 1 and discussed more in detail in section I.2. In the mouse a new nomenclature has been adopted (Klein, 1979). The products of the SD loci are now called class I antigens. Class II antigens are the former I-region associated (Ia) antigens. Class I and class II antigens are described in somewhat more detail below. In addition class III and class IV antigens have been recognized in mice. Amongst the class III antigens are serum proteins such as some complement components, and the class IV antigens are coded for by the former Tla (thymus leukaemia antigen) region (Snell, 1981). Class III and class IV antigens will not be considered here. Dog equivalents of these antigens have not yet been described. The dog equivalent of the class I antigens are the former SD antigens. The controversy whether lymphocyte defined (LD) antigens, immune response (Ir) gene products and I-region associated (Ia) antigens are identical has not yet been resolved. In Fig. 1 it is assumed that LD antigens are class II antigens, but formally they cannot be called class II.

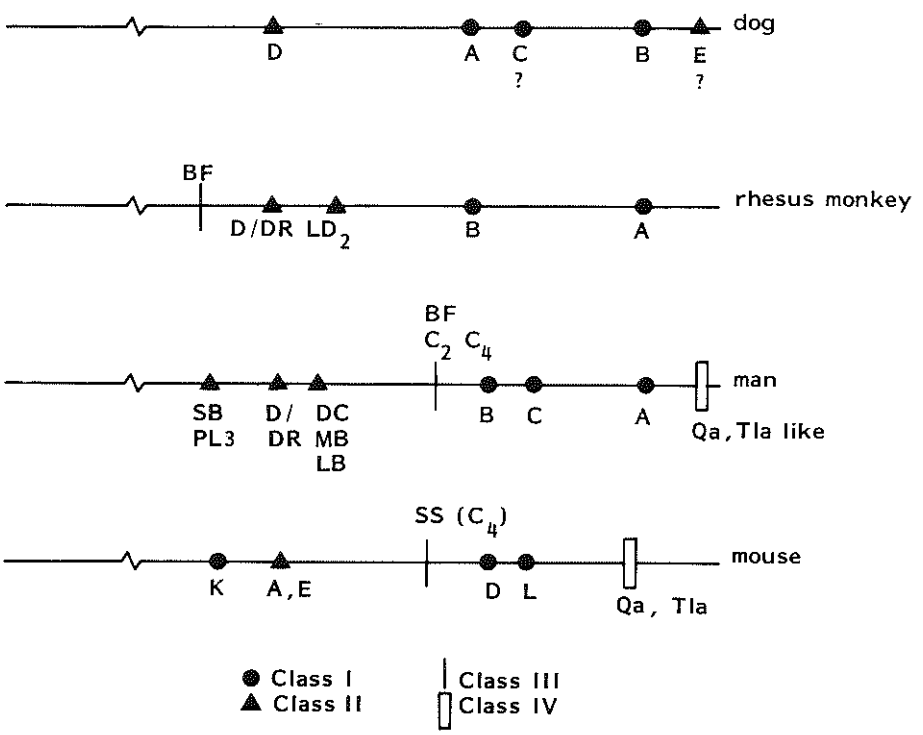

Figure 1. Simplified scheme of the genetic organization of the DLA complex, compared to the organization of the RhLA, HLA and H-2 complexes.

In all species investigated so far, great homology has been found for MHC products in function, structure, and genetic organization. Class I antigens consist of two polypeptide chains of 44,000 and 12,000 daltons. They are found on all tissues, and play a role in the recognition of self versus non-self by cells of the immune system. They are the principle targets for cell-mediated cytotoxicity in graft rejection (Snell et al., 1976) and they are involved in the recognition of viral and tumour antigens by syngeneic cytotoxic T cells (Schrader and Edelman, 1976, Zinkernagel, 1979). The heavy chain of class I antigens is coded for by MHC genes, but the light chain is coded for by a different chromosome. For example, in the human MHC, the HLA complex, the heavy chain of class I antigens is coded for by chromosome 6 (van Someren et al., 1974) and the light chain by chromosome 15 (Goodfellow et al., 1975).

Class II antigens have a more restricted tissue distribution than class I antigens. They occur mostly on B lymphocytes, macrophages, activated T cells and certain tumours. Class II antigens consist of polypeptide chains of 34,000 and 29,000 daltons. They are involved in the communication between various cells of the immune system, e.g. for antigen presentation (macrophage – T cells), helper function (T cells – B cells) and suppression (T cells – T cells) (McDevitt, 1976). The genes of the class II antigens are closely linked to immune response (Ir) genes that determine the outcome of the immune response against certain synthetic antigens, such as GT and GA (McDevitt and Landy, 1972).

The Major Histocompatibility Complex of the dog contains the genetic code for DLA antigens. Class I antigens and LD antigens have been defined in the dog (Vriesendorp et al., 1977). Detection of class I antigens is done by serological typing of peripheral blood lymphocytes (PBL) in the one-stage or the two-stage micro-lymphocytotoxicity test (Smid-Mercx et al., 1975). The antigens are defined by their reaction pattern to a panel of antisera. The existence of the LD antigens is concluded from the outcome of mixed lymphocyte reactions (MLR's) (Yunis and Amos, 1971). The reaction pattern in MLR with reference cells from dogs, homozygous for LD antigens, is used to define these antigens.

The expression of DLA class I and LD antigens is codominant. This means that the class I and LD antigens from both parents can be detected. The genes of the MHC of one chromosome, which segregate together in a family are called a haplotype. Class I antigens in the dog were identified before LD antigens, and therefore the first correlations between histocompatibility typing and transplant survival were made using class I profiles. The first evidence for the existence of a major histocompatibility complex in the dog was found in littermate beagles (Westbroek et al., 1971). Mean survival times of skin, small bowel and orthotopic heart transplants in that model were shown to be significantly prolonged in DLA identical littermates, as compared to littermates that differed in one or two haplotypes (Vriesendorp et al., 1971). In kidney transplantation between unrelated donor-recipient combinations in mongrel dogs, the LD antigens have more influence on transplant survival than the class I antigens (Bijnen et al., 1979a, Westbroek et al., 1975).

In our laboratory typing for both DLA class I antigens and LD antigens has been

put at the service of transplantation research. For organs that are technically difficult to transplant (e.g. liver, pancreas), donor and recipient are matched so as to minimize immunological reactions leading to rejection. Kidney transplantation, however, has been used as a model system to test the influence of matching on graft survival, using related and unrelated dogs. The MLR test, defining LD (in)compatibility was thoroughly investigated, and using this test the influence of class I, LD and minor histocompatibility antigens on the outcome of kidney grafting was assessed (Bijnen et al., 1979a and 1979b).
The history of canine histocompatibility testing has been summarized by Vriesendorp et al. in 1977. International co-operation in this field was started by a workshop held in Rotterdam in 1972, followed by a second workshop in 1974 in Portland, Oregon, U.S.A. (Joint reports, 1973 and 1976). A third workshop is planned for 1983 in Seattle, Washington, U.S.A.

I.2 **DLA genetics**

A model for the genetic organization of the DLA complex is reproduced in a simplified form in Fig. 1 (Vriesendorp, 1980). For comparison, simplified models of the MHC's of man, mouse and rhesus monkey are also depicted. In the dog it is not known on which chromosome the MHC is located. The MHC is located on chromosome 6 in man (van Someren et al., 1974) and on chromosome 17 in the mouse (J. Klein, 1975). In both cases the light chain is coded for by a different chromosome, chromosome 15 in man (Goodfellow et al., 1975) and chromosome 2 in the mouse (Cox et al., 1982).
DLA-A, -B and -C form the class I loci. Recombination between DLA-A and -B has been found (Vriesendorp, 1973). There is not enough information to establish the exact location of DLA-C, nor, for that matter, to indisputably establish the existence of DLA-C at all. In the most recent review on the subject (Vriesendorp, 1980) DLA-A is said to have 7 alleles, DLA-B 4 and DLA-C 3. The so-called "blank" alleles are as yet undefined, they comprise 27.6% for DLA-A, 42.2% for DLA-B and 55.2% for DLA-C.
Class I antigens are detected in the microlymphocytotoxicity test by means of tissue typing sera. Most typing sera have been collected from beagles, either by skin transplantation between dogs with a limited DLA difference (e.g. parent and sib) and lymphocyte boost, or from pregnancy sera. They are good typing reagents in beagles but not in other breeds or mongrel dogs. This is reminiscent of the situation in man, where some sera are better suited for typing a particular race than others (Histocompatibility testing 1972). Mongrel dogs are especially difficult to type; they show a high percentage (50%) of blanks. DLA-D and DLA-E are loci for dog LD antigens. They have been separated from the class I loci by recombination (van den Tweel et al., 1974).
Two aspects of typing for DLA antigens are notable. First there is the polymorphism. With five loci and at least 27 alleles the number of possible combinations is

large. However, in beagles it is observed that certain haplotypes (the genetic information on MHC antigens carried by one chromosome) occur more often than others. This so-called positive linkage disequilibrium between two alleles causes them to occur with a frequency higher than the product of the gene frequencies for each allele. In the beagle the linkage disequilibrium has a much greater delta value (the parameter of linkage disequilibrium) than in man. Vriesendorp (1973) suggests this could be due to the founder or bottleneck effect. This founder effect stands for the possibility that in some periods in the past only a limited number of dogs was available for breeding.
As mentioned before, class II antigens have a much more restricted tissue distribution than class I antigens. They are most easily isolated from B cells or B cell lines (Springer et al., 1977a). For their incontestable identification anti-class II antisera are necessary. These have so far not been recognized in the dog. One of the major reasons for this is that it is very difficult to separate dog B and T cells (Krakowka and Guyot, 1977).
Antisera are well defined reagents with which the purification of an antigen can be monitored. Typing sera are available for class I antigens, but not for class II antigens. Our biochemical analyses are therefore concerned with class I antigens only.

I.3 The biochemical characteristics of the HLA-B7 molecule as a model of a class I antigen

A schematic view of a human MHC class I molecule, the HLA-B7 molecule, containing only the most salient features is represented in Fig. 2. Most data mentioned in this paragraph are quoted from the review of Ploegh et al. (1981b). The HLA-B7 molecule consists of two polypeptide chains of 44,000 and 12,000 daltons. The light chain is not directly associated with the membrane, but it is non-covalently associated with the heavy chain. The light chain is indistinguishable from β_2-microglobulin (β_2m), a protein also found in plasma and urine (Berggård and Bearn, 1968). It contains one disulfide loop and it is identical in all HLA class I antigens investigated so far. The polymorphism detected by alloantisera is located in the heavy chain. This polypeptide chain spans the membrane. The greater part is found on the outside of the cell. It can be divided into three regions. From the NH_2-terminal end to the first papain cleavage site amino acids 1-90 form the α1 region, amino acids 91-180 the α2 and 181-271 the α3 region. The transmembrane portion comprises 24 residues and the intracellular part 31 residues. The α1 region contains a carbohydrate side chain, N-linked to amino acid 86 (asparagine). The α2 and α3 regions each contain a disulfide loop of about 60 amino acids. Two papain cleavage sites are present which allow the isolation of the water soluble parts of the heavy chain by limited papain digestion.
The division of the extracellular portion into three regions, approximately similar to the fragments obtained by chemical cleavage by Lopez de Castro (1979), is reminiscent of the immunoglobulin structure. The HLA class I molecule can be said

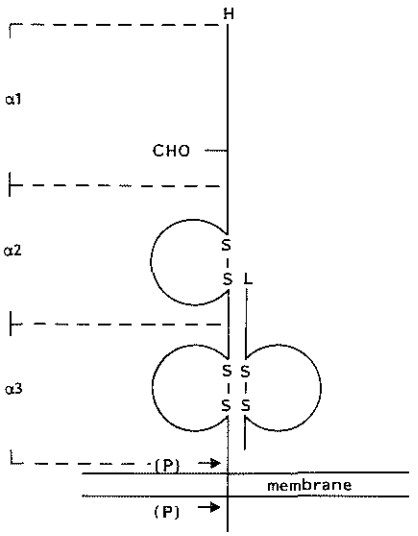

Figure 2. Schematic diagram of a class I antigen.
CHO: sugar side chain
$\alpha 1$-$\alpha 3$: domains
S-S: joining of the disulfide loop
H: heavy chain
L: light chain
P: papain cleavage site

to contain four extracellular domains, $\alpha 1$, $\alpha 2$, $\alpha 3$ and β_2m. Amino acid sequences of β_2m, the heavy chain and immunoglobulin point to a statistically significant homology between β_2m, the $\alpha 3$ region and the $C_H 3$ region of immunoglobulin. Comparison of physical-chemical properties of HLA and immunoglobulin, and comparison of the immunoglobulin crystalline structure with preliminary data on HLA crystals confirm this similarity.

The membrane binding region of the heavy chain is hydrophobic; it does not contain any polar or charged amino acid residues. Immediately after this stretch of neutral amino acids some basic residues are found which may play a role in anchoring the heavy chain to the membrane. The intracellular part can be phosphorylated (Pober et al., 1978). Both the orientation of the heavy chain, with the greater part of the molecule outside the cell, and the possibility of phosphorylation of the intracellular part suggests that HLA could have a function in transmembrane signalling.

The elucidation of amino acid sequences of several antigenically different HLA molecules was hoped to shed some light on the structural basis for the polymorphism. Comparison of (incomplete) sequences of HLA-A2 with HLA-B7 shows that amino acid differences are scattered throughout the molecule. Certain regions with greater variability are found in residues 65-80, around 110 and around 175. Differences between allelic products (e.g. HLA-A2 and HLA-A28) are somewhat less than those observed between the products of different loci. HLA-A2 and HLA-A28, however, are cross-reacting and not quite representative of "normal" alleles. No other (non-cross-reactive) alleles have been sequenced far enough to yield information on typical variability and typical homology regions. There is also extensive sequence homology with MHC class I products of other

species. Mouse H-2 class I antigens have been best investigated in this respect (Nathenson et al., 1981). For the papain solubilized extracellular parts of H-2K^b and HLA-B7, the sequence homology is 71%. The α3 region is the most conserved domain. This is the domain which shows homology with β_2m and immunoglobulin. It is possible to know by amino acid sequence alone if one has an H-2 or an HLA molecule. Distinction between products of different loci or alleles within one locus is not (yet) possible on the basis of amino acid sequence. Sequencing data can be used to speculate about the time of divergence of proteins of different species or within a species in evolution, and about mechanisms by which polymorphism has developed (Nathenson et al., 1981, Ploegh et al., 1981b, Robertson, 1982).
Studies of the crystal structure of papain solubilized HLA antigens have commenced in order to obtain more information on their three-dimensional structure. By comparison of three-dimensional structure and amino acid sequence data it will be possible to investigate whether or not the most variable regions fold together to form the "alloantigenic site". The possible involvement of the sugar side chain in the alloantigenic site is disputed by most authors (Parham et al., 1977, Ploegh et al., 1981a), defended by some (O'Neill et al., 1981).
Although dog class II antigens have not been described here, occasional references are made in this thesis to human and mouse class II products. Therefore a schematic view of an HLA class II antigen is given in Fig. 3. It consists of two polypeptide chains both of which span the membrane. Similarity between class I, class II and immunoglobulin molecules in a structural respect is obvious. Both class II polypeptide chains carry carbohydrate residues and polymorphism resides in the light chain. Recently, Kaufman et al. (1982) have shown that the extracellular region of the class II antigens consists of four domains (two per chain). Two of them, the carboxyl-terminal ones, have sequence homology with immunoglobulin. This is comparable to the situation in class I antigens, where the α3 region and β_2m have statistically significant sequence homology with immunoglobulin constant domains. The author suggests that there may have been a common ancestral gene for both class I and class II antigens that contained an immunoglobulin-like region. Whether the structural similarity of immunoglobulin and MHC molecules bears any relation to their function remains to be seen.

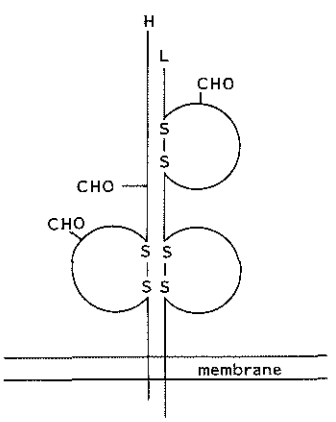

Figure 3. Schematic diagram of a class II antigen.
CHO: sugar side chain
S-S: joining of the disulfide loop
H: heavy chain
L: light chain

I.4 Isolation of MHC products

Methods for the isolation of MHC products must be designed to render the membrane bound hydrophobic part of the molecule soluble. Alternatively, only the water soluble part may be purified. Reviews have been published summarizing the history of the methods applied (Strominger et al., 1981, Walker and Reisfeld, 1982). One way to avoid difficulties in releasing MHC antigens from cell membranes is to look for them in body fluids. Possibly because of turnover of cells and membrane molecules HLA antigens can be found in soluble form in serum and urine (Billing and Terasaki, 1974, Reisfeld et al., 1976, Vincent et al., 1976) and be purified by conventional biochemical techniques and immuno-adsorbents.
For structural and functional studies, the isolation of MHC antigens from cell membranes is essential. Two techniques have been used, each with its own advantages and disadvantages (I.4.1, I.4.2).
The presence of the nonpolar transmembrane region prevents one from obtaining the whole molecule in water soluble form. The water soluble, external part of the heavy chain, together with the complete light chain, can be isolated by papain digestion.

I.4.1 *Isolation of MHC products by papain digestion*

When membrane bound HLA antigens are treated with papain only the extracellular portion of 34,000 MW is detected together with β_2m. The second papain cleavage site (Fig. 2) was discovered when HLA antigens were first isolated by detergent solubilization and treated with papain afterwards (I.4.2) (Springer et al., 1974). The molecules isolated by papain digestion retain their alloantigenic specificity. HLA molecules were isolated in this way from spleens (Sanderson and Batchelor, 1968), lymphoblastoid cell lines (Mann et al., 1968, Turner et al., 1975) and platelets (Gockerman and Jacob, 1979). In the mouse one has to resort to using organs and sacrificing a lot of animals because lymphoblastoid cell lines are not available. Papain digestion of H-2 antigens has been described for antigen from spleen (Shimada and Nathenson, 1969) and liver (Henriksen et al., 1979).
The advantage of using cell lines is that one has a reproducible source of cells of one tissue-type. Homozygosity of the cell line facilitates the separation of the products from different loci by reducing the number of different class I products from six to three. B cell lines transformed by Epstein-Barr virus can express about 36 times as much HLA as peripheral blood lymphocytes (McCune et al., 1975). Tissue culture, however, is laborious and expensive. In other species, no B cell lines exist so that organs must be used for large scale preparations. In small animals like mice, inbred strains are available to provide a reproducible source of tissue as starting material. Larger animals, like the miniature swine (Metzger et al., 1981) and the dog (van der Feltz et al., 1981) have spleens of a size large enough for the isolation of sufficient amounts of MHC products. The papain solubilized molecules can be purified by standard protein fractionation techniques. As they are water soluble they can be

used for amino acid sequencing and the preparation of crystals for the study of the three-dimensional structure. Their biological activity is unhampered as fas as the antigenic activity in serological assays is concerned.

I.4.2 *Isolation of MHC products by detergent solubilization*

Intact class I molecules can only be obtained by detergent solubilization. Detergent micelles replace the lipid bilayer around the hydrophobic transmembrane part. After purification of detergent solubilized products, molecules are found to consist of a heavy chain of 44,000 MW and a light chain of 12,000 MW. Hence the size of the light chain remains the same in both papain digested and detergent solubilized molecules, whereas the heavy chain size varies with the isolation procedure. HLA antigens have been isolated by detergent solubilization from platelets (Dautigny et al., 1973, Trägårdh et al., 1979) and lymphoid cell lines (Springer et al., 1974). Although no B cell lines are available in the mouse, tumour cell lines have recently been used for large scale isolation of H-2 antigens by detergent solubilization (Freed et al., 1979, Rogers et al., 1979).

The detergent mostly used is NP-40, which is a non-ionic detergent. Advantages and disadvantages of various detergents are discussed by Strominger et al. (1981). Because detergents may interfere with gel filtration and other physical-chemical techniques, alternative procedures have been developed for further purification such as lentil lectin affinity chromatography (Springer et al., 1977b, Snary et al., 1974) and affinity chromatography employing anti-β_2m serum (Robb et al., 1976) or anti-HLA monoclonal antibodies (Parham, 1979).

In the mouse, much work has been done on an analytical scale. Radiolabelled preparations have been characterized by immunoprecipitation with hyperimmune sera (Schwartz and Nathenson, 1971). In the case of HLA, class I and class II antigens have been studied by the same technique using heterologous antisera or monoclonal antibodies. The available alloantisera are usually too weak to be suitable.

A combination of detergent solubilization and papain digestion revealed the existence of the second papain cleavage site of HLA antigens (Springer et al., 1974). When detergent solubilized HLA antigens were subsequently digested with papain, molecules of 39,000 MW and then molecules of 34,000 MW appeared. NH_2-terminal amino acid sequence determination showed that the NH_2-terminal ends of both papain digested and detergent solubilized molecules are identical. It was concluded that papain cleaves the molecule towards the carboxyl-terminal end. The difference in MW between the two heavy chains is small, therefore papain cleaves the heavy chain close to the membrane.

A disadvantage of using detergent is that detergent must remain present to keep the molecules in solution. This precludes testing of the isolated molecules for biological activity and the use of standard protein purification techniques. Inhibition of cytotoxicity by detergent solubilized molecules can be tested, however, in the presence of 30% BSA (Springer et al., 1974).

The advantage of detergent solubilization is that one can study the complete molecule, including its mode of insertion into the plasma membrane. Functional studies can be done by introducing detergent solubilized MHC antigens into artificial membranes such as liposomes (Engelhard et al., 1978a, Trägårdh et al., 1979, Turner and Sanderson, 1978). First observations with these liposomes were of the orientation of HLA and H-2 antigens. It was similar to that observed in the native membrane of the cells. Liposomes containing HLA antigens bind to certain bacteria (Klareskog et al., 1978), and to Semliki Forest Virus (Helenius et al., 1978). Both HLA class I and class II antigens incorporated in liposomes can stimulate secondary cytotoxic T lymphocytes (Engelhard et al., 1978b, 1980).
Whatever the method of isolation of MHC antigens may be, at one stage or another it is dependent on antisera either for characterization, or for the isolation itself. Antisera are indispensable for monitoring the isolation procedure, for estimating the purity of the products obtained or for the isolation of MHC antigens by immunoprecipitation or immunoaffinity columns. Alloantisera are usually not suitable for isolation of MHC antigens. In man, either conventional heterologous antisera, or monoclonal antibodies can be employed. One of the early conventional heterologous antisera used was the anti-β_2m serum as described by Robb et al. (1976). Sera against urinary β_2m are the best sera to use for the isolation and characterization of MHC antigens from species like the dog in which no monoclonal antibodies are available. This antiserum potentially reacts with all class I antigens because of the non-covalent association of the heavy chain with β_2m.

I.5 **Recent developments in MHC research**

In man and in the mouse, the two best investigated species as far as the MHC antigens are concerned, the search for the biosynthetic pathways and the examination of the genetic organization of the MHC have dominated the scene during the last few years. It is important to study these subjects in other species as well in order to get to know more about the relationship between structure and function of the MHC.

I.5.1 *Biosynthesis*

The elucidation of the biosynthesis pathways of H-2 and HLA antigens started with investigations in model systems. Cell-free translation of mRNA yielded MHC heavy chains with an NH_2-terminal extension comparable to the signal sequence normally found in secretory and plasma membrane proteins (Dobberstein et al., 1979, Ploegh et al., 1979). The signal sequence is thought to locate the ribosomal complex on the endoplasmic reticulum. Glycosylation and cleavage of the signal sequence occurred cotranslationally when microsomal membranes had been added to the cell-free translation system. β_2-microglobulin is also synthesized with a signal

sequence. Transfer of β_2m into microsomal vesicles results in cleavage of the signal sequence (Dobberstein et al., 1979). In the endoplasmic reticulum (E.R.), β_2m associates with the heavy chain, as shown by in vivo experiments. During transport to the plasma membrane, the glycosylation pattern of the molecule is modified. Biosynthesis of HLA has been further studied by pulse-chase labelling in cell lines (Krangel et al., 1979, Owen et al., 1980, Ploegh et al., 1981a). The cell-free translation studies (Ploegh et al., 1979) were followed by in vivo studies of the assembly and maturation of HLA antigens in the B cell line T5-1 (Krangel et al., 1979). Initially the HLA heavy chains were not associated with β_2m and were glycosylated with "high mannose" residues. Ten to fifteen minutes after complete synthesis of the heavy chain β_2m was found complexed with it, in the E.R. Half an hour after synthesis the high mannose sugars had been modified to complex sugars in the Golgi complex and 30-60 minutes later the antigens appeared at the cell surface. Only complex sugar bearing molecules were found at the surface. Other cell lines show basically the same course of events although the timing might be slightly different (Owen et al., 1980, Ploegh et al., 1981a).

Although not all details are known, it is clear that the change in glycosylation from "high mannose" to "complex" sugars and the association of heavy chain and β_2m are crucial events in the biosynthesis of HLA molecules. The pathway of glycosylated molecules was compared to that of molecules synthesized in the presence of tunicamycin, an inhibitor of N-linked glycosylation. Conclusions were that glycosylation is not necessary for insertion in microsomal vesicles, intracellular transport or (timing of) surface expression (Owen et al., 1980, Ploegh et al., 1981a). Lack of carbohydrate does not affect the association of light chain and heavy chain or the conformation of the molecule as measured by the reaction with (allo-) antisera and the susceptibility to lysis with proteolytic enzymes (Ploegh et al., 1981a).

Ploegh et al. (1979) and Owen et al. (1981) have studied the role of β_2m by comparing biosynthesis in "normal" cell lines with that in the Daudi line which does not express HLA antigens on the cell membrane, presumably because it does not synthesize β_2m. From early studies on Daudi cells it was clear that they do synthesize glycosylated heavy chains with the signal sequence removed (Ploegh et al., 1979). These heavy chains, however, remain in the cell. In vivo association with β_2m in cell hybrids "rescues" the Daudi HLA antigens (Arce-Gomez et al., 1978). Together with β_2m, HLA heavy chains which were not those of the hybridization partner appeared at the cell surface of the hybrid cells. It was concluded that these must be the Daudi HLA heavy chains. Attempts to bring about in vitro association of heavy chains and β_2m have not met with succes (Ploegh et al., 1979). Glycosylation of Daudi cell heavy chains does not proceed further than the high mannose stage (Sege et al., 1981). The Daudi heavy chain remains sensitive to endoglycosidase H, an enzyme that can remove the sugar residues in the high mannose form but not the complex sugars. It seems probable that heavy chains in Daudi cells do not reach the Golgi complex but stay in the endoplasmic reticulum. By looking at the glycosylation in the biosynthesis of HLA class II antigens in Daudi cells, Sege

and colleagues (1981) concluded that class II antigens are synthesized normally, hence there is no general defect in glycosylation or modification in Daudi cells. Therefore it seems that β_2m is essential for the transport of the HLA heavy chain within the cell (e.g. from the E.R. to the Golgi complex) and from within the cell to the extracellular side of the cell membrane.
The association of heavy chain and β_2m can go wrong because of some defect in β_2m, as described above, but a failure in the heavy chain can also affect the association. One report describing a failure of association of β_2m and heavy chain because of a structurally altered heavy chain has recently appeared (Krangel et al., 1982). This also results in lack of processing and transport to the cell surface.
Association of β_2m with the heavy chain is necessary for the conformation of the final product. Certain antisera, which are directed against the heavy chain, can only react with the heavy chain when it is associated with β_2m. An example of such an antiserum is the monoclonal antibody W6/32, described by Parham et al. (1979). Only one group has described the phenomenon that the heavy chain kept its alloantigenic conformation after dissociation from β_2m (Nakamuro et al., 1975). Whether β_2m can associate with nascent chains or only with finished heavy chains is not clear. Generally there is an excess of β_2m intracellularly so that cotranslation of the two subunits is not necessary.

I.5.2 *The genetic organization of the MHC*

Lack of sufficient pure material has hampered the amino acid sequence determination of many different allelic products. Amino acid sequencing is also very time consuming. Cloning of the DNA sequences coding for these products and inferring the amino acid sequences from these is faster and more efficient. It does not suffer from the restriction imposed by scarcity of material. Moreover, when cDNA is available, the genomic organization of the MHC complex can be investigated. This may give some clues as to how the extreme polymorphism of the MHC has arisen. So by looking at the genes we can obtain information about their products and the function of those products.
A prerequisite for studies of genomic DNA is the availability of the right probes. Molecular cloning of cDNA for both HLA and H-2 antigens was performed by Ploegh et al. (1980) and Kvist and colleagues (1981). Investigations generally start from enriched mRNA. This is transcribed into cDNA. cDNA is inserted into plasmids and these are used to transform E. coli. After propagation, individual transformants are screened by isolation of their DNA and hybridization with HLA or H-2 mRNA. In vitro translation of mRNA hybridized to the DNA from the plasmids is used to detect those clones that contain DNA coding for HLA or H-2. Then a restriction map is constructed and the nucleotide sequence of the inserted HLA or H-2 DNA is determined.

The characterization of cDNA poses new problems. Because of the lack of sufficient amino acid sequence data, it is difficult to find out for which allelic product a particular DNA restriction fragment or recombinant DNA clone is

coding. In order to solve this problem, Orr et al. (1982) analysed HLA loss mutants with the pHLA-1 probe (Ploegh et al., 1980) by Southern blotting. Genomic DNA from a heterozygous cell line was digested with a restriction enzyme and hybridized to probe pHLA-1. DNA from variants of the same line, which had lost one or more alleles, was digested with the same restriction enzyme as the parent line and hybridized to the probe. By comparing Southern blot patterns individual restriction fragments could be correlated to the expression of specific HLA alleles. Another approach was taken by Korman et al. (1982). They purified polysomes carrying nascent polypeptides by an immunoaffinity column containing a monoclonal antibody against HLA determinants. This prepurified mRNA yields excellent starting material for the preparation of cDNA. As the antigenic specificity of the purified mRNA is known, full length cDNA prepared from this mRNA will have a nucleotide sequence related to that particular antigen.

cDNA probes have been used to investigate genomic DNA in man and in the mouse. An H-2 probe was found to hybridize to 15-20 restriction fragments on a Southern blot of mouse DNA that had been treated with *Bam*H1 and *Eco* R1, respectively. Therefore it was concluded that 15-20 copies of H-2 class I genes are present in the haploid genome (Cami et al., 1981). There is a discrepancy between the number of genes and the number of products. The 15-20 copies are more than one would expect to correlate with the different class I loci, but less than one would expect for all existing alleles.

The estimate from Malissen's experiments (Malissen et al., 1982) of the number of class I gene copies for HLA is about 30 copies per haploid genome. Some of these copies could be non-functional, so-called pseudogenes (Steinmetz et al., 1981).

The distribution of class I antigens into domains turns out to be parallelled in the DNA sequences. H-2 genes contain 8 exons, one for the signal sequence, three for the external domains, one for the transmembrane part and three for the cytoplasmic region (Steinmetz et al., 1981). In man, a similar picture is found, but there are two exons instead of three for the cytoplasmic region, and the transmembrane part and the cytoplasmic part are each partly coded for by the fifth exon (Malissen et al., 1982). Whether the complexity of the cytoplasmic region will reveal differentiation in function is not known.

As mentioned before, DNA sequencing can replace amino acid sequencing. In the case of the carboxyl-terminal end of the H-2 heavy chain, containing the hydrophobic transmembrane stretch, DNA sequencing was a good alternative from which the amino acid sequence could be deduced (Brégégère et al., 1981). Information on a recent meeting on the subject held in Oxford has been reported in Science (Marx, 1982) and Nature (Robertson, 1982). As a mechanism for the way in which the polymorphism in MHC products has arisen, gene conversion is favoured. Gene conversion is the process whereby genetic information can be transferred from one gene to another related gene anywhere in the genome. It seems to be the only way in which to account for stretches of variety in MHC antigens, as a number of point mutations seems unlikely. Pseudogenes could then function as a "reservoir of polymorphic variation".

I.6 Function of MHC antigens

The isolation (I.4) and the characterization (I.3) of MHC antigens, the experiments relating to the biosynthesis (I.5.1) and the genetic organization (I.5.2) of MHC antigens all have as an ultimate goal the examination of the function of the MHC antigens. Although MHC antigens have first been detected in transplantation experiments, their normal biological role cannot be in transplantation rejection, as organ transplantation is an artefact.

The best studied function of MHC class I antigens is the so-called MHC restriction (Doherty and Zinkernagel, 1975). The killing of virus-infected cells by cytotoxic T cells depends on the identity of the MHC class I antigens on the infected cells and the effector cells. The cytotoxic T cells are specific both for the foreign antigen and a "self" MHC product. MHC restriction has been demonstrated for the first time in the mouse in choriomeningitis infected target cells (Zinkernagel and Doherty, 1974). It has also been reported in man and mouse for cell-mediated lympholysis reactions against other viruses (Gardner et al., 1974, McMichael et al., 1977, Rickinson et al., 1980), chemically modified cells (Dickmeiss et al., 1977, Shearer, 1974) and cells expressing the H-Y antigen (Gordon et al., 1975, Goulmy et al., 1977). The requirement for MHC homology has also been suggested for lysis of cells expressing tumour-associated antigens (Schrader and Edelman, 1976, Germain et al., 1975, Kaneko et al., 1978). Zinkernagel has put forward a theory in which susceptibility to diseases associated with certain MHC haplotypes may directly reflect MHC restriction of T cells (Zinkernagel, 1979). This will be further discussed in I.7.

MHC restricted effector cells may recognize the foreign antigen and the self MHC product as two entities (dual recognition). Another hypothesis is that the foreign antigen on the cell surface interacts with the MHC antigen. The resulting change of the MHC antigen is then recognized by T lymphocytes (the "altered self" hypothesis). These two theories have led to experiments with the objective of solving the identity of the T cell receptor.

In the case of "altered self", MHC antigens could be a receptor for the foreign antigen. Helenius and colleagues (Helenius et al., 1978) have shown that HLA-A, -B and H-2K, -D antigens can function as receptors for Semliki Forest Virus. Daudi cells, which lack surface expression of HLA, were not susceptible to the virus. An association of rat MHC antigens with an adenovirus protein in cells transformed by this virus has been reported (Kvist et al., 1978). In their immunoprecipitation experiments, MHC antigens and a viral antigen were coprecipitated. More recently it was shown that the viral glycoprotein specifically binds to the heavy chain of class I antigens in both man and mouse (Signäs et al., 1982). The possibility of an association between H-2 and viral antigens is disputed by another group of investigators (Calafat et al., 1981). They did not find an association of viral antigens and H-2 on the virus-infected cell surface by patching and capping studies. HLA antigens incorporated into liposomes can bind to certain bacteria (Klareskog et al., 1978).

It has been shown that some combinations of foreign antigens + self markers are better recognized immunologically than others (McMichael, 1978). In this context, polymorphism at the level of the MHC could be very valuable. With the codominant expression, heterozygotes are at an advantage as far as fighting viral infections is concerned. Arguing on the level of the species, the greater the polymorphism, the greater the chance that the viral antigens wil be recognized in the context of a self product and that the infected cells will be eliminated.
The technique of gene transfer into mouse L cells has scope for introducing MHC antigens into cells at will and investigation of these together with foreign antigen as a target for MHC restricted T cells will be possible. This type of experiment has been performed and it has fulfilled expectations; a transferred H-2L^d gene brought about cell surface expression of an H-2L^d antigen which could serve as a restriction element in lymphocytic choriomeningitis virus specific killing (Örn et al., 1982). So it seems essential, in MHC restricted responses, that sufficient "self" MHC antigen is present on the target cell membrane. An increase in HLA synthesis was found in melanoma cells after culture in the presence of interferon (Basham et al., 1982). Different cell lines were studied and an increased cell surface expression of HLA correlated with the antiviral function of interferon. This is in agreement with a role for increased expression of HLA on infected cells, which would facilitate the killing of these cells by MHC restricted T cells.
The unusual strength of the allogeneic response could be explained by assuming that allogeneic MHC antigens are just a different form of "self + foreign antigen X". Alloreactive T cells were found to be highly cross-reactive with TNP (trinitrophenyl) modified syngeneic cells (Lemonnier et al., 1977).

I.7 **MHC and disease**

After the demonstration by Lilly et al. (1964) that susceptibility to Gross virus-induced leukaemia was influenced by H-2 linked genes, and the finding of McDevitt and Chinnitz (1969) that Ir genes, which determine the immune response to certain antigens are linked to the H-2 system, it was only natural that the relationship between MHC antigens and disease was investigated.
In the dog, some studies have been carried out concerning leucosis, diabetes mellitus and autoimmune hemolytic anaemia. With the particular antiserum used, it was found that DLA-B7 is more frequent than expected in leucosis dogs, and in dogs with diabetes mellitus DLA-A3 and DLA-B7 occur more often than expected. In autoimmune hemolytic anaemia no correlation with DLA was found. When the p values are corrected for the number of comparisons made, the correlations are no longer significant (Vriesendorp et al., 1973).
In man, statistically significant associations were mostly found between HLA and immunopathological diseases (Dausset and Svejgaard, 1977). The strength of the association can be tested by calculating the relative risk (R.R.): the chance to get the disease when a particular MHC antigen is present in relation to the chance to get the disease without the MHC antigen present. In man, the correlation with the highest

R.R. found so far is that between HLA-B27 and ankylosing spondylitis with a relative risk of 88 for Caucasoids.
Several hypotheses have been proposed for the way in which the MHC influences susceptibility to disease. These include the following mechanisms: 1) the MHC antigens themselves may function as cell surface receptors for particular pathogens. 2) MHC antigens and certain pathogens may cross-react. This is the so-called mimicry. In the case of cross-reaction between MHC products and infectious agents, disease could be a consequence of tolerance. 3) the MHC antigens might have nothing to do with the disease, but be closely linked to the relevant genes. No evidence for this has been found.
In man, most of the associations with (immunopathological) diseases are with HLA-DR. One is tempted to think here that immune response genes are directly involved (Oliver, 1978). Most HLA-linked diseases are chronic or subacute. Generally they do not influence the capacity for reproduction as they occur later in life and therefore, they are not important for "natural selection". Zinkernagel (1979) speculates that the MHC associated diseases are caused by autoaggressive MHC restricted T cells. It is true that most HLA associated chronic diseases have an autoimmune component. These diseases may be caused by non- or poorly cytopathic agents that cause chronic infections. If so, there may be a balance for immunoprotection versus damage caused either by an infectious agent or by the ensuing immune response. MHC restricted effector cells may turn autoaggressive. After an investigation of the relationship between HLA and infectious diseases, de Vries and van Rood (1982) conclude that maybe the association of immunopathological diseases with certain HLA antigens may be an unfortunate side effect of the occurrence of these antigens which are favourable for other reasons, e.g. the resistance to infectious diseases.
HLA-A1/B8/DR3 is a haplotype with which several immunopathological diseases are associated. Individuals positive for HLA-A1/B8/DR3 have enhanced immune responsiveness. This could be due to a decreased suppressor cell function (Ambinder et al., 1982). On the other hand, this haplotype may play an important role in recovery from viral infection and in survival of patients with lymphoma or leukaemia (Oliver, 1977).
Concluding, one can remark that each HLA determinant has its own capacity for immune response, which may be favourable in certain cases and less favourable in others. Polymorphism is maintained to maximize the chance that amongst the various individuals there are always some of the species who survive.

I.8 MHC antigens on tumour cells

Much attention has been focused on the phenomenon of alien MHC antigens on tumour cells. As tumour cells are abnormal, any difference between tumour cells and normal cells is worth investigating in order to find the reason for the tumour cell behaviour. The occurrence of alien MHC antigens could explain something

about tumour cell function, and at the same time it may explain something about MHC function.

Especially in inbred strains of mice, several tumours have been found which carry H-2 class I antigens that cannot be accounted for by the genetic make-up of the strain. The evidence for the presence of alien histocompatibility antigens on tumour cells was in most cases first found in transplantation studies (Martin and Imamura, 1980, Parmiani et al., 1979). Further investigations comprised serological assays (cytotoxicity, absorption, blocking) (Parmiani et al., 1979, Schirrmacher et al., 1980a, Schmidt et al., 1980), assays for cell-mediated immunity (Festenstein et al., 1980, Imamura and Martin, 1980, Parmiani et al., 1979, Schirrmacher et al., 1980b) and biochemical studies (Parmiani et al., 1979, Schmidt et al., 1980).

In spite of these indications, the presence of alien histocompatibility antigens on tumour cells has only been really established in a few cases. When more refined techniques were applied such as peptide mapping and immunoprecipitation, some "alien" histocompatibility antigens turned out to be caused by contamination with other cell lines (Robinson et al., 1981) or cross-reactive viral antibodies in the sera were responsible for the "extra" reactions (P.A. Klein, 1975). The H-2 antisera are induced by using normal cells so it is not surprising that they may behave differently when used on tumour cells. Even if the presence of real alien histocompatibility antigens is established, their function is unknown. It is doubtful whether they function as tumour associated transplantation antigens (Parmiani, 1980).

Robinson and colleagues found by cloning of a sarcoma tumour, MCG4, which had been shown to possess foreign H-2 specificities (Schirrmacher et al., 1980) that four different haplotypes could be demonstrated (Robinson et al., 1981). Typing for independent genetic markers (β_2m, gpi 1) identified one contaminant. So it is essential to use cloned tumours, homogeneous typing reagents, and to type for genetic markers not linked to the MHC. It is evident that biochemical studies, preferably at the DNA level, are required to give the ultimate proof of the presence of "alien" histocompatibility antigens. Reviews on the subject ar given in the Journal of Immunogenetics (1980), Transplantation Proceedings (1980, 1981) and by Festenstein and Schmidt (1981).

There are several possible mechanisms of expression of alien histocompatibility antigens: alien histocompatibility antigens as hidden normal antigens, mutation of H-2 structural genes or derepression of silent genes. Epigenetic mechanisms are also conceivable, like changes in translation, glycosylation, membrane insertion or specific association with additional molecules. In view of what is now known about the number of MHC class I genes per haploid genome, which is much greater than the number of class I loci previously defined (Cami et al., 1981, Malissen et al., 1982), the derepression hypothesis might be correct. DNA methylation patterns of the genes coding for MHC antigens should be compared in normal and tumour cells. There are many examples of an inverse correlation between the level of methylation and the transcriptional activity of a gene (Felsenfeld and McGhee, 1982). Derepression of genes for histocompatibility antigens as the cause of appearance of alien antigens is suggested by Festenstein and Schmidt (1981),

Parmiani et al. (1979) and Robinson and Schirrmacher (1979). The expression of alien H-2 antigenic specificities, as distinguished from actual alien H-2 molecules, may be much more common. Under certain circumstances, normal $H\text{-}2^k$ molecules on tumour cells can express alien alloantigenic specificities. This can only be explained by some conformational alteration in the $H\text{-}2^k$ molecules, which were the "normal" H-2 antigens in the tumour studied (Rogers et al., 1981).

Changes in histocompatibility antigens on tumour cells could have several implications for tumour immunology. Not only gain of "alien" histocompatibility antigens occurs, loss of the original histocompatibility antigens has been found, too. A loss of antigenic determinants might cause a less efficient associative recognition by MHC restricted cytotoxic T cells.

One can envisage that the presence of extra histocompatibility antigens might have different effects. The alien antigens might function as a target for the immune response. Alternatively, the alien histocompatibility antigens may not be suited to function as restricting elements, or they may interfere with associative recognition, so that the tumour cells can escape T cell cytotoxicity. They may also stimulate suppressor cells which would facilitate their escape. Normally, cells bearing alloantigens are eliminated by the immune system. With the alien antigen bearing tumour cells this is not always the case. Sondel and Hank (1981) have proposed a theory of "alien-selected escape". If tumorigenesis requires involvement of a pathogen which leaves a surface antigen X on the cells, the alien histocompatibility antigens may be altered by X in such a way that they look like "self" and cannot be recognized by syngeneic cytotoxic T cells. This is a different version from what happens normally, according to the "altered self" hypothesis (I.6) where the "self" antigens are changed by a viral antigen into "altered self", and where the "altered self" antigens are the target for MHC restricted T cells.

The data on alien histocompatibility antigens on human tumour cells are scarce. A structural relationship between β_2m and tumour associated antigens is suggested by Thomson et al. (1978), who could isolate tumour associated antigens by affinity chromatography employing an anti-β_2m serum. Some investigators report that most tumour cells do not display changes in HLA phenotype (Mayer et al., 1982, McAlack, 1980, Pollack et al., 1981). Ferrone and colleagues (1980) on the other hand, have found evidence for the expression of alien histocompatibility antigens on tumour cells of different histological origin together with other changes in surface markers on human and mouse tumour cell lines.

Several human tumour lines express DR alloantigens although these do not cause stimulation in primary mixed lymphocyte reactions (Pollack et al., 1981). What is now known to be anti-HLA-DR reactivity in HLA-A, -B, and -C typing sera may have caused "extra reactions" on tumour cells.

Manipulation of the immune response in favour of tumour destruction has been attempted by several groups. In vitro stimulation of human or mouse cells with a pool of allogeneic cells has been shown to give rise to T cells and non-T cells which could lyse autologous or syngeneic abnormal cells in vitro (Bach et al., 1980). This would imply that the antigens recognized by these effector cells on autologous

abnormal cells are alien histocompatibility antigens or cell surface antigens that are cross-reactive with alloantigens. In vivo experiments, also based on the assumption that tumour cells bear alien histocompatibility antigens, were performed by Truitt et al. (1980). Mice were immunized with a pool of allogeneic cells. Bone marrow from these mice was grafted into H-2 compatible leukaemic mice. This caused a significant graft versus leukaemia reactivity whereas lethal graft versus host disease was kept to a minimum.
These results do not necessarily mean that alloantigens on tumour cells are the target cells for the immune response.
The tumours used for the experiments of the two groups just mentioned were not described to contain alien histocompatibility antigens. Another example of the growth inhibition of a tumour after pool immunization with five different allogeneic cells is that of YC8 (Parmiani and Sensi, 1981). This tumour was shown to lack detectable amounts of alien H-2 antigens. The mechanism for the effect of pool immunization on tumour growth remains ill defined. Activation of NK cells is a possibility, or activation of cytotoxic T cells to alloantigens cross-reactive with tumour associated antigens.

In conclusion: Many of the early experiments on alien histocompatibility antigens on tumour cells have been shown to lack unambiguous evidence for the existence of these alien antigens. With the use of more refined techniques the number of cases of real alien histocompatibility antigens on tumour cells has dropped. The relevance of alien histocompatibility antigens on tumour cells as a target for the immune response is dubious. The presence of alien histocompatibility antigens on tumour cells has become a field of interest for the investigator of the MHC rather than for the tumour immunologist. Bone marrow transplantation or adoptive immunotherapy with cells after allogeneic stimulation may be clinically relevant, but their beneficial effect has probably nothing to do with the presence of alien histocompatibility antigens on tumour cells.

I.9 **Problem orientation, rationale of the thesis**

Most of the subjects in the previous sections, illustrating the main topics of MHC research, have not been studied in the dog. One of the reasons for this is that the biochemistry of DLA antigens has not yet been thoroughly investigated. As mentioned before, the dog as a laboratory animal has so far been mainly used for transplantation research. As a preclinical model the dog is very suitable, because of its size, temperament, longevity, large litter size and relatively short gestation period. Moreover it is outbred, as opposed to most other laboratory animals and therefore it reflects more realistically the human situation.
Tissue typing is a requirement for transplantation research. To broaden the basis upon which clinically relevant experiments can be done, it is necessary to further investigate the molecular nature of the MHC antigens which form, after all, the

main transplantation barrier. The problems encountered with organ transplantation have been brought to a cellular level by serological and MLR typing, and the next step is to investigate these antigens at the molecular level. Research into the biochemical properties of DLA antigens can benefit from the experience obtained and the techniques developed in studies on the H-2 and HLA antigens (I.3-I.5). It is necessary to know if the homology of the dog with other species on the level of organ transplantation, serology and MLR can be extended to homology on the molecular level. Class I antigens are investigated in this study as only class I specific antisera and anti-β_2m antiserum are available. As mentioned before, antisera are indispensable and must be used either for monitoring the isolation procedure or for the isolation itself.

Very little is known about the biochemical properties of DLA antigens. It is of interest to know whether DLA class I antigens are similar to class I antigens of other species. If for example the DLA alloantigenic determinant would turn out to be carbohydrate in nature, the DLA loci would have a regulatory function through glycosylating enzymes, rather than the structural function which they have when they code directly for a protein determinant. If the DLA antigenic determinant would be solely carbohydrate, this would be quite different from what has been found in other species, and it would have implications for the genetic organization of the DLA complex.

For DLA antigens the chemical composition, the tertiary structure, the role of β_2m and the location of the alloantigenic site are unknown.

The questions we wanted to answer were:

1. What is a good method for the isolation of DLA antigens?
   1a. papain digestion? (see chapter II)
   1b. detergent solubilization? (see chapter IV)
2. What is the molecular weight of the isolated molecules and what is their chemical composition? (see chapters II, IV, VI)
3. What is the role of β_2m? (see chapter III)
   3a. Is β_2m part of the DLA antigens? (see chapters II, IV)
   3b. Can an anti-β_2m serum be used for the immunoprecipitation of DLA antigens? (see chapter IV)
4. Do DLA antigens have a two chain structure like MHC antigens of other species?
   Do alloantisera and anti-β_2m sera give comparable immunoprecipitation patterns? (see chapter V)
5. What is the location and the nature of the alloantigenic determinant? (see chapter VII)
6. Is there a difference (quantitative or qualitative) in DLA antigens between tumour cells and PBL of the tumour bearer? (see chapter VII)

The rationale for investigating these questions can be understood from the

description of the isolation and characterization of HLA and H-2 antigens (I.4 and I.3). The outline of the thesis is such that the various experiments follow one another in a chronological order. At the end of each chapter the results are discussed in the light of the previous findings and in relation to the experiments in the following chapter.
Gaps in our knowledge of MHC antigens concern the relationship of structure and function and the organization of their genes. All species investigated so far have shown similar structures and genetic organization. More information about dog MHC antigen structure will provide evidence which either confirms previous data or contradicts them. In the first case yet another species will be added to the number of species which show homologous features and which can be used to draw up the evolutionary time scale.
Confirmation of the MHC class I antigen model by data on DLA class I antigens will give this model a more general validity.
If there is a discrepancy between previous data and the results of the investigation of dog MHC antigens, this may stimulate a closer examination of the difference(s) in order to clear up some of the unknown aspects of the MHC antigens.

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CHAPTER II

PURIFICATION AND CHARACTERIZATION OF PAPAIN SOLUBILIZED DLA CLASS I ANTIGENS*

II.1 Introduction

The Major Histocompatibility Complex antigens of many species have been found to be very similar to each other (Götze, 1977). In all species investigated so far the class I antigens consist of two non-covalently bound chains, one variant, the other invariant (Arnoux et al., 1974, Finkelman et al., 1975, Giphart et al., 1978, Katagiri et al., 1975, Kimball et al., 1979, Parham and Ploegh, 1980, Phillips et al., 1978, Strominger et al., 1981, Ziegler and Pink, 1975). The invariant chain is identical to β_2-microglobulin (β_2m). β_2m was first discovered in urine of patients with tubular dysfunction (Berggård and Bearn, 1968). It has a molecular weight of 11,800 daltons. The MW of the other chain varies with the isolation procedure used. For HLA it ranges from 34,000 daltons after papain digestion (Sanderson and Batchelor, 1968) to 44,000 daltons after detergent solubilization (Dautigny et al., 1973).

The two techniques used most for large scale isolation of class I antigens are detergent solubilization (Dautigny et al., 1973, Freed et al., 1979) and papain digestion (Sanderson and Batchelor, 1968, Shimada and Nathenson, 1969) of crude membranes. Large scale preparations of HLA have been obtained from Epstein-Barr virus (EBV)-transformed B cell lines (Strominger et al., 1975), spleens (Sanderson and Batchelor, 1968) and pooled platelets (Trägårdh et al., 1979b).

DLA antigens have not yet been studied at the molecular level. In order to isolate DLA class I antigens and to compare their biochemical properties with those of other MHC antigens, techniques that have been used to purify human (HLA) and mouse (H-2) histocompatibility antigens have been applied. There are no dog lymphoid cell lines available and dog lymphocytes do not have an Epstein-Barr virus receptor (G. Klein, personal communication). Therefore, spleens have been taken as a source of DLA antigens. In order to monitor the purification, it is necessary to test the biological activity of the product at the various purification stages. The biological activity is most easily measured when the DLA antigens are water soluble. Papain digestion has been chosen for the isolation of DLA antigens as it renders molecules water soluble. If DLA antigens are homologous to HLA and H-2 antigens, this procedure will release most of the molecule including the allo-antigenic site. The methods described by Parham et al. (1977) were adapted to suit the dog material.

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II.2 Materials and methods

II.2.1 *Preparation of crude spleen cell membranes*

Spleens were obtained from tissue-typed beagles and mongrel dogs. Small chopped pieces were passed through a sieve, and collected in 0.01 M Tris-HCl pH 8.0. The wet weight of the sifted pieces was determined, and they were washed six times with 15 ml of buffer per 10 grams of material. Each time they were centrifuged for 10 minutes at 3000 × g and 4°C. The low speed supernatants, containing the membranes obtained by hypotonic lysis, were combined and centrifuged for 1 hour at 100,000 × g and 4°C.

II.2.2 *Papain digestion*

After ultracentrifugation the membrane pellet was homogenized with an Ultra-Turrax TP 18-10 (Janke and Kunkel, Staufen i. Breisgau, F.R.G.). The pellet was resuspended to 6 mg protein/ml and treated with 60 units of papain/ml (type IV, Sigma Chemical Co., St. Louis, MO, U.S.A.) in 0.01 M Tris-HCl pH 8.0 containing 5 mM cysteine for 45 minutes at 37°C. The enzyme was inactivated by immediate cooling to 0°C. The solubilized proteins were separated from the membranes by centrifugation for 1 hour at 100,000 × g and 4°C.

II.2.3 *Column chromatography*

In order to remove the papain, the ultracentrifugation supernatant was applied to a DE 52 (Whatman, Maidstone, England) column under conditions in which HLA antigens are retained by the column whereas the papain appears in the flow-through, assuming that DLA antigens would behave similarly. A 10-15 ml DE 52 column, equilibrated with 0.01 M Tris-HCl pH 8.0 was used. After the column was washed with ten bed volumes of buffer, the putative DLA containing glycoproteins were eluted with 0.25 M NaCl in 0.01 M Tris-HCl pH 8.0. Fractions of 1.5-1.75 ml/half hour were collected. The first 5-10 fractions of the protein peak (as measured with an Uvicord II ultraviolet analyzer, LKB, Bromma, Sweden) were pooled and applied to a Sephadex G-150 column (Pharmacia, Uppsala, Sweden). A column of 68 × 2.6 cm was used with 0.14 M NH_4HCO_3 pH 8.0 as running buffer. G-150 fractions that contained polypeptide chains of 34,000-39,000 MW (as measured by SDS-polyacrylamide gel electrophoresis) were pooled and applied to a 1.5 × 3 cm Sepharose-lentil lectin column (Pharmacia, Uppsala, Sweden), equilibrated with 0.01 M Tris-HCl pH 7.8, 0.001 M $MnCl_2$, 0.0001 M $CaCl_2$, 0.14 M NaCl, 0.00025 M dithioerythritol (lectin column buffer). After application of the sample, the column was washed, equilibrated in lectin column buffer containing 2.5% α-methyl-D-mannoside and slowly eluted (flow rate 3-3.5 ml/hour). All chromatographic separations were performed at 4°C.

II.2.4 *Gel electrophoresis*

Electrophoresis was performed on 10 × 14 × 0.1 cm 12.5% SDS-polyacrylamide gels according to laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 as described by Vesterberg (1971). All chemicals for electrophoresis were from Bio-Rad, Richmond, CA, U.S.A.

II.2.5 *Protein assay*

Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, U.S.A.) with human serum albumin as a standard.

II.2.6 *Inhibition of cytotoxicity assay*

The ability of the purified material to inhibit the standard microlymphocytotoxicity test (Smid-Mercx et al., 1975) was assayed. Target cells were Ficoll-Isopaque purified peripheral blood lymphocytes (PBL) from tissue-typed beagles. Fractions of the Sephadex G-150 column and the lentil lectin eluate were dialyzed against 0.15 M NaCl. Typing serum was diluted two-fold in putative DLA containing column fractions and 40% normal dog serum (NDS). It was tested against appropriate target cells. Parallel to this, the same dilution range of typing serum in 0.15 M NaCl, 40% NDS, without G-150 or lentil lectin column fractions, was tested against the same target cells. As a control non-DLA containing fractions were also tested for inhibitory activity in the microlymphocytotoxicity test. Specificity control was a typing serum and target cells mismatched for the DLA-type of the spleen donor. All dilutions were left at 4°C for 16 hours before use.

II.3 Results

At first large scale preparation of DLA antigens was started from Ficoll-Isopaque purified spleen lymphocytes. We had thought that starting from selected material would facilitate the following purification. As the yields were very small, it was decided to use small chopped spleen pieces passed through a sieve, without bothering too much about the initial purity of the material. A summary of the methods used is given in Fig. 1. DE 52 ion exchange chromatography mainly separates papain from the digested material. The choice of DE 52 fractions for further purification was consequently based upon protein concentration only. A profile of a representative G-150 column, which separates the digested material according to MW, is shown in Fig. 2. Fractions eluted from this column were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions (Fig. 3). Fractions 29-33 contained polypeptide chains of 37,000 MW and 12,000 MW, more or less contaminated with a few other proteins. As a MW of 34,000-37,000 daltons is what one would expect for a papain-solubilized MHC molecule, these fractions, together with some others, were tested for their ability to inhibit a standard microlymphocytotoxicity assay (Table 1).

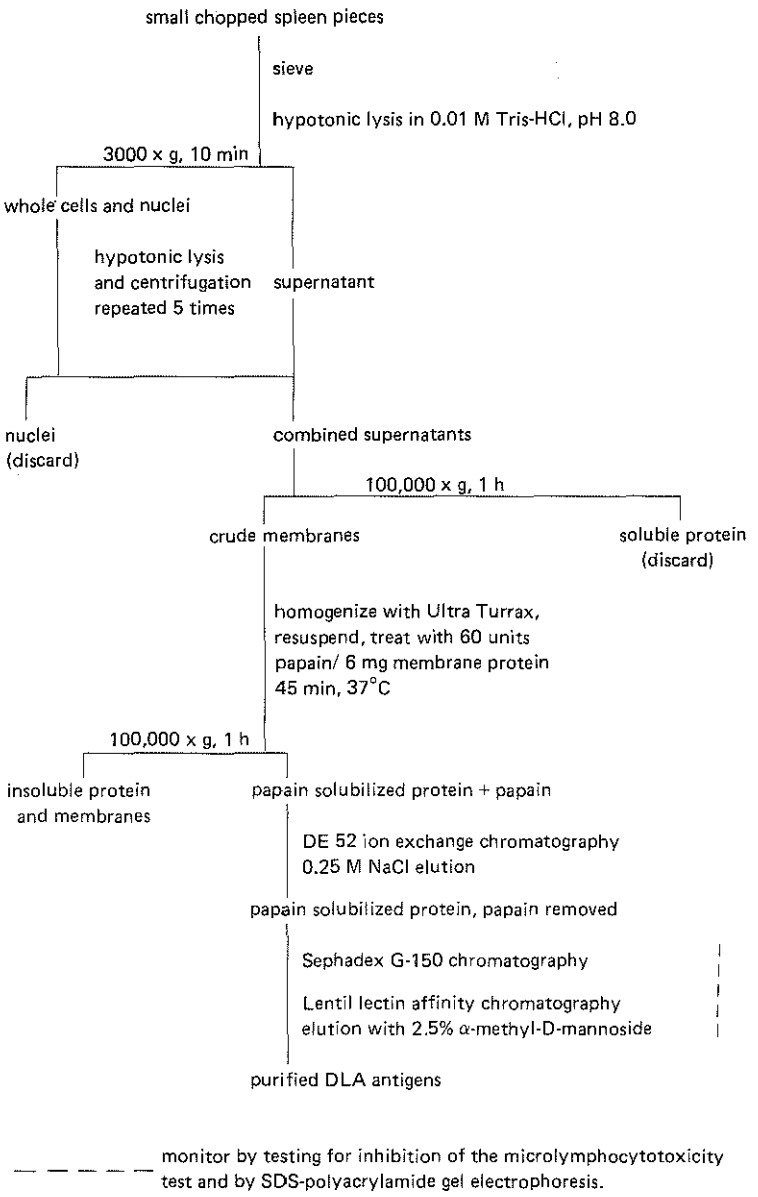

Figure 1. Method used for the isolation of papain solubilized DLA antigens from spleens.

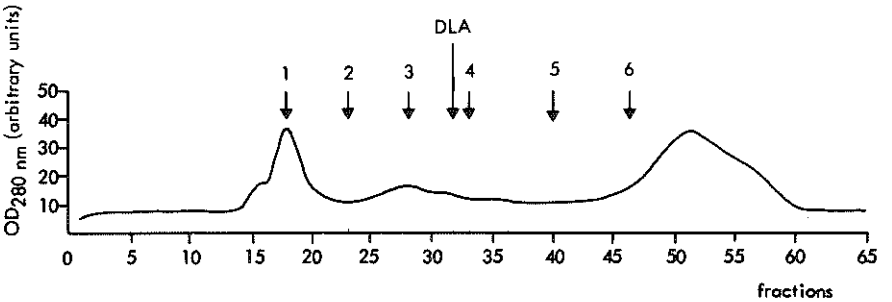

Figure 2. Gel chromatography on a Sephadex G-150 column (68 × 2.6 cm) of papain digested membrane proteins eluted with 0.25 M NaCl from a DE 52 column.
6 ml fractions, flow rate 12 ml/h, eluent 0.14 M NH_4HCO_3.
The arrows denote the marker substances: 1: Blue Dextran; 2: aldolase (MW 158,000); 3: albumin (MW 67,000); 4: ovalbumin (MW 43,000); 5: chymotrypsinogen A (MW 25,000); 6: cytochrome c (MW 12,300).

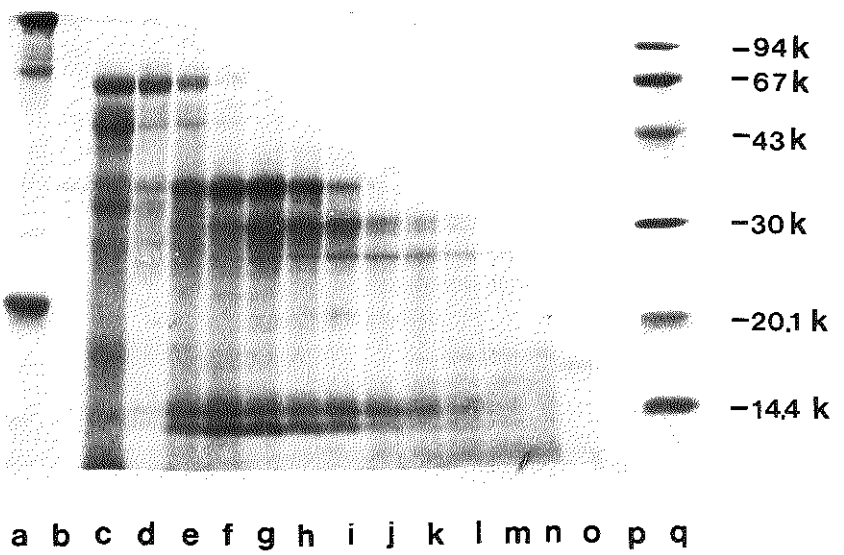

Figure 3. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of fractions from a Sephadex G-150 column (Fig. 2).
lanes a-p: fractions 18, 22, 27, 29-39, 42, 52.
lane q: molecular weight standards: 94,000 phosphorylase b; 67,000 albumin; 43,000 ovalbumin; 30,000 carbonic anhydrase; 20,100 trypsin inhibitor; 14,400 lactalbumin.

Table 1. Inhibition of cytotoxicity by DLA containing column fractions[a]

Antigen preparation (made to 40% NDS)	Dilution of an anti-DLA-A2 serum in 40% NDS/0.15 M NaCl			
	1/1	1/2	1/4	1/8
0.15 M NaCl	—[b]	—	—	—
G-150 fraction 18	—	—	—	—
G-150 fraction 23	—	—	—	+
G-150 fraction 26	—	—	—	+
G-150 fraction 29	—	—	+	++
G-150 fraction 30	—	—	++	+++
G-150 fraction 31	—	±	+++	+++
G-150 fraction 32	—	+	+++	+++
G-150 fraction 33	—	—	+±	++±
G-150 fraction 36	—	—	—	+
G-150 fraction 51	—	—	—	±
lentil lectin eluate	—	—	++	+++

Antigen preparation (made to 40% NDS)	Dilution of an anti-DLA-B6 serum in 40% NDS/0.15 M NaCl			
	1/1	1/2	1/4	1/8
0.15 M NaCl	—	—	—	—
G-150 fraction 18	—	—	—	—
G-150 fraction 31	—	—	—	—
G-150 fraction 51	—	—	—	—

[a]target cells: peripheral blood lymphocytes typed as DLA-A2, B5, C R20/A9, B6, C12. Peripheral blood lymphocytes from the spleen donor were DLA-A2, B5, C R20/A9, B4, C-. The anti-DLA-B6 serum is a specificity control.

[b]scores:

-	no inhibition	++	37.5%-50% inhibition
±	0-12.5% inhibition	++±	50%-62.5% inhibition
+	12.5%-25% inhibition	+++	62.5%-75% inhibition
+±	25%-37,5% inhibition		

The preparation described here was isolated from a beagle with tissue-type A2,B5,R20/A9,B4,C-. For testing specific inhibition, an anti-DLA-A2 serum was chosen and, as a control, an anti-DLA-B6 serum. Target cells were PBL from a beagle of tissue-type A2,B5,R20/A9,B6,C12.

The anti-DLA-A2 serum was titrated to see at which dilution the serum would no longer be cytotoxic. The anti-DLA-A2 serum in a dilution of 1/8 killed 100% of the target cells; in a dilution of 1/16 it killed only 50% of the target cells. In Table 1 the inhibition of cytotoxicity is shown. When cytotoxicity was 100%, inhibition was 0% and vice versa. Dilutions of the anti-DLA-A2 serum up to 1/8 caused 100% cytotoxicity (Table 1, line 1). Thereafter, from 1/16, less cytotoxicity was observed solely because of the dilution of the serum. Real inhibition therefore took place when serum diluted less than 1/16 was not able to kill all cells. This real inhibition

could have been caused by soluble DLA antigen present in the column fractions with which the antiserum was diluted during the 16 hour preincubation period. As serum dilutions for the microlymphocytotoxicity test in the dog have to be in 0.15 M NaCl, 40% NDS, fractions of the Sephadex G-150 column (Figs. 2,3) had to be dialyzed against 0.15 M NaCl, and made to 40% NDS.

The absolute amount of DLA antigens in the column fractions was not known. As judged from the SDS-PAGE patterns, the 37,000 MW band of the highest intensity was present in fraction 32. The minimum amount of fraction 32 protein which would inhibit the (100%) cytotoxicity caused by the 1/8 diluted antiserum (Table 1, line 1) for at least 75% was determined (Table 1, line 8). Similar volumes of the other column fractions were added to the anti-DLA-A2 serum in 0.15 M NaCl, 40% NDS in such a way that a 1/1-1/8 dilution range was obtained. They were tested in the assay. Fraction 32 turned out to be the most inhibitory fraction, fraction 18 did not inhibit at all, and all other fractions had intermediate inhibitory activity. A 100% inhibition has not been observed, possibly attributable to the presence of other antibodies in the antiserum used, e.g. anti-class II antibodies. Specificity controls were performed by using an anti-DLA-B6 serum, mismatched for the DLA type of the spleen donor. This serum was not inhibited (Table 1). Sephadex G-150 fractions 29-33 were pooled and applied to a Sepharose-lentil lectin column. Some purification was achieved, as judged by the SDS-PAGE pattern (compare Fig. 3 to Fig. 4).

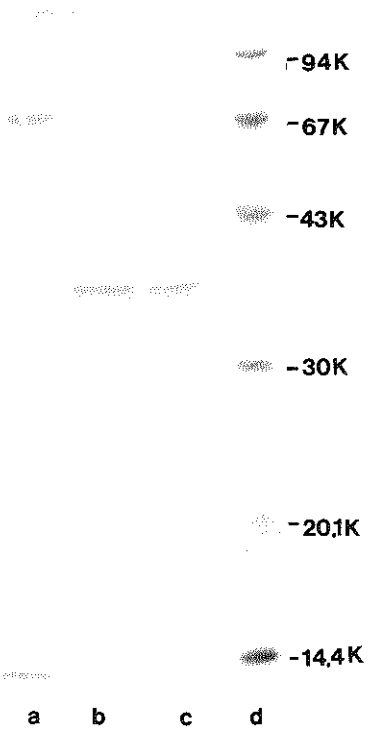

Figure 4. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of fractions from a Sepharose-lentil lectin column.
Fractions 29-33 from a Sephadex G-150 column (Figs 2,3) were applied to the lentil lectin affinity column. The 2.5% α-methyl-D-mannoside eluate was pooled into two fractions and samples were applied to the slab gel. lane a: flow-through of the column, lane b: eluate fraction 5-8, lane c: eluate fraction 9-10, lane d: molecular weight standards as described for Fig. 3.

Based on the results of the inhibition test, an amount of lentil lectin eluate as inhibitory for the anti-DLA-A2 serum as fraction 32 was calculated. In fact, this amount of lentil lectin eluate was slightly less inhibitory (Table 1), probably because there was some loss of DLA antigens in the last purification step.
The molecular weight of the polypeptide chains was estimated by SDS-PAGE (Fig. 4) to be 37,000 and 12,000 daltons, respectively.

II.4 **Discussion**

DLA antigens can be isolated from spleens by methods that have been developed for large scale preparation of HLA and H-2 antigens (Sanderson and Batchelor, 1968, Shimada and Nathenson, 1969). They behave like proteins in the isolation procedure and must have a carbohydrate moiety because of their binding to lentil lectin. Lentil lectin binds to α-D-glucosyl and α-D-mannosyl residues (Sharon and Lis, 1972). Under reducing conditions the isolated molecules appear to be composed of two polypeptide chains of an apparent molecular weight of 37,000 and 12,000 daltons (Fig. 4). SDS-PAGE is not an accurate method for the determination of the MW of glycoproteins (Segrest et al., 1971). For the present studies, however, a rough estimate was sufficient.

The recovery of DLA antigens was about twice as much as that published by Trägårdh et al. (1979a) from human spleens; 2.7 mg from 21,800 mg of crude membranes. It should be possible to get a better yield, even without using B lymphoid cell lines, which are known to have an increased expression of HLA antigens (McCune et al., 1975). Parham et al. (1977) obtained 4 mg HLA antigens from 1750 mg of crude membranes from a B cell line. It is possible to get a similarly high yield from spleens, as shown by Metzger et al. (1981) who obtained 3.6 mg SLA (swine MHC antigens) from 735 mg of crude membranes. One of the reasons for differences in recovery of MHC antigens from spleens could be that Trägårdh et al. need more purification steps than the other group, with the unavoidable loss of material at each step.

The DLA antigens were obtained by applying conventional biochemical techniques, without the use of antisera. Their biological activity was demonstrated by their capability to react with the alloantisera with which they were originally defined. The alloantisera available have been characterized by their reaction in a microlymphocytotoxicity test with beagle and mongrel dog PBL as target cells (Vriesendorp et al., 1977). The isolated molecules were shown to inhibit this cytotoxicity in a specific way, i.e. they inhibited only a reaction of a typing serum (anti-DLA-A2) matched with the spleen donor (A2,B5,R20/A9,B4,C-); this is shown in Table 1. DLA preparations isolated from other spleens by the same methods as those described here were also able to specifically inhibit the microlymphocytotoxicity test (results not shown). It was not possible to test earlier steps in the isolation procedure than the G-150 fractions, due to the presence of non-specific inhibition.

Another technique which can be used to investigate the alloantigenic state of the isolated molecules is immunoprecipitation of radiolabelled cell surface antigens with various antisera. This will be described in chapter V. This technique has also been used to demonstrate that β_2-microglobulin probably forms the light chain of the MHC class I antigens (Grey et al., 1973, Rask et al., 1974). The experiments described here indicate only that a polypeptide chain of 12,000 daltons is isolated together with a polypeptide chain of 37,000 daltons. The MW of β_2m is approximately 12,000 daltons as well, so the DLA light chain could very well be identical to β_2m. Immunoprecipitation of DLA antigens with an antiserum against β_2m will be necessary to really prove this. Such experiments are described and discussed in chapter IV.
Lyophilization of DLA antigens causes dissociation of the two chains. It is then possible, by lentil lectin affinity chromatography, to separate the heavy chain from the light chain, as the latter contains no carbohydrate. A rabbit antiserum was raised against the heavy chain. Immunoprecipitations with this antiserum are described in chapter V.
Further evidence for the identity of the molecules isolated here, and for possible homologies with HLA and H-2 antigens, will come from amino acid sequence analyses as described in chapter IV.
In the isolation procedure described here it is not possible to discriminate between an intracellular or a cell surface location for the DLA antigens. However, the microlymphocytotoxicity test can only work when the target antigens are on the cell surface. Immunoprecipitation experiments, referred to above, can be designed in such a way that only surface proteins are detected. In chapter IV, further confirmation of the cell surface location of the DLA antigens will be presented.
In the HLA system, it is possible to separate different antigens by ion exchange chromatography (Turner et al., 1975). So far this has not been described for DLA antigens. SLA antigens from inbred miniature swine isolated by papain digestion of spleen cells are relatively homogeneous (Metzger et al., 1981), whereas SLA antigens from non-inbred swine show substantial biochemical differences (Arnoux et al., 1974). For the separation of different SLA antigens, DEAE chromatography is the method of choice.
Many gel patterns of lentil lectin eluates of DLA antigens show an additional band of 30,000 MW. Possibly these are the dog equivalents of HLA-DR antigens. HLA-DR antigens bind to lentil lectin through their carbohydrate chains (Springer et al., 1977). As no class II specific antisera are available it is not possible to further characterize the 30,000 MW molecules.

The DLA antigens described in this chapter resemble MHC class I antigens of other species in the molecular weight of the polypeptide chains and their behaviour in biochemical and immunological procedures. They form a sufficiently pure starting material for amino acid sequence studies, which will make comparison with MHC antigens from other species even more valuable.

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through a filter of sand and NaN_3 was added to a concentration of 0.02%. The urine was dialyzed extensively at 4°C against tap water in Thomas (Philadelphia, PA, U.S.A.) dialysis tubing with a cut-off at 8000 MW. After dialysis the urine was lyophilized. It was dissolved in 40 ml of 0.01 M Tris-HCl pH 7.4, 1 M NaCl, 0.02% NaN_3, to a protein concentration of 25 mg/ml (reading the absorbance at 280 nm, using bovine serum albumin as a standard). The mixture was stirred at 4°C for 30 minutes and centrifuged for 10 minutes at 10,000 × g. The supernatant was divided into 10 ml aliquots and applied to a Sephadex G-150 (Pharmacia, Uppsala, Sweden) column of 68 × 2.6 cm in 0.1 M NH_4HCO_3 pH 7.8, 0.02% NaN_3 at a flow rate of 20 ml/hour, with 10 ml fractions. Low molecular weight fractions were pooled and concentrated in an Amicon (Lexington, MA, U.S.A.) 400 ml ultrafiltration cell, equipped with a UM 2 (MW cut-off 1000) filter. Aliquots of the pooled low MW fractions were applied to a Sephadex G-75 superfine column (Pharmacia, Uppsala, Sweden) of 1.5 × 90 cm in 0.1 M NH_4HCO_3 pH 7.8, 0.02% NaN_3, at a flow rate of 9 ml/hour, with 1.8 ml fractions. Fractions were analysed by SDS-polyacrylamide gel electrophoresis (III.2.2) and enzyme linked immunosorbent assay (ELISA, III.2.4). All chromatographic separations were performed at 4°C.

III.2.2 *Gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was conducted according to Laemmli (1970) on 12.5% acrylamide gels of 10 × 14 × 0.1 cm. Gels were stained with Coomassie Brilliant Blue R-250 (Vesterberg, 1971). All chemicals for electrophoresis were from Bio-Rad, Richmond, CA, U.S.A..

III.2.3 *Antisera*

A rabbit anti-dog-β_2m serum was kindly given to us by Dr. M.D. Poulik (Royal Oak, MI, U.S.A.). More antiserum was raised in two rabbits. Aliquots of purified β_2m (III.2.1) (0.5 mg in 0.5 ml 0.9% NaCl) were emulsified in 0.5 ml complete Freund's adjuvant (Difco, Detroit, MI, U.S.A.) and injected subcutaneously at five different sites on the back. Seven boosts, in incomplete Freund's adjuvant (Difco, Detroit, MI, U.S.A.), were performed at three week intervals. After each boost the sera were tested in the ELISA (III.2.4).

III.2.4 *ELISA*

The ELISA was performed by the (modified) procedure of Engvall and Ruoslahti (1979). Terasaki trays (Greiner, Nürtingen, F.R.G.) were coated with 50 μg/ml poly-L-lysine (Sigma, St. Louis, MO, U.S.A.). Each well was filled with 10 μl of a 100 μg/ml protein solution to be tested for the presence of β_2m. After 30 minutes the trays were washed with 2% bovine serum albumin in phosphate buffered saline (BSA/PBS) and 5 μl of dilutions of rabbit anti-dog-β_2m in 4% BSA/PBS/0.05% NNP10 (a non-ionic detergent from Servo, Delden, the Netherlands) were added. After one hour of incubation, the trays were washed again and 5 μl of a goat anti-rabbit-IgG coupled to horse radish peroxidase (Nordic, Tilburg, the Netherlands),

diluted 1:200 in 4% BSA/PBS/0.05% NNP10 was added for one hour. After another wash, 5-aminosalicylic acid (Merck, Darmstadt, F.R.G.), 0.8 mg/ml, pH 6.5, and 0.05% H_2O_2 were added in a 9:1 ratio, 10 μl per well. The reaction was terminated after 30 minutes by the addition of 1 M NaOH, and the intensity of the brown colour was recorded for each well. As a control non-immune rabbit serum and non-β_2m containing fractions or lysozyme (Sigma, St. Louis, MO, U.S.A.) were used.

III.2.5 *Immunochemical techniques*

Double diffusion tests according to Ouchterlony (1958) were performed in 1% agar (Difco, Detroit, MI, U.S.A., agar noble) in PBS. The immunoelectrophoresis method of Scheidegger (1955) was used. After serum incubation, both Ouchterlony and immunoelectrophoresis plates were washed extensively with 0.9% NaCl and distilled water. The slides were dried and they were stained with 0.05% thiazine red and 0.05% amido black in 10% acetic acid and 90% methanol. For destaining a solution of 10% acetic acid and 50% methanol was used.

III.2.6 *Cytotoxicity testing*

The cytotoxicity of the sera, raised against dog β_2m was tested in the two-stage microlymphocytotoxicity test (Smid-Mercx et al., 1975).

III.2.7 *Analytical isoelectric focusing*

Isoelectric focusing was performed on slab gels of 10.5×0.15 cm with a length according to the number of samples analysed. The gels contained 6.75 M Urea (Merck, Darmstadt, F.R.G.), 7% acrylamide, 0.19% N,N'-methylene-bis-acrylamide (Bio-Rad, Richmond, CA, U.S.A.), 2% v/v NNP10 (Servo, Delden, the Netherlands) and 2.5% w/v Ampholine carrier ampholytes (LKB, Bromma, Sweden) consisting of 11.2% pH 4-6, 11.2% pH 5-7, 5.2% pH 9-11 and 73.2% pH 3.5-10 ampholytes. The urea was deionized and filtered on a 0.45 μm filter before use. The gels were polymerized with 0.025% ammoniumpersulphate and 0.025% TEMED (Bio-Rad, Richmond, CA, U.S.A.). Electrode buffers were 1 M NaOH at the cathode and 1 M H_3PO_4 for the anode. For the isoelectric focusing an LKB (Bromma, Sweden) 2117 Multiphor flat bed gel apparatus was employed. The glass plate was water-cooled to a temperature of 7°C. After focusing, the pH gradient was determined by cutting out equal pieces of gel across the width, soaking them in distilled water for 2 hours at room temperature and measuring the pH.

III.3 Results

In order to isolate dog urinary β_2m, we have used methods most of which work by separation on a molecular weight basis. A summary of the procedure is given in Fig. 1.

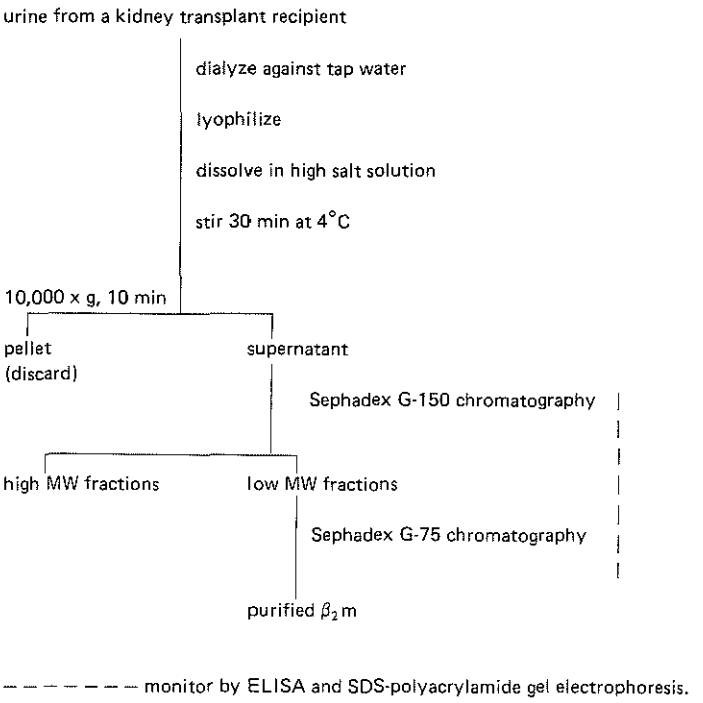

Figure 1. Method used for the isolation of dog β_2m from urine.

The isolation of β_2m by gel chromatography (Fig. 2) was monitored by SDS-polyacrylamide gel electrophoresis (Fig. 3). On these gels, human β_2m was used as a reference. Fractions 23-34 of the Sephadex G-150 column contained a protein of the same molecular weight as human β_2m (12,000 daltons). The protein concentration of aliquots of these and some adjacent non-β_2m containing fractions was adjusted to 100 μg/ml and they were tested for reactivity with Dr. Poulik's antiserum against dog β_2m in the ELISA. Fractions 26-33 were positive, whereas fractions 15, 18, 23, 34-36 hardly reacted. The reactive fractions were pooled, concentrated and further purified on Sephadex G-75. Some G-75 fractions were rechromatographed on the same column. The isolated β_2m was pure as far as it could be detected by SDS-PAGE.

This purified β_2m was used to prepare rabbit anti-dog-β_2m antisera (III.2.3). After each boost, the titre of the sera was determined in the ELISA (III.2.4). For the ELISA, the β_2m used for injection was taken as the antigen and Dr. Poulik's antiserum as a positive control. Non-immune rabbit serum and G-150 fraction 18 or lysozyme were used as a negative control. After seven boosts, the sera of both rabbits employed reacted with β_2m above background up until a dilution of $1:10^5$. The sera produced were compared to the serum given to us by Dr. Poulik in an

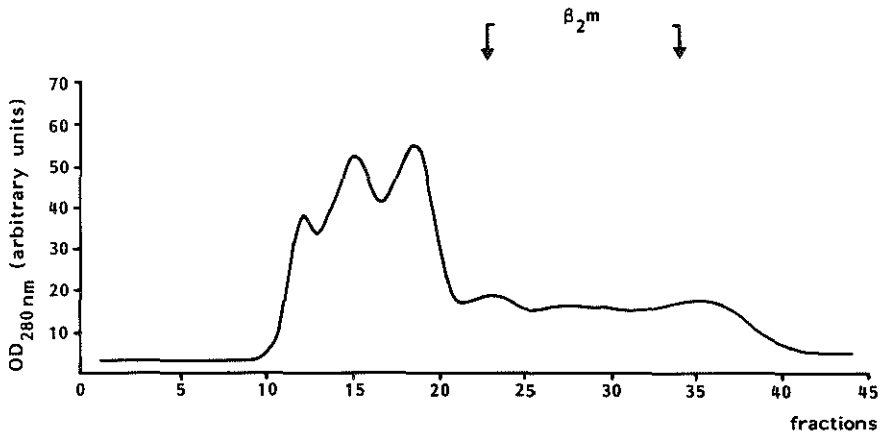

Figure 2. Gel chromatography on a Sephadex G-150 column (68 × 2.6 cm) of dialyzed and concentrated urinary proteins from a kidney transplant recipient.
10 ml fractions, flow rate 20 ml/h, eluent 0.1 M NH_4HCO_3, 0.02% NaN_3. Molecules of a MW similar to that of human β_2m are found in the fractions between the arrows.

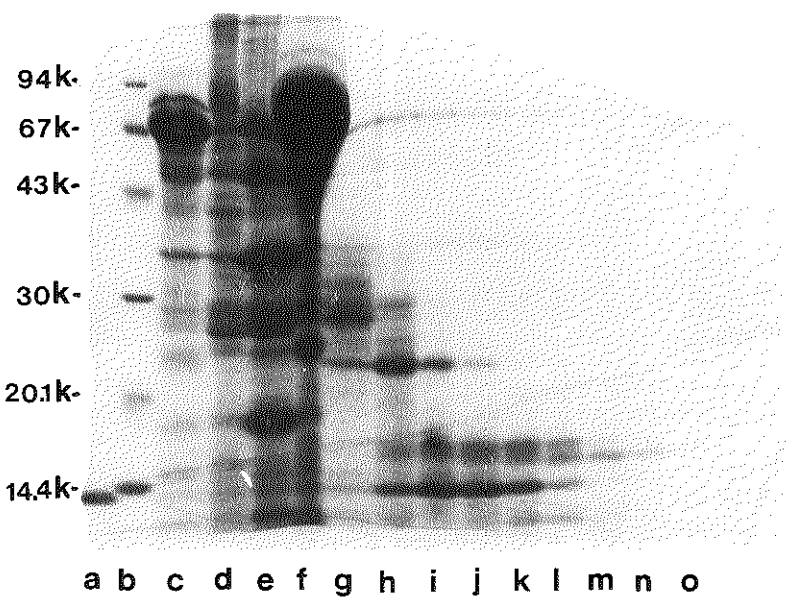

Figure 3. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of fractions from the Sephadex G-150 column of Fig. 2.
lane a: human β_2m
lane b: molecular weight standards: 94,000 phosphorylase b; 67,000 albumin; 43,000 ovalbumin; 30,000 carbonic anhydrase; 20,100 trypsin inhibitor; 14,400 lactalbumin;
lanes c-o: fractions 12, 15, 18, 23, 26, 29-36 of the G-150 column of Fig. 2.

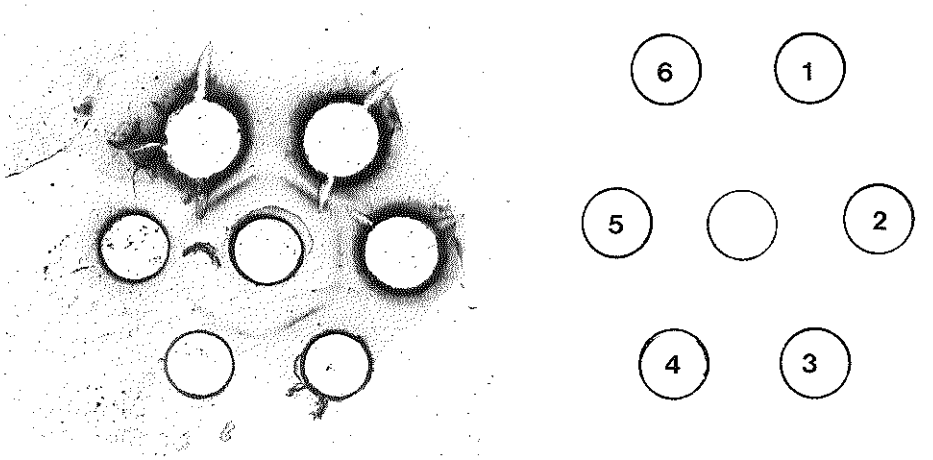

Figure 4. Ouchterlony double diffusion test of two rabbit anti-dog-β_2m sera. The centre well contained dog β_2m, concentrated G-75 fraction 24. The other wells contained: well 1: anti-dog-β_2m serum from rabbit 1; well 2: rabbit anti-dog-β_2m serum from Dr. Poulik; well 3: anti-dog-β_2m serum from rabbit 1, diluted 1/2; well 4: as well 3; well 5: rabbit anti-dog-β_2m serum from Dr. Poulik, diluted 1/2; well 6: anti-dog-β_2m serum from rabbit 2.

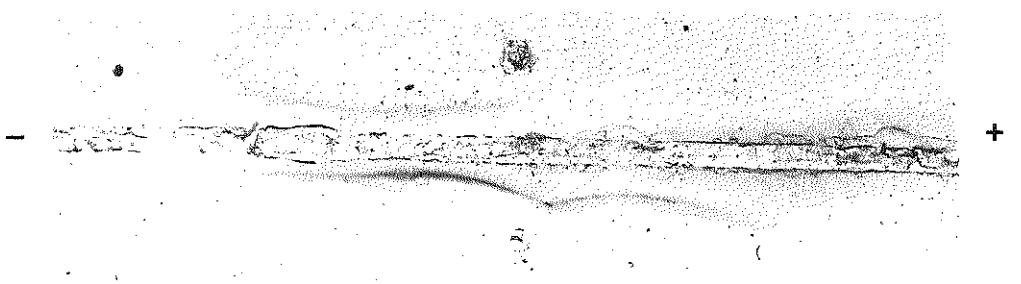

Figure 5. Immunoelectrophoretic analysis of purified β_2m. Buffer: 0.12 M sodium barbital pH 8.6. Upper well: G-75 fraction 23; bottom well: G-75 fraction 24; trough: anti-dog-β_2m serum from rabbit 2.

Ouchterlony test (Fig. 4). The antisera were used undiluted or diluted 1/2. A G-75 fraction containing pure β_2m as judged by SDS-PAGE was concentrated by lyophilization and redissolution in 1/10 of the original volume. Lines of identity were observed between Dr. Poulik's antiserum (well 2 and 5) and the two newly raised antisera (well 1, 3, 4 and 6). Immunoelectrophoresis (Fig. 5) was done to investigate whether the dog β_2m had the same mobility in this system as human β_2m. Several G-75 fractions were tested in immunoelectrophoresis at pH 8.6. G-75 fraction 24 (Fig. 5, bottom well) contained β_2m which had a mobility similar to that described by Berggård and Bearn (1968), in the β_2-globulin region. G-75 fraction 23

(Fig. 5, upper well) was contaminated by two components other than β_2m. Unfortunately, no antiserum against total urinary proteins was available, so only impurities that react with the unpurified anti-β_2m sera could be detected. The isoelectric point reported for dog β_2m is 5.1-5.3 in preparative isoelectric focusing (Poulik, 1975). Various G-75 column fractions were tested in analytical isoelectric focusing on urea containing slab gels (Fig. 6). As a reference, human β_2m was also applied; the isoelectric point detected was pI 6.9 and the dog β_2m had an isoelectric point of 6.4. One other major band was visible both in human and in dog β_2m preparations.

If the antisera are to be used for the characterization of DLA antigens, they must react with (the DLA antigens on) viable peripheral blood lymphocytes. This was tested for in the two-stage microlymphocytotoxicity test (Table 1). The anti-β_2m serum was indeed cytotoxic, albeit to a different degree, to PBL from dogs of

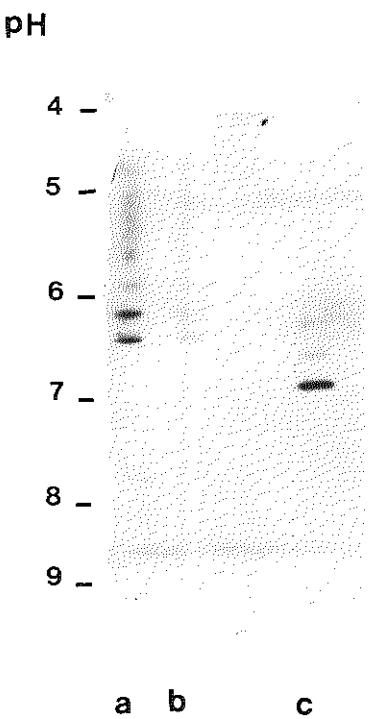

Figure 6. Analytical isoelectric focusing of β_2m. Aliquots of purified β_2m were applied to a thin layer isoelectric focusing gel in a pH gradient of 3.5-10. Isoelectric focusing was performed for 1½ h, at 1000 V.

lane a: dog β_2m G-75 fraction 21
lane b: dog β_2m G-75 fraction 22
lane c: human β_2m.

Table 1. The microlymphocytotoxicity test with anti-dogβ_2m antisera.

dog peripheral blood lymphocytes typed as:	anti-DLA-A9	reaction with anti-β_2m (1)	anti-β_2m (2)	anti-β_2m, no C′
A9, B6, C12 /A2, B5, C11	1/16*	1/8	ND	no dead cells
A3+10, B-, C- /A9, B6, C-	1/2	1/128	1/128	no dead cells
A2, B5, C11, R20 /A9, B4, C12	1/2	1/16	1/128	no dead cells
A9 B6, C12 /A3+10, B-, C-	ND	1/2	ND	ND
A7 B-, C- /A9, B4, C12	ND	1/16	ND	ND
A7, B-, C- /A9 B4, C12	ND	1/2	ND	ND
A1, B13, C- /A9, B6, C12	ND	1/128	ND	ND
A9, B6, C12 /A7, B-, C-	ND	1/2	ND	ND
A9, B6, C12 /A7, B-, C-	ND	1/128	ND	ND

*the highest dilution of antiserum whereby 75% of the cells is dead. ND: not done.

different tissue-type. As a positive control, an alloantiserum was used, and a control without rabbit complement was added to exclude toxic factors in the anti-β_2m serum other than antibodies. The fact that the antiserum reacted with lymphocytes of different tissue-type is an indication that the antigenic determinant on β_2m reacting with the antiserum is the same in different DLA molecules.

III.4 Discussion

The main evidence for the assumption that the isolated protein is indeed β_2m is the reaction of the protein with rabbit anti-dog-β_2m serum from Dr. Poulik in the ELISA and the Ouchterlony test. It is further supported by our observation that the serum reacts with lymphocytes of different DLA types. The ELISA was used to identify dog β_2m. Column fractions containing β_2m by this criterion and of sufficient purity, as judged by SDS-PAGE, were pooled and used to raise anti-β_2m antisera in two rabbits. After each boost, the sera were tested in the ELISA to monitor the titre and specificity. In an Ouchterlony test lines of identity were formed between the antiserum given to us and the antisera we raised ourselves (Fig. 4). The immunoelectrophoresis (Fig. 5) of dog β_2m indicates that the mobility of dog β_2m is the same as that of human β_2m tested in similar conditions. Berggård and Bearn (1968) reported that after immunoelectrophoresis at pH 8.6, β_2m would be found in the β_2-globulin region, with a mobility slightly lower than that of transferrin. Two extra curves were seen when G-75 fraction 23 (Fig. 5, upper well) was investigated. This implies that fraction 23 contains impurities, and that the antiserum can react with them and is therefore not directed exclusively against β_2m. Whether this has any implications for the possible reaction of the anti-β_2m antisera with DLA antigens remains to be seen.

The isoelectric point (pI) of dog β_2m determined here (6.4, Fig. 6) differs from that reported before: 5.1-5.3 (Poulik, 1975). The pI of human β_2m (6.9, Fig. 6), however, is also different from the value reported (5.4-5.6, Poulik, 1975). The absolute difference between the two values reported and the two values found here is about the same, 0.5 pH units. The values established by Poulik were found after preparative isoelectric focusing in a column, which is known to be more accurate than the method used here, where cathodic drift can occur. Two homologues of β_2m of different pI have been found in man (Hall et al., 1977), the rat (Lödgberg et al., 1979), the rabbit (Björck and Berggård, 1981) and the guinea pig (Cigén et al., 1978). In Fig. 6 both human and dog β_2m have two components, a major one of pI 6.9 and 6.4, respectively, and another one of slightly lower pI.

The antisera react with dog peripheral blood lymphocytes (Table 1) of different tissue-type. If the determinant recognized is part of the DLA molecules, it is unlikely that it will be polymorphic as the reaction was not dependent on typing for class I antigens. The antisera raised in the two rabbits will be used to investigate if DLA antigens can be immunoprecipitated with them, either from detergent cell lysates or from papain solubilized DLA antigens prepared from dog spleens

(described in chapter IV). Inhibition of cytotoxicity of the antisera by purified β_2m did not give clear-cut results. This is probably due to the extra specificities present in the serum (Fig. 5). When it can be demonstrated that DLA antigens are purified β_2m can be tested. Reaction of DLA antigens with anti-β_2m sera indicates that β_2m is the light chain of these molecules. If an immunoprecipitation reaction between DLA antigens and an anti-β_2m serum can be inhibited by purified urinary β_2m, the evidence becomes even stronger. Experiments along these lines have been performed and they are discussed in chapter IV. One group has described dissociation of MHC antigens after incubation with anti-β_2m antiserum (Nakamuro et al., 1975). If the antisera against dog β_2m behave similarly, it may be difficult to obtain immunoprecipitates containing intact DLA molecules.
From one anti-β_2m antiserum, IgG has been isolated using Sepharose-protein A. This IgG has been coupled to CNBr-activated Sepharose. Small amounts of purified β_2m could be bound by this affinity column, but it was extremely difficult to elute the β_2m again, either with 3 M KSCN, 3.5 M $MgCl_2$ or 0.5 M glycine pH 2.8. Robb et al. (1976) eluted their anti-β_2m affinity column with purified β_2m. Due to lack of material, we could not do this. Another, as yet untried, possibility is elution with a high pH buffer as described for elution from affinity columns with monoclonal anti-MHC antibodies (Parham, 1979). Therefore, even if β_2m turns out to be the light chain of the DLA antigens, lentil lectin chromatography remains for the time being a better method for large scale isolation of DLA antigens than anti-β_2m affinity chromatography.

The function of β_2m is not known. Membrane-bound β_2m may have an influence on the control of cellular functions. Indirect evidence for this is found in experiments that show that xenoantisera to β_2m affect mixed lymphocyte reactivity and PHA stimulation (Bach et al., 1973) and B cell stimulation (Möller and Persson, 1974). Investigations on Daudi cells, which produce HLA heavy chains, but are devoid of β_2m, indicate that β_2m may be important for the transport of HLA to the membrane and for the creation of determinants recognized by alloantibodies (Ploegh et al., 1979, Sege et al., 1981). This is discussed more extensively in chapter I.5.1 and chapter VIII.3.1.
So far, we did not develop the dog system far enough to investigate similar questions in this species.

Concluding, we can say that β_2m has been isolated from dog urine and that antisera have been raised against it. For large scale isolation of DLA antigens by affinity chromatography the antiserum is not as useful as lentil lectin (chapter II). Small scale isolation and characterization of radiolabelled DLA antigens by the anti-β_2m sera will be discussed in chapters IV, VI and VII.

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CHAPTER IV

CHARACTERIZATION OF DLA CLASS I ANTIGENS BY IMMUNOCHEMICAL TECHNIQUES USING AN ANTI-β_2-MICROGLOBULIN SERUM*

IV.1 Introduction

Like other MHC antigens, DLA class I antigens have originally been characterized in vitro by serological means. Antisera can be used both for characterization and for isolation of the antigens to which they are directed. In chapter II the isolation of DLA antigens by conventional biochemical techniques was described. Their biological activity was determined by their ability to inhibit the reaction of alloantisera with peripheral blood lymphocytes in the microlymphocytotoxicity test. In this chapter and the following one, antisera were used for the isolation of DLA antigens. This was done by immunoprecipitation; subsequently they were analysed on SDS-polyacrylamide gels. For a number of species immunochemical methods have been successfully applied in order to further elucidate the structure of the MHC antigens (Finkelman et al., 1975, Giphart et al., 1978, Katagiri et al., 1975, Kimball et al., 1979, Lunney and Sachs, 1978, Parham and Ploegh, 1980, Phillips et al., 1978, Schwartz and Nathenson, 1971, Wernet and Kunkel, 1973, and Ziegler and Pink, 1975).
The MHC antigens consist of two polypeptide chains of MW 43,000-47,000 and 12,000. Antisera against MHC antigens can be directed against determinants on the light chain and/or on the heavy chain. In this chapter only one antiserum was used. It was raised against purified urinary β_2m (chapter III.2.3).

In all species studied so far, β_2m forms the light chain of the MHC class I antigens. Our first aim was to investigate if the rabbit-anti-dog-β_2m serum would react with DLA antigens in the immunoprecipitation technique. The molecules isolated by papain digestion of crude spleen cell membranes had been shown to have DLA-like activity (chapter II.3), so they were used for the first immunoprecipitation experiments with the anti-β_2m serum. At the same time, NH_2-terminal amino acid sequences of both heavy and light chain of the papain solubilized molecules were determined to verify their identity. Once it is established that anti-dog-β_2m serum can be employed for the isolation of papain solubilized DLA antigens by immunoprecipitation, more experiments can be done using this technique to examine the structure and the location of the DLA antigens.
For these experiments peripheral blood lymphocytes were radiolabelled with ^{35}S-methionine or by lactoperoxidase-catalysed iodination. These are typical methods for protein labelling. In this way the protein nature of the DLA antigens could be

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confirmed. Detergent was used to release membrane integrated molecules from peripheral blood lymphocytes. If the anti-β_2m serum would react with detergent solubilized molecules, these would most probably be DLA antigens. The two chain structure could then be corroborated and the MW of the heavy chain should be somewhat larger than that found for papain solubilized DLA antigens (37,000 daltons, chapter II.3).
The location of the DLA antigens could be examined using the immunoprecipitation technique. Lactoperoxidase-catalysed iodination is supposed to exclusively label proteins on the cell surface, as lactoperoxidase cannot pass the cell membrane. When ^{35}S-methionine is incorporated, both surface and intracellular proteins are labelled. When intact cells are incubated with the antiserum, however, immune complexes can only be formed with antigens on the outside of the cell. Both lactoperoxidase-catalysed iodination and incubation of intact cells with antiserum have been tested in order to assess the location of the DLA antigens.

IV.2 **Materials and methods**

IV.2.1 *Antisera*

For the anti-β_2m serum rabbits were immunized with dog β_2m as described in chapter III.2.3. Normal rabbit serum was a pool of serum from non-immunized rabbits. Goat anti-rabbit-IgG was obtained from Miles, Slough, England.

IV.2.2 *Radiolabelling of prepurified DLA antigens from spleen cells*

DLA antigens were isolated from spleen cells and purified as described in chapter II.2. The procedure of Fraker and Speck (1978) was modified to label dog MHC products. Small glass test tubes were coated with 2 μg of 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (IODOGEN^{Tm}, Pierce, Rockford, IL, U.S.A.). A reaction mixture consisting of 0.11 μg KI, 30 μg protein and 14 μCi Na^{125}I (Amersham International plc, Amersham, England) in a volume of 100 μl sodium phosphate buffer (0.05 M, pH 7.4) was added to the coated test tube. After 15 minutes at room temperature the mixture was placed into a clean tube and applied to a 0.9 $\times$ 15 cm Sephadex G-75 column (Pharmacia, Uppsala, Sweden). The running buffer was phosphate buffered saline pH 7.4, 1% bovine serum albumin. The labelled protein fractions were pooled.

IV.2.3 *Immunoprecipitation of radiolabelled DLA antigens from spleen cells*

Antigen preparations were incubated with rabbit anti-dog-β_2m serum or normal rabbit serum for 1 hour at room temperature. An excess of goat anti-rabbit-IgG was added and the precipitates were left at 4 °C for 16 hours. The precipitates were washed once in 0.2 M Tris-HCl pH 8.0, 0.5% BSA, 0.5% NNP10 (Servo, Delden, the Netherlands), once in 0.05 M Tris-HCl pH 8.0, 0.05% BSA, 0.05% NNP10, once in 0.0625 M Tris-HCl pH 6.8 and once in 0.1% SDS. After washing, the precipitates were boiled in Laemmli sample buffer (Laemmli, 1970) for 5 minutes.

IV.2.4 *Radiolabelling of DLA antigens from peripheral blood lymphocytes*

Peripheral blood lymphocytes from tissue-typed beagles were isolated from heparinized blood and purified by centrifugation on Ficoll-Isopaque. They were surface labelled using lactoperoxidase-catalysed iodination (Vitetta et al., 1971). Alternatively, they were labelled metabolically using ^{35}S-methionine (Amersham International plc, Amersham, England; specific activity 800-1000 Ci/mMol). To increase the metabolism of the lymphocytes, they were stimulated by PHA-M (Difco, Detroit, MI, U.S.A.) for 72 hours in a 5% CO_2 humidified atmosphere at 37 °C. PHA-M, final dilution 1/360-1/960 (v/v) out of a stock solution of 1% in 0.85% saline, was added to 10^6 cells per ml in RPMI 1640 medium supplemented with glutamine to a final concentration of 2 mM, penicillin and streptomycin to a final concentration of 50 U/ml and 50 μg/ml (all from Gibco Europe, Paisley, Scotland) and 10% normal dog serum. After stimulation, the percentage and the viability of lymphoblasts was determined (both generally about 90%), the cells were washed and resuspended at 10^6 cells per ml in methionine-deficient RPMI 1640 supplemented with glutamine, penicillin, streptomycin and 10% dialyzed foetal calf serum (Gibco Europe, Paisley, Scotland). Preincubation of the cells in this medium for 30 minutes was necessary to deplete the endogenous methionine pool. After 30 minutes, ^{35}S-methionine was added, 0.1-1 mCi to 10^7 cells. The cultures were incubated for 16 hours at 37°C in the CO_2 incubator. Incorporation was measured by TCA precipitation of aliquots from cell suspensions or membrane extracts.

IV.2.5 *Antigen preparation from radiolabelled peripheral blood lymphocytes*

As ^{35}S-methionine is incorporated into both intracellular and cell surface proteins, prepurification was carried out by preparing membranes from the cells before detergent solubilization. Cells were washed and lysed in 0.01 M Tris-HCl pH 8.0 and centrifuged for 10 minutes at 1000 $\times$ g. This was repeated five times. The supernatants were collected and centrifuged for 1 hour at 50,000 $\times$ g and 4°C. The resulting pellet, containing the crude membranes, was solubilized, with occasional shaking, in 0.75% non-ionic detergent NNP10 (Servo, Delden, the Netherlands) in PBS containing 0.125% EDTA and 0.02% NaN_3, for 30 minutes on ice. The membrane extract was centrifuged for 1 hour at 100,000 $\times$ g in a Beckman airfuge at 4°C and the pellet was discarded. The supernatant contained the crude antigen preparation. Crude membranes from unlabelled cells of irrelevant DLA specificity were added to reduce non-specific interactions in immunoprecipitation. Further purification of membrane extracts was done by lentil lectin-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography. For 2.5 $\times$ 10^6 cell equivalents 0.5 ml of packed lentil lectin-Sepharose beads was used. The beads were washed in 0.5% NNP10 in PBS. Antigen preparation and lentil lectin-Sepharose were incubated for 30 minutes at room temperature. After thorough washing the antigen was eluted with 0.1 M α-methyl-D-mannoside in 0.5% NNP10 in PBS. The eluted material was dialyzed for 16 hours at 4°C against 0.5% NNP10 in PBS and then used for immunoprecipitation.

Samples from the experiments described under IV.2.7 and IV.2.8 were reduced and alkylated (Dobberstein et al., 1979) and analysed on 7.5-15% linear gradient gels of 15 × 30 cm.
All chemicals for electrophoresis were from Bio-Rad, Richmond, CA, U.S.A. Marker proteins are listed in the relevant figure legends. ^{14}C-labelled marker proteins were obtained from NEN, Dreieich, F.R.G.

IV.3 **Results**

Cell surface molecules were isolated either by papain digestion from spleens or by detergent solubilization from peripheral blood lymphocytes. Both types of cell extract and intact cells were used for the following immunoprecipitation studies. A summary of immunoprecipitation schedules is given in Fig. 1a.

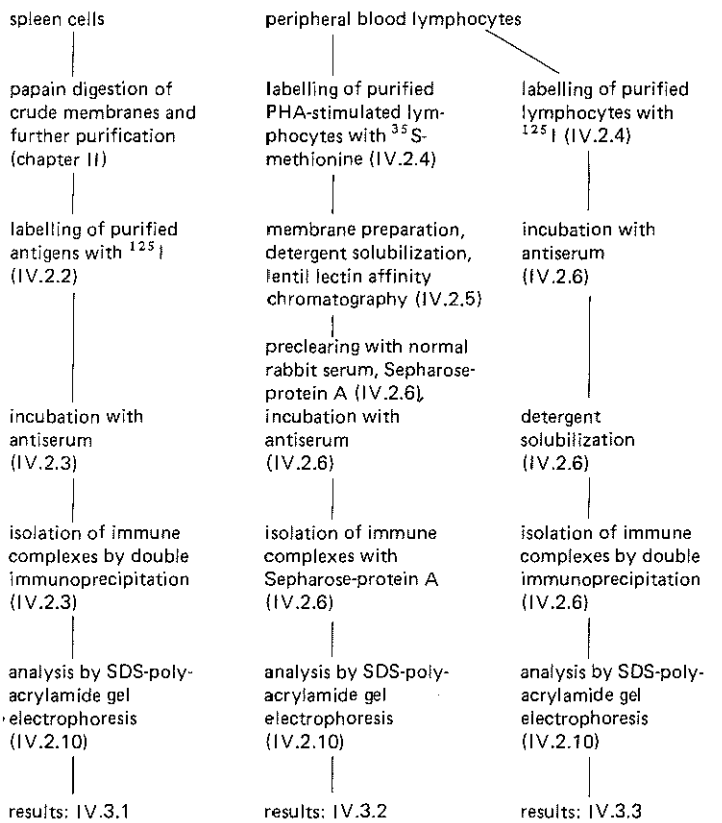

Figure 1a. Summary of the immunoprecipitation schedules used.

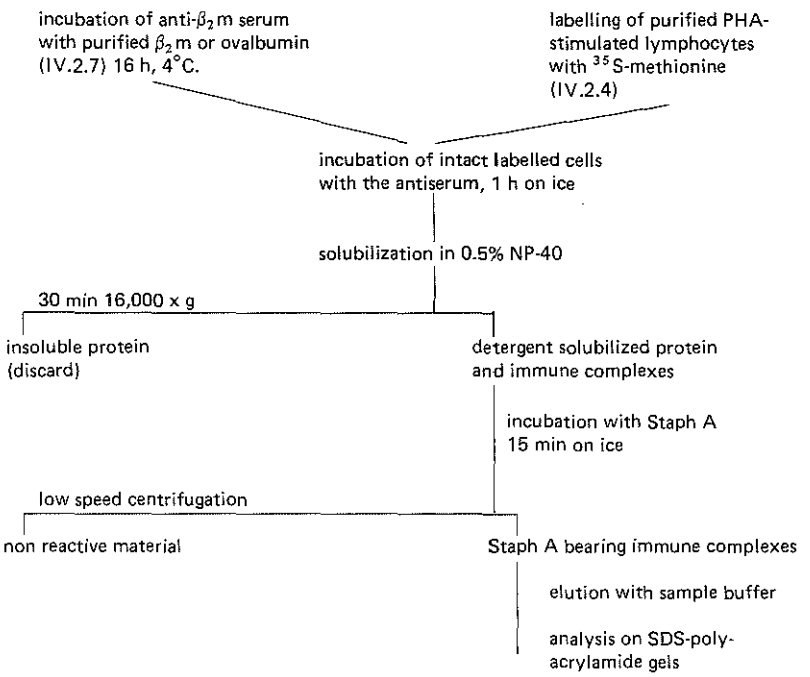

Figure 1b. Inhibition of immunoprecipitation (IV.2.7).

IV.3.1 *Immunoprecipitation of ^{125}I labelled DLA antigens obtained from spleens by papain digestion*

DLA antigens were isolated and purified as described in chapter II.2. After purification on Sepharose-lentil lectin they were radiolabelled and incubated with rabbit anti-dog-β_2m serum (see section IV.2.2, IV.2.3). The antiserum should react with all DLA molecules independent of tissue-type. Most sera raised against urinary β_2m will precipitate the whole class I molecule, i.e. both heavy and light chain (Poulik et al., 1979).

In Fig. 2 the results of the immunoprecipitation are shown. Normal rabbit serum, used as a control, does not precipitate any labelled material (lane a). The anti-β_2m serum brings down two components of an aproximate MW of 37,000 and 12,000. The biochemically purified DLA antigens (chapter II.2) show exactly the same pattern on SDS-polyacrylamide gels after staining for protein (chapter II, Fig. 4). So, the DLA antigens that were isolated by conventional biochemical techniques can react with an antiserum against β_2m.

Knowing this, we could use the technique of immunoprecipitation of radiolabelled DLA antigens by anti-β_2m serum to perform several experiments on an analytical scale. First, we wanted to establish whether DLA antigens could be isolated from peripheral blood lymphocytes by detergent solubilization.

Figure 2. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of ^{125}I labelled, papain solubilized DLA antigens after immunoprecipitation with: normal rabbit serum (lane a) or rabbit anti-dog-β_2m serum (lane b). Molecular weight standards: 94,000 phosphorylase b, 67,000 albumin, 43,000 ovalbumin, 30,000 carbonic anhydrase, 20,100 trypsin inhibitor, 14,400 lactalbumin.

IV.3.2 *Immunoprecipitation of ^{35}S-methionine labelled DLA antigens obtained from peripheral blood lymphocytes by detergent solubilization*

The molecular weight of the detergent solubilized MHC molecules is always greater than that of papain digested molecules, as detergent solubilizes the whole molecule, including the transmembrane and intracellular part. DLA molecules were precipitated from ^{35}S-methionine labelled, detergent solubilized cells (see section IV.2.4, IV.2.5, IV.2.6). ^{35}S-methionine incorporation was used as an alternative to ^{125}I labelling as the iodination procedure modifies the tyrosine residues in the protein. Metabolic labelling only incorporates a radiolabelled amino acid where normally the same, but non-radioactive amino acid would be.
The anti-β_2m serum brings down two polypeptide chains of 41,700 and 12,000 MW (Fig. 3, lane b). Normal rabbit serum (lane a) precipitates a band which appears to be heavy chain, but in a much smaller amount than in lane b. This band could

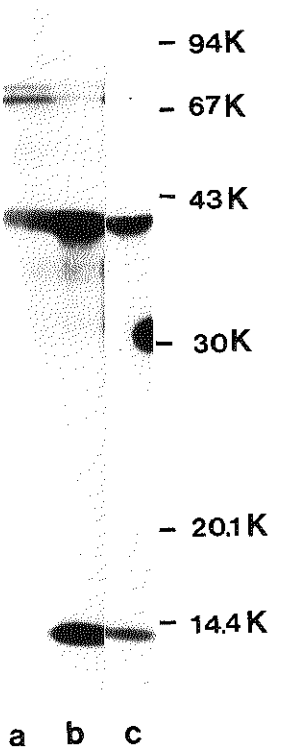

Figure 3. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after immunoprecipitation with: normal rabbit serum (lane a) or rabbit anti-dog-β_2m serum (lanes b, c).
lanes a,b: ^{35}S-methionine labelled membrane extract.
lane c: ^{35}S-methionine labelled membrane extract after lentil lectin-Sepharose purification.
Molecular weight standards as described for Fig. 2.

represent actin, a common contaminant of immunoprecipitates from lymphocytes (Barber and Delovitch, 1979). The 41,700 MW band in lane b would then be a mixture of actin and heavy chain. A relatively pure cell extract was used for Fig. 3, lane a,b, as membranes were prepared from the cells before detergent was added. Further purification on lentil lectin-Sepharose separates actin, which appears in the flow-through, from the MHC antigen heavy chains. A lentil lectin purified antigen preparation and the anti-β_2m serum were used for the precipitation in lane c. The same two bands as in lane b are visible.
In order to distinguish between an intracellular and an extracellular location of the DLA antigens experiments were done using intact cells rather than cell extracts for the incubation with anti-β_2m serum.

IV.3.3. *Immunoprecipitation of ^{125}I labelled DLA antigens from intact peripheral blood lymphocytes reacted with antiserum before detergent solubilization*

Both lactoperoxidase-catalysed iodination and incubation of intact cells with antiserum are ways of demonstrating the presence or absence of proteins on the outside of the cell membrane. ^{125}I labelled peripheral blood lymphocytes were incubated with anti-β_2m serum (see section IV.2.4 and IV.2.6) and solubilized afterwards. In Fig. 4 lane b the resulting gel pattern is shown. Here again, two polypeptide chains of 41,700 and 12,000 daltons are visible. Normal rabbit serum (lane a) did not precipitate any material.

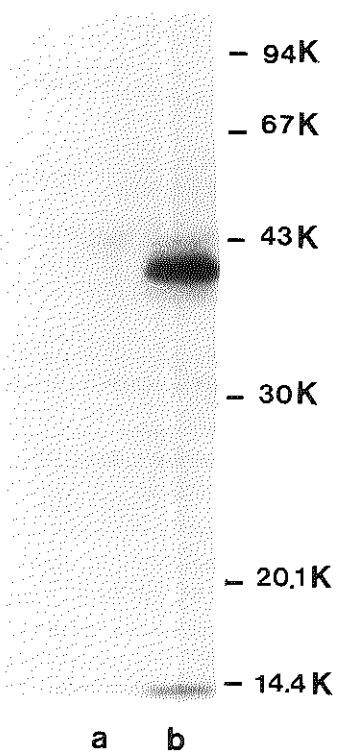

Figure 4. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of ^{125}I labelled, detergent solubilized DLA antigens after incubation of the intact cells with: normal rabbit serum (lane a) or rabbit anti-dog-β_2m serum (lane b).
Molecular weight standards as described for Fig. 2.

IV.3.4 *Inhibition of immunoprecipitation*

The reaction of anti-β_2m serum with intact peripheral blood lymphocytes was inhibited by purified β_2m. After detergent solubilization and reaction with Staph A no DLA antigens could be detected by SDS-polyacrylamide gel electrophoresis. The method of inhibition of immunoprecipitation is shown in Fig. 1b (p. 67). The gel is pictured in Fig. 5. In lane a undiluted antiserum was used and the resulting pattern contains two major bands of 43,000 and 12,000 daltons. Actin and the heavy chain have fused to form one broad band. In lanes b and c no light chain is observed at all, and in the heavy chain area only actin remains. Dilution of the anti-β_2m serum 1/3 and 1/9 with purified β_2m (IV.2.7) inhibited immunoprecipitation completely. Control dilutions in ovalbumin (lanes d and e) did not have this effect.
Another way to show that the anti-β_2m serum precipitates two-chain molecules by reacting with one chain is to incubate the serum with separated light and heavy chains.

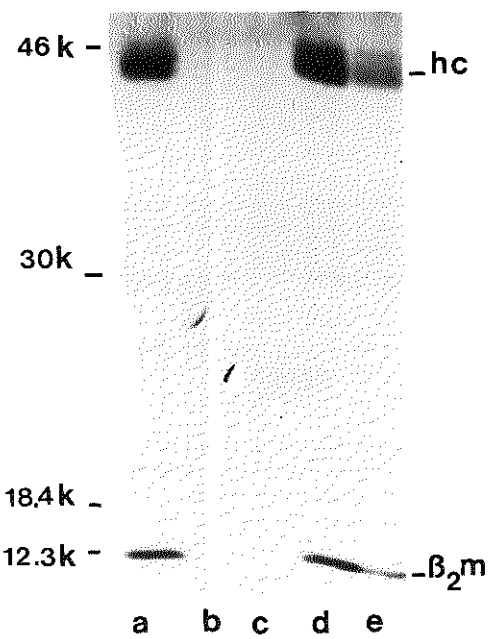

Figure 5. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after incubation of the intact cells with: rabbit anti-dog-β_2m serum (lane a), rabbit anti-dog-β_2m serum diluted 1/3 and 1/9 with a solution of purified β_2m (lanes b,c) or rabbit anti-dog-β_2m serum diluted 1/3 and 1/9 with a solution of ovalbumin (lanes d,e).
Molecular weight standards: 46,000 ovalbumin, 30,000 carbonic anhydrase, 18,367 lactoglobulin A, 12,300 cytochrome c. hc: heavy chain; β_2m: β_2-microglobulin.

IV.3.5 *Immunoprecipitation of denatured DLA polypeptide chains*

Anti-β_2m serum was incubated with ^{35}S-methionine labelled detergent solubilized DLA antigens which had been dissociated by boiling in 1% SDS (IV.2.8). Figure 6 shows the resulting gel pattern; only the light chain is precipitated. No heavy chain is visible, only a faint band representing actin. This is an indication for the fact that the antiserum reacts with the light chain and that in the case of precipitation of native DLA antigens the heavy chain is merely coprecipitated. Apart from dissociation, 1% SDS also causes denaturation. Therefore this experiment does not preclude that the anti-β_2m serum can react with the native heavy chain.

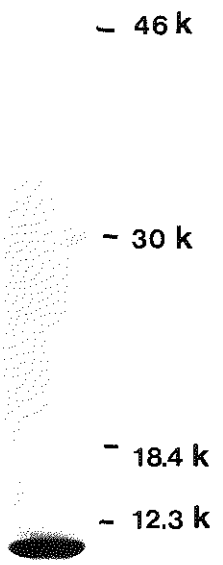

Figure 6. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after dissociation and denaturation, and subsequent immunoprecipitation with rabbit anti-dog-β_2m serum.
Molecular weight standards as described for Fig. 5.

IV.3.6 *NH_2-terminal amino acid sequence determination, amino acid composition*

Unequivocal proof of the identity of a protein is only given by its amino acid sequence. NH_2-terminal sequences of the two component chains of papain solubilized DLA molecules were determined (IV.2.9).
Papain solubilized, lentil lectin purified DLA molecules from several spleens were pooled. The heavy and light chains were separated by gel filtration in 1 M propionic acid. The light chain was obtained in sufficient quantity and purity to identify 24 amino acids. In Fig. 7a these residues are shown, compared to the NH_2-terminal

	1									5										10										15		
dog urinary β_2m	V	–	Q	–	H	–	P	–	P	–	K	–	I	–	Q	–	V	–	Y	–	S	–	R	–	H	–	P	–	A	–	
DLA light chain	V/Y	–	Q	–	L H	–	P/M G	–	P/M N/S	–	K W	–	V/Y I	–	Q	–	V/Y A	–	V/Y A	–	K N/S (H?) (R?)	–	F	–	E H	–	P/M	–	A N/S	–	

					20								
	E/Q	–	N/D	–	G	–	K	–	P	–	N/D	–	F	–	L	–	N/D
	E F	–	N/S	–	G (T?)	–	K (Q?)	–	P/M (A?)	–	N/S	–	F	–	L	–	N/S (P/M?)

Figure 7a. NH_2-terminal amino acid sequence of DLA light chains compared to the NH_2-terminal amino acid sequence of dog urinary β_2m (Smithies and Poulik, 1972).
Amino acid symbols: A: alanine, R: arginine, N: asparagine, D: aspartic acid, B: asparagine and/or aspartic acid, C: cysteine, Q: glutamine, E: glutamic acid, Z: glutamine and/or glutamic acid, G: glycine, H: histidine, I: isoleucine, L: leucine, K: lysine, M: methionine, F: phenylalanine, P: proline, S: serine, T: threonine, W: tryptophan, Y: tyrosine, V: valine.
V/Y: no distinction could be made between valine and tyrosine.
$\begin{matrix} L \\ H \end{matrix}$: the amino acid in this position can be either leucine or histidine.
(H?): histidine cannot be excluded in this position.

Table 1. Amino acid composition of the DLA light chain compared to the amino acid compositions of human (Berggård and Bearn, 1968) and bovine (Groves and Greenberg, 1982) β_2m.

	DLA light chain*	human β_2m**	bovine β_2m**
D+N	11.5	12	11
T	6.0	5	2
S	7.0	10	8
E+Q	13.7	11	12
P	7.5	5	9
C	0.4	2	2
G	5.3	3	3
A	3.3	2	1
V	5.8	7	5
M	0.7	1	—
I	3.3	5	6
L	7.6	7	8
Y	3.1	6	6
F	5.7	5	4
H	3.9	4	4
K	6.3	8	9
W	ND	2	2
R	3.4	5	5

ND: not determined.
Amino acid symbols: see Fig. 7.
*: residues/100 residues
**: residues/molecule

sequence published for dog urinary β_2m (Smithies and Poulik, 1972). Under the experimental conditions used no distinction could be made between respectively valine and tyrosine, proline and methionine, and asparagine and serine. Serine, threonine, histidine and arginine PTH-amino acid derivatives give relatively small yields on sequencing and are therefore hard to detect. The amino acid composition of the light chain (Table 1) agrees well with the amino acid composition of human and bovine β_2m.

A double sequence was found at most positions; both possible amino acids appeared with nearly identical intensity. In spite of all these restrictions sufficient homology is found of the sequence for the light chain with that published for urinary β_2m to justify the conclusion that the DLA light chain is identical to β_2m. The heavy chain was less pure, it was estimated that only 25-30% of the reaction mixture consisted of heavy chain. The impurities did not interfere with the NH_2-terminal sequence determination and they represent therefore small quantities of several proteins. Seven positions could be identified. In Fig. 7b these residues are compared with the NH_2-terminal sequences of HLA-A2, HLA-B7, H-2K^b, H-2K^d, H-2D^b and H-2D^d. Homology with either one is found in 6 out of 7 positions determined.

	1				5
H-2K^b	G – P	– H	– S		– L – R – Y – F – V
H-2K^d	G – P	– H	– S		– L – R – Y – F – V
H-2D^b	G – P	– H	– S		– M – R – Y – F – E
H-2D^d	G – S	– H	– S		– L – R – Y – F – V
HLA-A2	G – S	– H	– S		– M – R – Y – F – F
HLA-B7	G – S	– H	– S		– M – R – Y – F – Y
DLA papain digested heavy chain	G – P/M N/S	– P/M?	– N/S (P/M?)		– L – X – X – F – V/Y

Figure 7b. NH_2-terminal amino acid sequence of DLA heavy chains, solubilized by papain digestion, compared to the NH_2-terminal amino acid sequences of H-2K^b, H-2K^d, H-2D^b, H-2D^d, HLA-A2 and HLA-B7. Data are from Nathenson et al. (1981) and Orr et al. (1979).
P/M?: possibly proline or methionine are in this position.
X: the amino acid in this position is not known.
Further legends: see Fig. 7a.

IV.4 Discussion

An antiserum raised against urinary β_2m has been shown to react with molecules far larger than β_2m, consisting of two polypeptide chains of 12,000 and 37,000 or 41,700 daltons according to the isolation procedure. Most anti-β_2m sera in other species can be used in this way, but sometimes such an antiserum dissociates the two component chains (Nakamuro et al., 1975). With our antiserum, this did not happen. The 37,000/12,000 dalton molecules were precipitated from dog spleen cell

membrane preparations which had been treated with papain and purified (Fig. 2). A similarly prepared membrane protein had the capacity to inhibit a microlymphocytotoxicity test of dog peripheral blood lymphocytes and tissue typing sera (chapter II.3). This protein also consisted of two polypeptide chains of 12,000 and 37,000 MW as measured from SDS-polyacrylamide gels after staining for protein. The inhibition of the microlymphocytotoxicity test, which was specific, indicates that the isolated molecules are DLA antigens; i.e. dog MHC class I antigens. The fact that a similarly purified protein reacts with an anti-β_2m serum strongly suggests that β_2m is identical to the light chain of the DLA class I antigens, as has been shown for the MHC class I antigens of all species so far investigated (references for this are given in chapter III.1).

The same anti-β_2m serum has been used to immunoprecipitate molecules from peripheral blood lymphocytes after detergent solubilization (Fig. 3). Like other MHC antigens, DLA antigens can be released from the cell membrane by detergent solubilization. The MW of the heavy chain is approximately 41,700. This is in accordance with data on detergent solubilized MHC class I antigens of other species where a molecular weight of around 43,000 daltons is found for detergent solubilized heavy chains, somewhat larger than the MW of the papain solubilized heavy chain. In the dog the difference in MW between the papain and detergent solubilized heavy chains is approximately 5000 daltons.

The protein nature of the DLA antigens which had been indicated by their behaviour in the biochemical isolation procedure (chapter II.2) is corroborated by the fact that they incorporate a radiolabelled amino acid, ^{35}S-methionine. Lactoperoxidase-catalysed iodination is also a typical procedure for protein labelling, by which ^{125}I becomes attached to tyrosine residues.

DLA antigens most probably contain a carbohydrate moiety, because they are retained on a lentil lectin affinity column (chapter II.2.3). Incorporation of radiolabelled sugars as described in chapter VI is needed to confirm this.

From the microlymphocytotoxicity test it is clear that DLA class I antigens are located on the outside of the cell membrane. This location is confirmed by the experiments described here. Lactoperoxidase-catalysed iodination of peripheral blood lymphocytes, incubation of the intact cells with the anti-β_2m serum and detergent solubilization resulted in a gel pattern (Fig. 4) similar to that obtained with a cell extract (Fig. 3). ^{35}S-methionine labelled cells, which are internally labelled as well as externally, gave an identical result when incubated with the antiserum before detergent solubilization (Fig. 5).

Thus it is shown that DLA antigens from different sources (spleen, PBL cell or membrane extract) isolated by different means (papain digestion or detergent solubilization) can be immunoprecipitated by an anti-β_2m serum. Contamination by actin is sometimes found in control precipitates with normal rabbit serum (Fig. 3). Actin can be removed from the antigen preparation by lentil lectin affinity chromatography. It is generally present in immunoprecipitates from peripheral blood lymphocytes (Barber and Delovitch, 1979).

A clear distinction of heavy chain and actin can be obtained by analysing samples

on gradient gels rather than uniform gels. This type of gel has been used for the experiments shown in Figs. 5 and 6. The molecular weight of the DLA heavy chain is estimated to be 43,000 daltons on these gels.

The assumption that the DLA light chain is identical to β_2m is confirmed by the fact that immunoprecipitation of anti-β_2m serum and detergent solubilized DLA antigens can be inhibited by purified β_2m (Fig. 5). The determinants which are normally recognized on DLA molecules are blocked by this urinary protein.

More direct proof of the anti-β_2m serum reacting with the light chain was obtained from the experiments with dissociated DLA chains. The antiserum could react with single, denatured light chains (Fig. 6) without reacting with the heavy chain. This does not preclude the possibility that the antiserum can react with the native heavy chain or the whole complex. It is unlikely, however, that the antiserum only reacts with the light chain when it is denatured and dissociated. Most probably the anti-β_2m serum normally reacts with detergent solubilized DLA molecules through the light chain.

As only papain solubilized DLA antigens had been isolated on a large scale, these were chosen for the sequence determinations. Sequencing of small quantities of protein is possible, but it requires a lot of time and expense (Nathenson et al., 1981). Although the DLA light chain yielded a partly double sequence, comparison with the sequence published for urinary dog β_2m (Smithies and Poulik, 1972) showed that in 23 out of 24 positions sequenced, it was possible to detect the same amino acid both in urinary β_2m and in the DLA light chain (Fig. 7a). As the light chain from both papain digested and detergent solubilized DLA antigens reacts with the same anti-β_2m serum, we can safely claim that they will be identical to each other and to β_2m. Formal proof of the identity of proteins is only given by the identity of their amino acid sequences. For this purpose a stretch of 24 NH_2-terminal amino acids is enough. In man, identity of the HLA light chain and urinary β_2m was shown by agreement of their 16 NH_2-terminal amino acids (Bridgen et al., 1976). Similar evidence has now been obtained in the dog. The amino acid composition of the DLA light chain is not very different from the amino acid composition determined for β_2m in other species.

The sequence of the NH_2-terminal end of the heavy chain was more difficult to establish. Still, 6 out of 7 positions determined are identical to the amino acids in the corresponding positions of HLA-A2, HLA-B7, H-2K or H-2D. Comparison with heavy chains of other species (Nathenson et al., 1981) shows similar homology. The yield of PTH-amino acids was 25-30% of the amount determined by analysis of the amino acid composition. The identity of the contaminating material is not clear. In addition to the NH_2-terminal glycine, at least four other NH_2-terminal amino acids were present. As the preparation was purified by lentil lectin chromatography, it is unlikely that actin is still there. The NH_2-terminal end of the MHC class I molecules investigated so far is not a region of great variability. Although DLA molecules of different tissue-type were pooled for the sequence determination they are expected to have similar NH_2-terminal ends, and they would therefore not contribute to the variety of amino acids detected. The presence of previously

undetected DLA class I or class IV antigens cannot be excluded. The heavy chains of class IV antigens have a molecular weight very close to that of the class I heavy chain (Michaelson et al., 1977, Vitetta et al., 1976). Although DLA heavy chain was estimated to be only 25-30% of the reaction mixture, the 7 positions determined were clearly detected above the background of amino acids from contaminating proteins.
All in all these amino acid sequence data are sufficient reason for us to believe that we are indeed working with DLA antigens, and that these DLA antigens can be isolated by immunoprecipitation with an anti-β_2m serum.

The two chain structure of DLA antigens precipitated with anti-light chain serum has to be confirmed by using antisera directed against the heavy chain (chapter V). An advantage of the technique used is that little material is needed which can be screened with small amounts of antiserum. The immunoprecipitation technique is further used for an investigation into the nature of the alloantigenic determinant(s) of the DLA antigens (chapters VI, VII).

In conclusion: DLA antigens can be solubilized by detergent. They consist of two polypeptide chains of 41,700 and 12,000 daltons. The light chain is identical to β_2m. DLA class I antigens are located on the outside of the cell membrane. Preliminary amino acid sequence data point to homology of DLA antigens with HLA and H-2 antigens.

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CHAPTER V

CHARACTERIZATION OF DLA CLASS I ANTIGENS BY IMMUNOCHEMICAL TECHNIQUES USING ANTISERA DIRECTED AGAINST THE HEAVY CHAIN

V.I Introduction

In chapter IV experiments were described which formed the basis of the investigations reported here. It was established that DLA antigens could be solubilized from the cell membrane by detergent and that they could be immunoprecipitated with an anti-β_2m serum. This antiserum is directed against the light chain of the DLA antigens, and the heavy chain is co-precipitated. After dissociation of the two chains in 1% SDS, the anti-β_2m serum only precipitated the light chain.

If β_2m and the heavy chain are really associated, immunoprecipitation with anti-heavy chain antisera should also yield two chains. In this case the light chain would be co-precipitated with the heavy chain. There are two types of anti-heavy chain antisera. One type is monomorphic, or "anti-backbone", i.e. directed against an invariable part of the heavy chain. The other type is polymorphic, directed against the alloantigenic determinant. Tissue typing sera, or "allo"-antisera belong to the latter category. Monomorphic anti-heavy chain sera should react with DLA antigens of all specificities, just like (monomorphic) anti-β_2m serum. Alloantisera only react with DLA antigens of the corresponding tissue-type. In order to exclude the possibility that the determinant recognized by the anti-heavy chain sera is located on the light chain, inhibition of immunoprecipitation by purified urinary β_2m was tested.

The location of the DLA antigens on the cell surface was confirmed by the same experiments as those described in chapter IV, using anti-heavy chain sera instead of anti-β_2m serum.

With the two types of anti-heavy chain serum it is possible to perform sequential immunoprecipitations. When a cell extract is first incubated with an anti-backbone serum, no further DLA antigens can be precipitated in a second round by alloantisera. When the experiment is done the other way around, the second precipitation does yield DLA antigens. Sequential immunoprecipitation can also be done with alloantisera directed against products of different loci, as a way to prove that these products are indeed located on different molecules. This technique has been employed to distinguish H-2K from H-2D glycoproteins (Nathenson and Cullen, 1974) and to identify two different rat class I loci (Natori et al., 1979).

Alloantisera are difficult to work with in the immunoprecipitation technique. Their

titre is generally low and their binding weak. Only in the mouse have they been applied successfully (Schwartz et al., 1973). In the mouse, however, it is possible to raise hyperimmune sera because of the availability of congenic and recombinant mice. One of the many dog alloantisera tested could be used to immunoprecipitate DLA antigens.
In this chapter DLA immunoprecipitation patterns obtained with one monomorphic and one polymorphic antiserum are described.

V.2 **Materials and methods**

V.2.1 *Antisera*

Normal rabbit serum, rabbit anti-β_2m serum and goat anti-rabbit-IgG serum were as described in chapter IV.2.1. The preparation of the anti-heavy chain backbone (anti-HC) antiserum was carried out using DLA antigens isolated by papain digestion (chapter II.2). After partial purification, the DLA antigens were lyophilized and passed over a Sepharose-lentil lectin column (Pharmacia, Uppsala, Sweden). Lyophilization dissociates the two polypeptide chains. Lentil lectin does not have affinity for β_2m, so the heavy DLA chain could be obtained from the eluate of the lentil lectin column whereas β_2m was not retained by the column.
Two rabbits were immunized. A quantity of 0.5 ml of 1 mg/ml DLA heavy chain in 0.15 M NaCl was emulsified with 0.5 ml of complete Freund's adjuvant (Difco, Detroit, MI, U.S.A.). It was injected subcutaneously at five sites on the back. Four boosts, every two weeks, were given with the same amount of antigen emulsified in 0.5 ml of incomplete Freund's adjuvant. The titre of the antiserum was determined in the ELISA as described in chapter III.2.4.
Normal dog serum was a pool of serum from non-immunized dogs. Rabbit anti-dog-IgG was obtained from Miles, Slough, England. Alloantiserum 1302 was prepared as described (Vriesendorp et al., 1971). It was characterized in the second workshop on canine immunogenetics (Joint report, 1976). It reacts with DLA-C11 and it cross-reacts with DLA-A2 and DLA-B5.

V.2.2 *Immunoprecipitation of radiolabelled DLA antigens from spleen cells*

Radiolabelled DLA antigens from spleen cells were prepared as described in chapter IV.2.2. The immunoprecipitation with anti-HC serum was done as described for anti-β_2m serum in chapter IV.2.3.

V.2.3 *Immunoprecipitation of radiolabelled DLA antigens from peripheral blood lymphocytes*

Peripheral blood lymphocytes were labelled with ^{35}S-methionine as described in chapter IV.2.4. For some experiments antigens were prepared from membranes (chapter IV.2.5), for others a direct cell extract was made in lysis buffer (chapter IV.2.7). Immunoprecipitation with the anti-HC serum was done with intact cells

and membrane extracts (chapter IV.2.6). Inhibition of immunoprecipitation by purified urinary β_2m and immunoprecipitation of denatured DLA polypeptide chains were performed as described before (chapter IV.2.7 and IV.2.8).

V.2.4 *Immunoprecipitation of radiolabelled DLA antigens from peripheral blood lymphocytes by alloantisera*

Peripheral blood lymphocytes were labelled with ^{35}S-methionine as described in chapter IV.2.4. A direct cell extract was made in lysis buffer (chapter IV.2.7). Precipitation was done as described in chapter IV.2.7 with modifications. The cell extract was made to 0.1% SDS. Preclearing was done twice with 5 μl rabbit anti-dog-IgG and 100 μl Staph A per 2×10^6 cell equivalents. After preclearing the antigen preparation was centrifuged for 30 minutes at 100,000 $\times$ g and 4°C in a Beckman airfuge. Specific incubations were performed with 10 μl alloantiserum and 100 μl Staph A per 2×10^6 cell equivalents, for 90 minutes on ice. The immune complex bearing Staph A were washed as described. All buffers contained 0.1% SDS. Elution was according to Dobberstein et al. (1979). As alloantisera require a modification of the usual method, where preclearing is important, all tests were done with cell extracts rather than with intact cells, where preclearing is impossible. Therefore, inhibition of immunoprecipitation with urinary β_2m was also done using precleared cell extracts. The alloantiserum, and anti-β_2m serum as a control, were incubated for 16 hours at 4°C with dilutions of urinary β_2m or ovalbumin as described in chapter IV.2.7. For immunoprecipitation 10 μl of antiserum dilution was used per 10^6 cell equivalents. Immune complexes were reacted with 100 μl Staph A per incubation. Washing and elution were as described (chapter IV.2.7).

V.2.5 *Sequential immunoprecipitation*

Aliquots of 10^6 cell equivalents were precleared twice with the same antiserum and then reacted with a different antiserum, or with the first one as a control. Immunoprecipitations were done as described previously (chapter IV.2.7).

V.2.6 *Platelet absorption of antisera*

Platelets were prepared from beagle blood collected in anticoagulant citrate glucose solution. For the absorptions, 10^{10} thrombocytes were incubated with 1 ml of antiserum for 1 hour at room temperature. Thereafter 1 ml of fresh antiserum was incubated with the same thrombocytes. Elution was done with 1.0 ml 0.5 M glycine pH 3.0, 1% BSA. The eluates were neutralized and they were stored at -20°C until use.

V.2.7 *Gel electrophoresis*

Samples were analysed on $10 \times 14 \times 0.1$ cm, 12.5% acrylamide gels (Laemmli, 1970) or on 7.5-15% linear gradient gels in the system of Dobberstein et al. (1979). Fluorography was done according to Bonner and Laskey (1974). Marker proteins

are listed in the relevant figure legends. All chemicals for electrophoresis were from Bio-Rad, Richmond, CA, U.S.A. ^{14}C-labelled marker proteins were obtained from NEN, Dreieich, F.R.G.

V.3 **Results**

In Fig. 1 a summary is given of the methods used for the studies described in this chapter.
The anti-HC serum reacted with DLA antigens both from spleens and from peripheral blood lymphocytes (V.3.1-V.3.3).

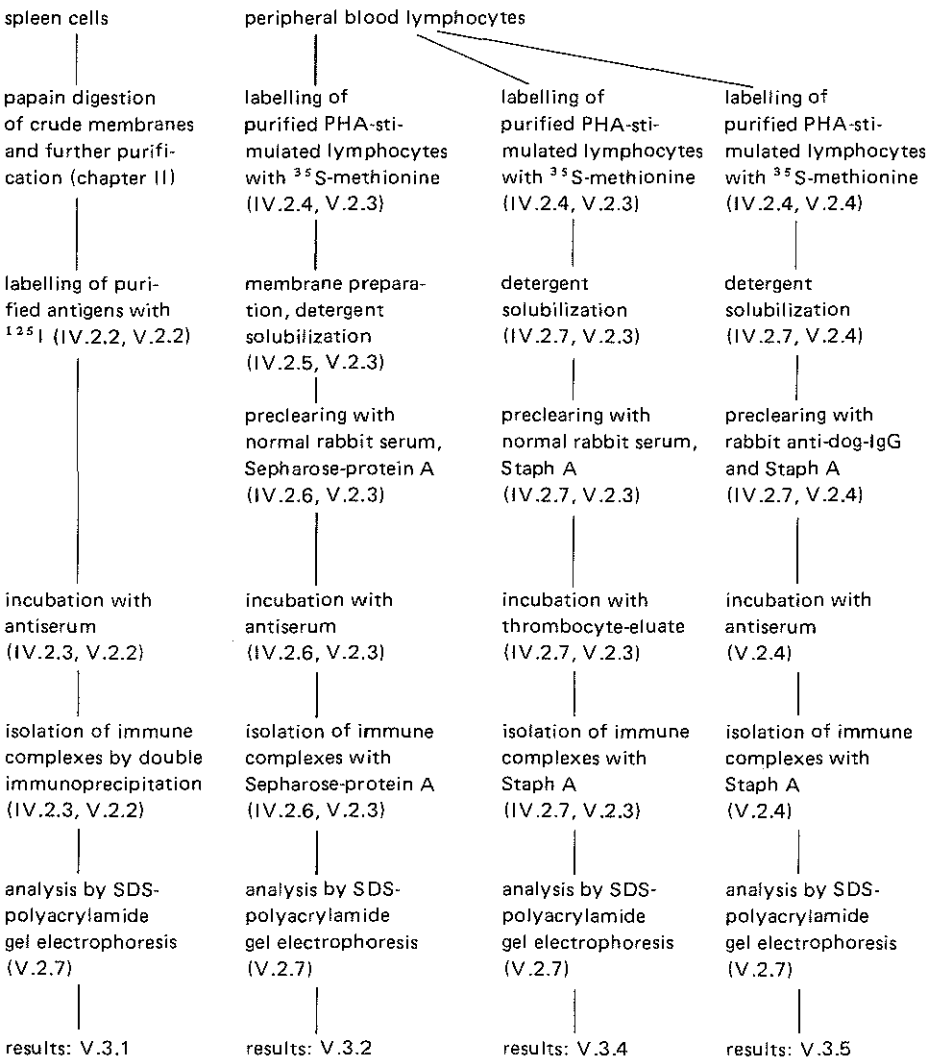

Figure 1. Summary of the immunoprecipitation schedules used.

V.3.1 *Immunoprecipitation of ^{125}I labelled DLA antigens obtained from spleen cells by papain digestion*

In Fig. 2 an analysis is shown of the immunoprecipitation of papain solubilized DLA antigens with the anti-HC serum. As a reference the anti-β_2m precipitation pattern is also given (lane a). The proteins recognized by the anti-HC serum appear in four major bands of 39,000, 37,000, 19,000 and 12,000 daltons (lane b). The anti-β_2m serum precipitated polypeptide chains of 37,000 and 12,000 daltons. The material used for immunization may have contained the 39,000 and 19,000 MW products although they were not detectable on SDS-polyacrylamide gels after Coomassie Brilliant Blue staining. A diffuse band of a MW of approximately 30,000, which is also visible in the precipitate by the anti-HC serum, could represent a breakdown product of the heavy chain or the dog equivalent of the class II antigens. Products of this MHC region are retained by lentil lectin and could have been present in the preparation used for immunization and in the labelled preparation. The possible identity of the 39,000, 30,000 and 19,000 MW molecules is further considered in the discussion (V.4).

Figure 2. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of ^{125}I labelled, papain solubilized DLA antigens after immunoprecipitation with: rabbit anti-dog-β_2m serum (lane a) or rabbit anti-HC serum (lane b). Molecular weight standards: 94,000 phosphorylase b, 67,000 albumin, 43,000 ovalbumin, 30,000 carbonic anhydrase, 20,100 trypsin inhibitor, 14,400 lactalbumin.

V.3.2 *Immunoprecipitation of ^{35}S-methionine labelled DLA antigens obtained from peripheral blood lymphocytes by detergent solubilization*

A membrane extract was used as a source of DLA antigens for precipitation with the anti-HC serum. From Fig. 3 it is clear that the anti-HC serum, which was raised against papain solubilized heavy chains of 37,000 MW, can also react with detergent solubilized heavy chains of 41,700 MW (lane a). The anti-β_2m serum pattern is shown for comparison (lane b). Bands of 30,000-39,000 MW are present again similar to those precipitated from spleen-derived DLA antigen preparations.

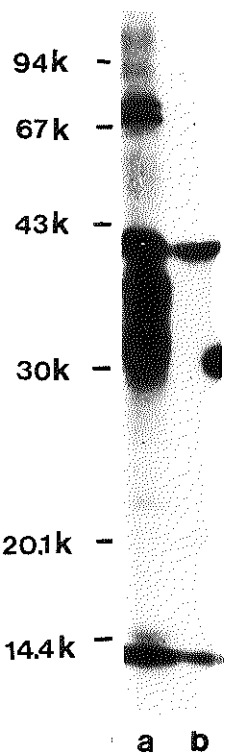

Figure 3. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after immunoprecipitation with: rabbit anti-HC serum (lane a) or rabbit anti-dog-β_2m serum (lane b). Molecular weight standards as described for Fig. 2.

V.3.3 *Immunoprecipitation of ^{35}S-methionine labelled DLA antigens from intact peripheral blood lymphocytes reacted with antiserum before detergent solubilization*

In order to confirm the cell surface location of the DLA antigens, lymphocytes were incubated with the anti-HC serum prior to detergent solubilization. It was attempted to inhibit the reaction between DLA antigens and the anti-HC serum

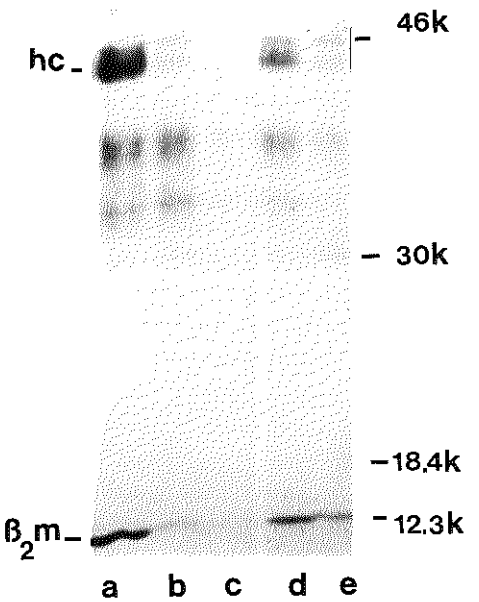

Figure 4. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after incubation of the intact cells with: rabbit anti-HC serum (lane a), rabbit anti-HC serum diluted 1/3 and 1/9 with a solution of purified β_2m (lanes b,c) or rabbit anti-HC serum diluted 1/3 and 1/9 with a solution of ovalbumin (lanes d,e). Molecular weight standards: 46,000 ovalbumin, 30,000 carbonic anhydrase, 18,367 lactoglobulin A, 12,300 cytochrome c. hc: heavy chain, β_2m: β_2-microglobulin.

with urinary β_2m, to exclude the possibility that the anti-HC serum recognizes light chain determinants. Similar experiments have been described in chapter IV, where the reaction of anti-β_2m serum with DLA antigens was inhibited by urinary β_2m (chapter IV, Fig. 1b, 5). Figure 4 represents the gradient gel to which the various samples have been applied. Lane a shows the pattern obtained with undiluted anti-HC serum. The two DLA polypeptide chains are present, as well as intermediate bands of 34,000 and 37,000 MW. Urinary β_2m inhibits the reaction slightly (compare lane b with lane d). The anti-β_2m serum was inhibited completely with the same amount of urinary β_2m (chapter IV, Fig. 5). So, although the anti-HC serum may have some anti-light chain activity, it seems to react mainly with the heavy chain. Immunoprecipitation of denatured and dissociated DLA antigens with the anti-HC serum did not yield a specific band (results not shown). Unlike the anti-β_2m serum, the anti-HC serum is not able to react with its determinants after denaturation of the antigen.

V.3.4 *Immunoprecipitation of detergent solubilized ^{35}S-methionine labelled DLA antigens by purified anti-HC serum*

One way to render the anti-HC serum more specific for DLA class I antigens was to absorb it onto thrombocytes and to elute the reacting antibodies from the thrombocytes afterwards. As reported by Colombani et al. in 1976, thrombocytes do not carry class II antigens. Therefore, antisera can be rendered class II specific by absorption with thrombocytes. We wanted to render the antiserum specific for class I antigens, so rather than using the absorbed serum we isolated the antibodies that had reacted with the thrombocytes and used those. Both the anti-β_2m serum and the anti-HC serum were tested after absorption onto and elution from thrombocytes. In Fig. 5 both antisera give exactly the same pattern; a heavy chain with slight heterogeneity and the light chain. No intermediate bands of appreciable intensity are present.

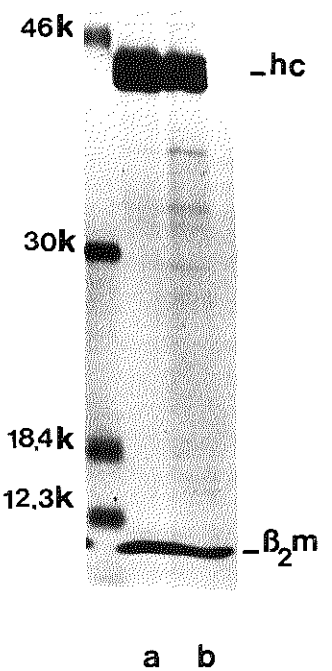

Figure 5. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after immunoprecipitation with: rabbit anti-dog-β_2m serum purified by absorption onto and elution from thrombocytes (lane a) or rabbit anti-HC serum purified by absorption onto and elution from thrombocytes (lane b). Molecular weight standards as described for Fig. 4. hc: heavy chain, β_2m: β_2-microglobulin.

V.3.5 *Immunoprecipitation of detergent solubilized ^{35}S-methionine labelled DLA antigens by alloantisera*

Of the available alloantisera, many were tested for activity in the immunoprecipitation technique. Those sera of all specificities which gave the best results in the one- or two-stage microlymphocytotoxicity test were tested. These were the strongest and most specific alloantisera. They were used untreated, or absorbed with erythrocytes or mismatched PBL. As a source of DLA antigens cell extracts, membrane extracts or lentil lectin purified membrane extracts were employed. The final procedure is described in V.2.4. One antiserum precipitated polypeptide chains of a MW comparable to those precipitated by the anti-β_2m serum (Fig. 6, compare lane d with lane b). Microheterogeneity of the heavy chain is clearly visible and the heavy chain precipitated by the alloantiserum is slightly different from that precipitated by the anti-β_2m serum. No other alloantiserum gave this pattern. Examples are shown in lane e and f. Absorption onto and elution from

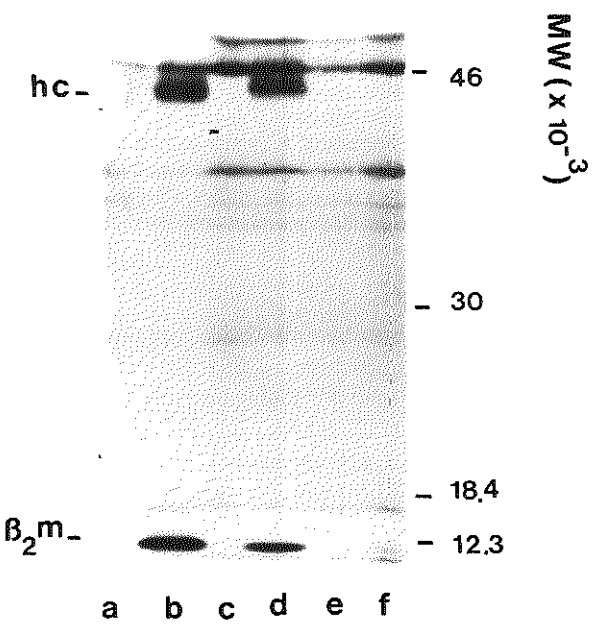

Figure 6. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized dog peripheral blood lymphocyte membrane antigens after immunoprecipitation with: normal rabbit serum (lane a), rabbit anti-dog-β_2m serum (lane b), normal dog serum (lane c), alloantiserum 1302, anti-DLA-C11 (lane d), alloantiserum 057, anti-DLA-C12 (lane e) or alloantiserum 5453, anti-DLA-B4 (lane f). Molecular weight standards as described for Fig. 4. hc: heavy chain, β_2m: β_2-microglobulin.

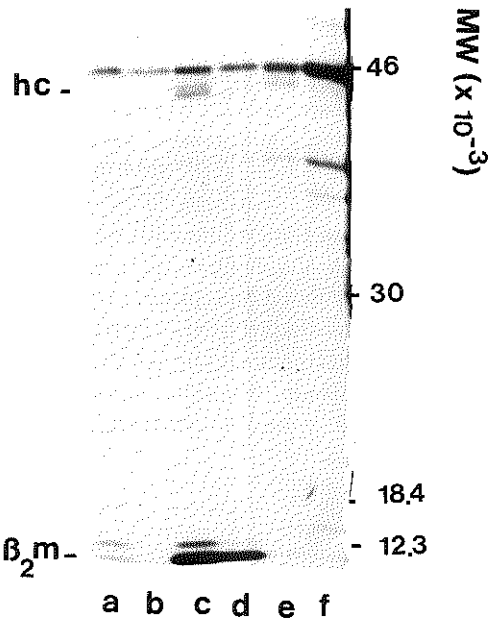

Figure 7. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after immunoprecipitation with: rabbit anti-dog-β_2m serum diluted 1/3 and 1/9 with a solution of purified β_2m (lanes a,b), rabbit anti-dog-β_2m serum diluted 1/3 and 1/9 with a solution of ovalbumin (lanes c,d) or alloantiserum 1302 diluted 1/3 and 1/9 with a solution of purified β_2m (lanes e,f). Molecular weight standards as described for Fig. 4. hc: heavy chain, β_2m: β_2-microglobulin.

thrombocytes, as performed with the anti-HC serum, could not be employed as it resulted in loss of all antiserum activity.

The DLA antigens precipitated by alloantiserum 1302 (anti-DLA-C11) were from a dog typed as A9,B6,C12/A2,B4,C11. No precipitate was found after reaction of the antiserum with DLA antigens from two control dogs homozygous for A9,B6,C12 and A9,B4,C12, respectively (results not shown).

The immunoprecipitation of DLA antigens by the alloantiserum could not be inhibited by urinary β_2m (Fig. 7). As a control the inhibition of the anti-β_2m serum by urinary β_2m is shown. The anti-β_2m serum diluted 1/3 and 1/9 with a solution of ovalbumin (lanes c and d) can precipitate DLA class I antigens; bands of 43,000 and 12,000 MW are clearly visible. The same antiserum diluted 1/3 and 1/9 in a solution of urinary β_2m, however, no longer precipitates similar amounts of 43,000 and 12,000 MW polypeptide chains (lanes a and b). The light chain is far less intense and the heavy chain is no longer visible. When the alloantiserum was diluted in the same amount of urinary β_2m, it was still capable of precipitating DLA heavy and light chains (compare lanes e,f with lanes a,b).

Sequential immunoprecipitations were performed with different combinations of antisera. The anti-β_2m serum could still precipitate DLA antigens after preclearing twice with the alloantiserum or normal rabbit serum, but not after preclearing with anti-β_2m serum. The alloantiserum could only precipitate DLA antigens after preclearing with normal rabbit serum; preclearing with an appropriate alloantiserum or anti-β_2m serum had removed all DLA antigens reactive with serum 1302 (results not shown).

V.4 Discussion

The main object we pursued in the investigations presented here was to confirm the two chain structure of the DLA antigens. If all experiments performed with the anti-β_2m (anti-light chain) serum could be repeated with anti-heavy chain sera this would be firm evidence for the structure of DLA antigens being the same as that of MHC antigens of other species. As experiments with alloantisera turned out to be difficult, we started with raising an antiserum against papain solubilized heavy chains in rabbits. This type of immunization was expected to result in a strong anti-heavy chain backbone (anti-HC) serum.

Firstly, papain solubilized DLA antigens, similar to those used for immunization but now consisting of both chains, were radiolabelled and incubated with the antiserum. The antiserum precipitated DLA heavy and light chains, like the anti-β_2m serum, but it recognized extra proteins of MW 19,000, 30,000 and 39,000 (Fig. 2).

The anti-HC serum could also recognize detergent solubilized DLA antigens. The papain solubilized heavy chain, against which the antiserum was raised, comprises the largest part of the extracellular portion of the DLA antigens. Therefore it was not surprising that the antiserum would also precipitate detergent solubilized DLA antigens.

From detergent solubilized membrane molecules, the anti-HC serum precipitated extra polypeptide chains, too, of 30,000-39,000 daltons (Fig. 3). Even when the anti-HC serum was incubated with intact cells did extra bands appear (34,000 and 37,000 daltons; Fig. 4).

The 39,000 MW band (Fig. 2) could be an early cleavage product of papain, as has been shown for HLA (Springer, 1974). In the HLA molecule, however, this papain cleavage site is located intracellularly and it is only detected after papain digestion of detergent solubilized molecules. If the 39,000 MW band would represent a similar product in the dog, there would have to be two papain cleavage sites extracellularly. If the 39,000 MW polypeptide chain is indeed an early cleavage product it remains unexplained that the anti-β_2m serum does not precipitate it.

The bands of MW other than 37,000 and 12,000 (Fig. 2) or 41,700 and 12,000 (Fig. 3) could all be breakdown products of DLA antigens. For the molecules of 34,000 MW and 37,000 MW (Fig. 3) this is unlikely, however, as they were also demonstrated on intact cells (Fig. 4). We consider it more likely that some of them

represent the dog equivalent of the class II antigens. There are no dog class II specific antisera and the purification of B cells is difficult in the dog (Krakowka and Guyot, 1977), so it is difficult to investigate this further. The obervation that after absorption onto and elution from thrombocytes the anti-HC serum no longer contains antibodies reacting to the "extra" polypeptide chains indicates that some of them are class II antigens (Fig. 5). Immunoprecipitation with the absorbed anti-HC serum should yield the missing products again.
Preliminary experiments were done with pokeweed mitogen or Staphylococcal protein A stimulated cells. Immunoprecipitation of membrane antigens from these with the anti-HC serum did not selectively enhance the class II-like bands. Still, Staphylococcal protein A could be useful as in the dog it behaves differently from PHA, a typical T cell mitogen (Betton et al., 1980).

The smallest protein which was recognized by the anti-HC serum and not by the anti-β_2m serum has a MW of 19,000 daltons, identical to T3, a molecule defined by van Agthoven et al. (1981). It could also be a breakdown product of the DLA class I antigens. It was only detected in spleen cell preparations after papain digestion; on PBL membranes or in detergent solubilized PBL extracts it was not found. This would argue against homology with T3. The fact that in addition to class I antigens, other chains were precipitated, could mean that the heavy chain preparation used for immunization contained small amounts of other proteins. The heavy chain preparation was only pure as far as it could be judged from Coomassie Brilliant Blue stained SDS-polyacrylamide gels.

Further evidence for the two chain structure of the DLA antigens was given by immunoprecipitation with alloantiserum 1302. This serum is used for typing and must therefore be directed against the alloantigenic determinant on the heavy chain. The gel pattern obtained with this serum consisted of two polypeptide chains of 43,000 and 12,000 MW, just like that of the anti-β_2m serum (Fig. 6). The MW of 41,700 for the heavy chain was found on 12.5% gels (Figs. 2, 3); on gradient gels the MW seems somewhat larger. SDS-PAGE is not an ideal method for MW determination. Some heterogeneity was observed in the heavy chain. This could be due to differences in amount of sialic acid; it is further discussed in chapter VI.4.
Control experiments to confirm that both types of anti-heavy chain sera recognized determinants on the heavy chain and not on the light chain were done by diluting the antisera with urinary β_2m. Immunoprecipitation of DLA antigens by anti-heavy chain sera was not inhibited much by β_2m (Figs. 4, 7), as opposed to immunoprecipitation by the anti-β_2m serum. DLA heavy chains, separated from the light chain by lyophilization, should be radiolabelled and used for immunoprecipitation by the anti-HC serum. It would then become clear whether the anti-HC serum is truly anti-heavy chain.
The anti-HC serum could not react with the dissociated denatured heavy chain (or light chain). It probably needs the native heavy chain conformation for reaction. It is difficult to obtain dissociated native heavy chains. Nakamuro et al. (1975) have described an anti-human-β_2m serum with which they can dissociate the two HLA chains without loss of alloantigenic activity of the heavy chain, but in most studies

this has not been found to be possible. The DLA heavy chain preparation with which we raised the anti-HC serum contained impurities. For this reason no attempt was made to inhibit the reaction between the anti-heavy chain sera and DLA antigens by isolated heavy chains. For the same reason we did not prove by immunoprecipitation that alloantiserum 1302 is directed against the heavy chain only.
The location of DLA antigens on the cell surface was confirmed in that anti-HC serum could react with DLA antigens on intact cells (Fig. 4).
Sequential immunoprecipitation experiments confirmed that alloantiserum 1302 reacts with some DLA antigens (DLA-C11) whereas anti-β_2m serum reacts with all, or certainly more, DLA antigens. As only one alloantiserum could be used in the immunoprecipitation experiments, no further investigation into the DLA antigens from different loci or alleles could be performed.

In conclusion: The two chain structure and cell surface location of the DLA antigens were confirmed by experiments with antisera directed against the heavy chain. The anti-heavy chain serum prepared after immunization with papain solubilized heavy chain may contain antibodies against class II antigens.

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CHAPTER VI

DLA CLASS I ANTIGENS CONTAIN A CARBOHYDRATE MOIETY

VI.1 Introduction

One of the most striking characteristics of the products of the MHC is their extreme polymorphism. The alloantigenic site(s) can in theory be protein, carbohydrate or both in nature, as the MHC antigens are glycoproteins. The contribution of carbohydrate to the antigenic activity of DLA antigens is unclear. It has not even been firmly established that DLA antigens contain a carbohydrate group.
Carbohydrate analysis of H-2 (Shimada and Nathenson, 1969) and HLA class I antigens (Mann et al., 1969) has revealed that they contain about 90% protein and 10% carbohydrate. H-2 antigens include two heterosaccharide chains per molecule (Nathenson and Muramatsu, 1971) whereas HLA antigens have one sugar chain (Strominger et al., 1981). Both are sugar chains of the complex form, and therefore susceptible to cleavage by endo-β-N-acetylglucosaminidase D from Diplococcus pneumoniae (Muramatsu, 1971). HLA and H-2 class I antigens have been synthesized by cells in the presence of tunicamycin, an inhibitor of N-linked glycosylation (Kuo and Lampen, 1974). The molecular weight of the HLA and H-2 heavy chain synthesized in these circumstances is slightly lower than that observed in control cases without tunicamycin. Therefore the HLA and H-2 class I antigens must contain N-linked oligosaccharides (Dobberstein et al., 1979, Ploegh et al., 1981).
The only indication that DLA antigens may contain a carbohydrate group is the fact that DLA antigens, like H-2 (Kvist et al., 1977) and HLA (Snary et al., 1974) antigens, can be isolated from a relatively crude membrane preparation by lentil lectin affinity chromatography (van der Feltz et al., 1981 and Chapter II.3). Lentil lectin has specificity for α-D-glucose and α-D-mannose residues, and DLA antigens can be eluted from lentil lectin with 2.5% α-methyl-D-mannoside.
In this chapter some more direct evidence for the presence of a carbohydrate chain in DLA antigens is presented. PHA-stimulated peripheral blood lymphocytes were grown in the presence of ^{3}H-fucose, and immunoprecipitation of cell lysate proteins was performed with a rabbit anti-dog-β_2m serum. DLA antigens were also radiolabelled in their protein moiety in the presence or absence of tunicamycin and analysed for possible changes in the molecular weight of the heavy chain.

VI.2 Materials and methods

VI.2.1 *Preparation of ^{3}H-fucose labelled cell extracts*

Peripheral blood lymphocytes from beagles were isolated from heparinized blood by centrifugation on Ficoll Isopaque. PHA stimulation was carried out for 72 or 96 hours in a 5% CO_2 humidified atmosphere at 37°C. PHA-M (Difco, Detroit, MI, U.S.A.), final dilution of 1/360 out of a stock solution of 1% in 0.85% saline, was added to 10^6 cells per ml in RPMI 1640 medium supplemented with glutamine, final concentration 2 mM, penicillin and streptomycin, final concentration 50 U/ml and 50 μg/ml (all from Gibco Europe, Paisley, Scotland) and 10% normal dog serum. Just before labelling, the percentage of lymphoblasts was determined (generally about 90%) and the viability was checked by trypan blue exclusion (usually the viability was around 90% as well). The cells were washed and resuspended at 10^6 cells/ml in RPMI 1640 supplemented with glutamine, penicillin and streptomycin, and 10% dialyzed foetal calf serum (Gibco Europe, Paisley, Scotland). ^{3}H-fucose was added (Amersham International plc, Amersham, England), 50 μCi to 10^7 cells. The cultures were incubated for 16 hours at 37°C in the CO_2 incubator. Either whole cells or membranes (see IV.2.5), prepared from cell lysates, were solubilized for 30 minutes on ice by a non-ionic detergent: 0.75% NNP10 (Servo, Delden, the Netherlands) in phosphate buffered saline, containing 0.125% EDTA and 0.02% NaN_3. After solubilization, the preparations were centrifuged in a Beckman airfuge at 100,000 $\times$ g for 60 minutes at 4°C. The supernatant contained the solubilized membrane proteins.

VI.2.2 *Preparation of ^{35}S-methionine labelled cell extracts in the presence or absence of tunicamycin*

Lymphocytes were purified and stimulated with PHA-M as described above. After 3.5 days of PHA-stimulation cells were spun down and resuspended at 6×10^6 cells/ml in RPMI 1640 without methionine (Gibco Europe Select-Amine kit) supplemented with glutamine, penicillin and streptomycin, and 10% foetal calf serum. One half of each culture was preincubated with 3 μg/ml tunicamycin (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) for one hour at 37°C, 5% CO_2; the other half was preincubated without tunicamycin. After one hour, ^{35}S-methionine was added (Amersham International plc, Amersham, specific activity 800-1100 Ci/mMol), 100 μCi per 3 ml culture, for a total of 8 hours. Thereafter cells were spun down and solubilized in 0.5 ml lysis buffer per culture. This lysis buffer consisted of 0.5% NP-40 (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 0.01 M Tris-HCl pH 7.3, 0.005 M $MgCl_2$, 0.02 M NaCl and 0.001 M PMSF). After 30 minutes on ice lysates were centrifuged at 16,000 $\times$ g in an Eppendorf centrifuge for 15 minutes at 4°C. The supernatant contained the solubilized membrane proteins.

VI.2.3 *Antisera and immunoprecipitation*

A rabbit anti-dog-β_2m serum was prepared as described (chapter III.2.3). As a

control, normal rabbit serum was used. For the ^{3}H-fucose labelled cell extracts the procedure was as follows: Sepharose-protein A (Pharmacia, Uppsala, Sweden) was used as an immunoadsorbent in a 10% (w/v) suspension. All incubations were carried out at room temperature. Of the ^{3}H-fucose labelled molecules, 5×10^{6} cell equivalents were incubated with either 50 μl normal rabbit serum or 50 μl anti-β_2m serum, which had previously been reacted for 30 minutes with a tenfold volume of Sepharose-protein A solution. The incubation of solubilized membrane proteins with Sepharose-protein A-attached antiserum took 60 minutes. After this incubation, the Sepharose-protein A beads, now carrying immune complexes, were washed thoroughly with each of the following buffers: twice with 0.5 M Tris-HCl pH 8.0, 1% NNP10, twice with 0.01 M Tris-HCl pH 8.0, 0.1% NNP10, and twice with 0.01 M Tris-HCl pH 8.0. The beads were eluted with dilute acetic acid of pH 2.8 and the eluates were lyophilized. The ^{35}S-methionine labelled cell extracts were pretreated with normal rabbit serum (5 μl per 9×10^{6} cell equivalents). As immunoadsorbent protein A bearing Staphylococcus aureus Cowan 1 was used in a 10% suspension prepared as described by Kessler (1975). Preclearing was ended by adding Staph A, 75 μl per incubation, for 15 minutes on ice. After centrifugation the extracts were treated with 3 μl anti-β_2m serum per 3×10^{6} cell equivalents for 1 hour on ice, and 50 μl Staph A for 15 minutes on ice. The immune complex carrying Staph A were washed and eluted as described (chapter IV.2.7).

VI.2.4 *Gel electrophoresis*

^{3}H-fucose labelled samples were analysed on $10 \times 14 \times 0.1$ cm 12.5% polyacrylamide gels according to Laemmli (1970). Before application to the gel, the lyophilized samples (VI.2.3) were dissolved in sample buffer and boiled for 5 minutes. ^{35}S-methionine labelled samples were analysed on $15 \times 30 \times 0.1$ cm, 7.5-15% linear gradient gels according to Dobberstein et al. (1979). Radioactive polypeptides were detected by fluorography (Bonner and Laskey, 1974). All chemicals for electrophoresis were from Bio-Rad, Richmond, CA, U.S.A. ^{14}C-labelled marker proteins were obtained from NEN, Dreieich, F.R.G.

VI.3 **Results**

We incubated peripheral blood lymphocytes from different tissue-typed beagles with ^{3}H-fucose in order to investigate whether DLA class I antigens incorporate this sugar. In Fig. 1, left column, a summary of the immunoprecipitation schedule is shown. As a control, lymphocytes were also labelled with ^{35}S-methionine, a procedure which is known to result in radiolabelling of DLA antigens (van der Feltz et al., 1982 and Chapter IV.3). After labelling, cell extracts were made and immunoprecipitation with anti-β_2m serum was performed. A representative fluorograph is presented in Fig. 2. Fig. 2a shows the heavy chain clearly labelled with ^{3}H-fucose (lane a), whereas the light chain, β_2m, is not. Identical results were obtained both with directly solubilized cells and with solubilized membranes.

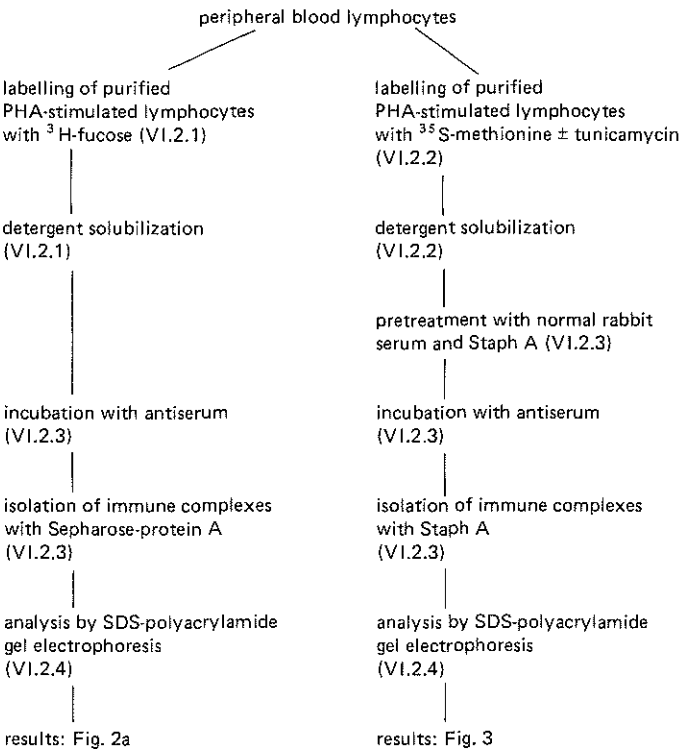

Figure 1. Summary of the immunoprecipitation schedules used.

Normal rabbit serum did not precipitate the DLA molecules. For comparison an immunoprecipitate of anti-β_2m serum and ^{35}S-methionine labelled antigen is shown in Fig. 2b. The light chain, β_2m, is clearly visible. The heavy chain has approximately the same molecular weight as the ^{3}H-fucose labelled heavy chain; about 42,000 to 44,000 daltons.

For the investigation of the effect of tunicamycin on the synthesis of DLA class I antigens, tissue-typed beagles were used. If DLA antigens contain N-linked sugars, labelling with ^{3}H-fucose in the presence of tunicamycin might result in unlabelled DLA molecules, as no sugar chain would be attached to the protein chain in these circumstances. The absence of a protein band cannot be positively interpreted. Therefore, cells were labelled with ^{35}S-methionine. Immunoprecipitation was carried out with anti-β_2m serum. The methods used are summarized in Fig. 1, right column. In Fig. 3 immunoprecipitates of cells, grown in the presence or absence of tunicamycin are shown. The whole procedure was very similar to that which resulted in the gel of Fig. 2b, but in Fig. 3 a linear gradient gel of larger dimensions was used, which affords better resolution.

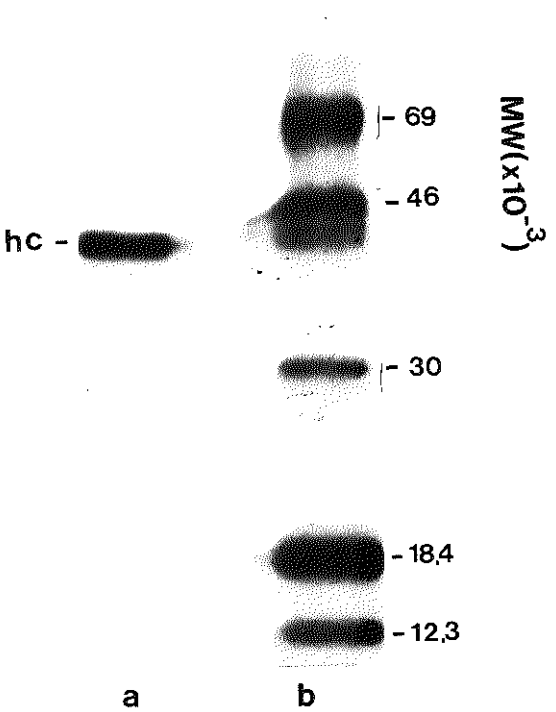

Fig. 2a

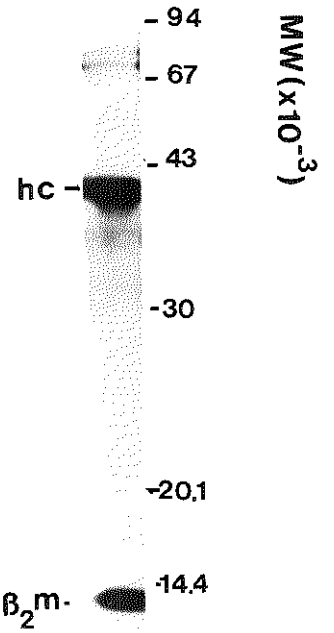

Fig. 2b

Figure 2a. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of ^{3}H-fucose labelled DLA antigens after immunoprecipitation with rabbit-anti-dog-β_2m serum (lane a). Molecular weight standards (lane b): 69,000 albumin, 46,000 ovalbumin, 30,000 carbonic anhydrase, 18,367 lactoglobulin A, 12,300 cytochrome c. hc: heavy chain.

Figure 2b. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) of ^{35}S-methionine labelled DLA antigens after immunoprecipitation with rabbit anti-dog-β_2m serum. Molecular weight standards: 94,000 phosphorylase b, 67,000 albumin, 43,000 ovalbumin, 30,000 carbonic anhydrase, 20,100 trypsin inhibitor, 14,400 lactalbumin. hc: heavy chain, β_2m: β_2-microglobulin.

A band co-migrating with the heavy chain of IgG, and actin, a common contaminant of immunoprecipitates from lymphocytes (Barber and Delovitch, 1979), is present in all precipitates, including the control precipitate with normal rabbit serum (lane a). Two other major bands are precipitated from the cell lysates by the anti-β_2m serum. In the case of cells grown in the absence of tunicamycin (lane b) they have apparent molecular weights of 43,000 and 12,000. Some heterogeneity of the heavy chain is observed. This could be due to different

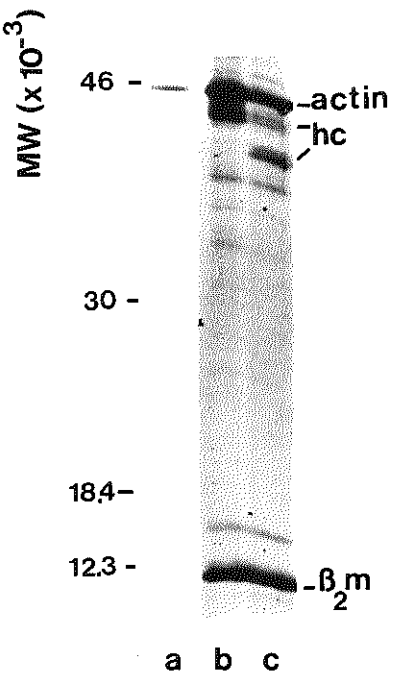

Figure 3. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after immunoprecipitation with normal rabbit serum (lane a), or rabbit anti-dog-β_2m serum (lanes b,c).
lanes a,b: labelling in the absence of tunicamycin.
lane c: labelling in the presence of tunicamycin.
MW markers as described for Fig. 2a.
hc: heavy chain, β_2m: β_2-microglobulin.

numbers of sialic acid moieties per heavy chain, as was shown to be the case for HLA antigens (Parham et al., 1974). In lane c the gel pattern of an immunoprecipitate from cells grown in the presence of tunicamycin is shown. The difference from lane b is evident. The apparent MW of the DLA heavy chain is clearly reduced as compared to the actin band. Almost complete inhibition of N-linked glycosylation must have occurred at the concentration of tunicamycin used (3 μg/ml). Two faint bands were found at the original MW. In both glycosylated and nonglycosylated heavy chains heterogeneity remains. This heterogeneity will be further dealt with in chapter VII.
From the data presented here one can conclude that DLA class I antigens consist of a nonglycosylated light chain, β_2m, and a heavy chain which shows heterogeneity and which most likely contains an N-linked carbohydrate chain.

VI.4 Discussion

As fucose is a component of HLA (Strominger et al., 1981) and H-2 class I antigens (Nathenson and Muramatsu, 1971), this sugar was the first choice for the investigation of the existence of a DLA sugar residue. From Fig. 2a it is evident that DLA antigens can be labelled by ^{3}H-fucose. Up till now, the only evidence for sugar residues in DLA antigens came from the isolation experiments with Sepharose-lentil lectin (van der Feltz et al., 1981 and Chapter II). As lentil lectin has specificity for α-D-glucose and α-D-mannose residues, this meant that DLA antigens contained either mannose or glucose, or both. The ^{3}H-fucose incorporation means that DLA antigens also contain fucose. However, the band which represents the heavy chain is the only one visible in the gel. The polypeptide of MW 12,000, which is normally seen when DLA antigens are labelled in their protein moiety (Fig. 2b), is absent in Fig. 2a. Incorporation of radioactive sugars in HLA and H-2 antigens gave identical results (Cresswell et al., 1974, Nathenson and Muramatsu, 1971). This is not surprising, as β_2m is devoid of carbohydrate (Berggård and Bearn, 1968).

Tunicamycin, an inhibitor of N-linked glycosylation, can be used to investigate the presence and size, or number of N-linked carbohydrate chains in many types of glycoproteins, e.g. MHC antigens (Dobberstein et al., 1979, Ploegh et al., 1981). In HLA and H-2 antigens synthesized in the presence of tunicamycin the heavy chain has a molecular weight which is lower than that of fully glycosylated heavy chain. The MW change is compatible with the presence of one N-linked carbohydrate chain in HLA and two in H-2 antigens, as had been found earlier by glycopeptide analysis (Nathenson and Muramatsu, 1971, Parham et al., 1977). O-linked glycosylation, which might also contribute to heterogeneity of histocompatibility antigens has not been found in MHC products.

DLA antigens from cells, grown in the presence of tunicamycin have a heavy chain which is approximately 3000 daltons lower in MW than that observed in control cells. This difference in MW is of the same order of magnitude as the difference in MW of glycosylated and nonglycosylated HLA heavy chains analysed under similar circumstances (Ploegh et al., 1981). It is therefore justified to assume that DLA class I antigens carry one N-linked carbohydrate side chain of a size similar to that seen for HLA antigens.

Limited attempts were made to show that DLA antigens would be susceptible to cleavage by endo-β-N-acetylglucosaminidase D. No clearcut results were obtained. In the case of HLA this enzyme can remove most of the carbohydrate side chain (Parham et al., 1977).

Susceptibility of a glycoprotein to tunicamycin means that an N-linked sugar is present; susceptibility to endo-β-N-acetylglucosaminidase D means that this sugar is of the complex type. Radiolabelling in the presence of tunicamycin and incubation with endo-β-N-acetylglucosaminidase D are both ways of estimating the size and number of carbohydrate chains present in a molecule. As for DLA antigens, it is likely that there is one N-linked sugar chain, but whether this chain is of the complex type or not is uncertain.

Biosynthetic experiments on nonglycosylated HLA antigens (Ploegh et al., 1981) have shown that glycosylation does not affect the association of β_2m and heavy chain. From the experiments described here it is clear that the process of glycosylation is not required for the association of DLA heavy and light chains either. The same has been reported for HLA, as mentioned above, H-2 (Dobberstein et al., 1979) and OMLA (owl monkey) antigens (Parham and Ploegh, 1980). Glycosylation is not necessary for membrane insertion and transport to the plasma membrane of HLA (Ploegh et al., 1981) and H-2 (Dobberstein et al., 1979) antigens. Biosynthetic experiments will be needed to explore these processes for DLA antigens. The conformation of the HLA antigens is not influenced by lack of glycosylation in terms of susceptibility to proteolytic enzymes and the reactivity with various alloantisera (Ploegh et al., 1981). In contrast, Wilson and colleagues (1981) have described a monoclonal antibody which fails to react with nonglycosylated HLA antigens, although the determinant recognized is supposedly protein in nature. The difference between the reagents used, a monoclonal antibody or a highly complex alloantiserum probably accounts for the different results. Unfortunately, only one alloantiserum was able to precipitate DLA antigens in the system used here (chapter V.3). It has not yet been reacted with tunicamycin-treated cell extracts. It can probably be used to precipitate nonglycosylated DLA antigens, but its broad specificity (anti-C11, cross-reacting with A2 and B5) makes it difficult to draw conclusions on the influence glycosylation may have on the alloantigenicity of DLA molecules. The old controversy on the biochemical nature of the alloantigenic determinant (carbohydrate: O'Neill et al., 1981, Sanderson et al., 1971; protein: Nathenson and Muramatsu, 1971, Parham et al., 1977, Ploegh et al., 1981) can at present not be directly resolved in the dog model.

In conclusion: The DLA heavy chain is sensitive to tunicamycin and must therefore have an N-linked sugar side chain. The difference in MW of glycosylated and nonglycosylated heavy chains is enough to accomodate one complex sugar side chain. The DLA light chain is unlikely to contain any sugar residue.

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CHAPTER VII

THE ALLOANTIGENIC SITE OF DLA CLASS I ANTIGENS

VII.1 Introduction

In the preceding chapters, the groundwork was laid for the experiments described here. DLA class I antigens were found to be glycoproteins with one N-linked sugar side chain (chapter VI). They can be isolated from detergent solubilized, radiolabelled peripheral blood lymphocytes by immunoprecipitation with anti-β_2m serum (chapters IV, VI).

From DLA serology, it is clear that DLA class I antigens are polymorphic. On the basis of serological data it was decided that there are three DLA class I loci which each consist of a variety of alleles (Vriesendorp et al., 1977). In this chapter an attempt is made to find a molecular basis for the polymorphism observed. In other species, there is usually no polymorphism at all or very limited polymorphism in the light chain (Michaelson et al., 1980) and the alloantigenic site is assigned to the heavy chain.

The first question we thus have to answer for the DLA antigens is: on which polypeptide chain lies the determinant with which the alloantisera react? The second question is: can we identify the products of different loci or alleles? A biochemical approach to these questions is to analyse DLA antigens by two-dimensional (2D) gel electrophoresis. This technique has been used to distinguish different MHC class I products by their heavy chain patterns (Jones, 1977, Vasilov et al., in press), and to establish that in HLA-DR antigens the light chain, rather than the heavy chain, is the variable one (Charron and McDevitt, 1979, Shackelford and Strominger, 1980). The DLA antigens to be analysed were isolated by immunoprecipitation with the rabbit anti-dog-β_2m serum as described in chapter VI, assuming that the antiserum will react with β_2m associated DLA class I antigens of all tissue-types, no matter where the alloantigenic site is located. As it is not known whether the alloantigenic determinant is protein, sugar or both in nature, experiments were carried out with DLA antigens labelled in the presence or absence of tunicamycin, an inhibitor of N-linked glycosylation (Chapter VI, Kuo and Lampen, 1974). After immunoprecipitation, DLA antigens were analysed by one- and two-dimensional gel electrophoresis.

The second question relating to the identification of different haplotypes or alleles is more difficult to answer. DLA antigens were isolated from two dog families with the anti-β_2m serum. Within a family, certain dogs have certain haplotypes in common and by comparison of different two-dimensional gel patterns tentative correlations of spots on the autoradiograms to tissue-type can be made. For one dog which was positive for DLA-C11, the pattern obtained with the one usable

alloantiserum (chapter V) was compared to the pattern obtained with the anti-β_2m serum.
Using the same methodology, DLA antigens on tumour cells and peripheral blood lymphocytes of the tumour bearer were analysed. It has been reported by several groups that "alien" histocompatibility antigens can be found on tumour cells, that is to say, specificities can be found on tumour cells, usually in the mouse, in addition to, or instead of, the original specificities of the tumour bearer (J. Immunogenet., 1980, Transplant. Proc., 1980, 1981). A biochemical way to investigate this is immunoprecipitation of radiolabelled detergent solubilized membrane antigens with anti-β_2m serum and subsequent analysis by two-dimensional gel electrophoresis. Immunoprecipitation patterns of alien histocompatibility antigens show that the light chain has a MW of 12,000 daltons, so that it is likely that the light chains are identical to β_2m (Parmiani et al., 1979, Schmidt et al., 1980). Most of the research concerning (alien) histocompatibility antigens on tumour cells has been performed with induced tumours in inbred rodents. We made an attempt to study spontaneous tumours in (outbred) dogs.

VII.2 **Materials and methods**

VII.2.1 *Cells*

Peripheral blood lymphocytes from beagles and mongrel dogs were purified by centrifugation on Ficoll Isopaque. Peripheral blood lymphocytes from beagles were always used fresh, whereas mongrel, tumour bearer peripheral blood lymphocytes were cryopreserved and stored in liquid nitrogen until use. Tumour cell lines were derived from solid tumours of different histological type in mongrel dogs. Tumour material was given to us by drs. P. Lansdorp from our laboratory, presently working at the Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis in Amsterdam and drs. G. Rutteman from the Kliniek voor kleine huisdieren in Utrecht. Tumour cells were grown in RPMI 1640 supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% foetal calf serum (all from Gibco Europe, Paisley, Scotland). Alternatively they were cultivated in Iscove's modified Dulbecco's medium supplemented with 5% foetal calf serum (both from Gibco Europe, Paisley, Scotland) and antibiotics. In one case the cells from an early passage were transplanted into nude mice, and the solid tumours obtained were used to establish a cell line. Cell lines were checked for mycoplasma infection at regular intervals.

VII.2.2 *Preparation of labelled cell extracts*

PHA stimulation and ^{35}S-methionine labelling of peripheral blood lymphocytes was done as described in chapter VI (VI.2.2). In the case of one dog family (family 4, Table 1), 200 μCi ^{35}S-methionine/3ml culture was used instead of 100 μCi. For ^{35}S-methionine labelling of tumour cells, half confluent monolayers in 75 cm^2 flasks

were incubated with 10 ml RPMI 1640 with the usual supplements but lacking methionine, for 1 hour in a CO_2 incubator at 37°C. ^{35}S-methionine was added, 100 μCi per flask and after 16 hours at 37°C, 5% CO_2 cells were trypsinized, washed and solubilized as described for peripheral blood lymphocytes. Tunicamycin (Calbiochem-Behring Corp., La Jolla, CA, U.S.A) was added to the cultures at a concentration of 3-5 μg/ml where required.

VII.2.3 *Antisera and immunoprecipitation*

The rabbit anti-dog-β_2m serum described in chapters III, IV and VI was used. Normal rabbit serum served as a control. Immunoprecipitation was basically as described in chapter VI (VI.2.3). For two-dimensional gel electrophoresis, the lysate of 10^7 cells was pretreated with 5 μl normal rabbit serum and 75 μl Staphylococcus aureus Cowan 1 (Staph A) and then incubated with 3 μl anti-β_2m serum and 50 μl Staph A. After washing, the pellets were stored dry at -70°C.
For one-dimensional gel electrophoresis, the pellets were eluted by boiling for 3 minutes with sample buffer followed by alkylation (Dobberstein et al., 1979). For two-dimensional electrophoresis, pellets were suspended in isoelectric focusing sample buffer (O'Farrell, 1975), kept at room temperature for 5-10 minutes and centrifuged. The resulting supernatant was applied to the isoelectric focusing gels.

VII.2.4 *Gel electrophoresis*

One-dimensional gel electrophoresis was performed on $15 \times 30 \times 0.1$ cm, 7.5-15% linear gradient gels according to Dobberstein et al. (1979). Two-dimensional gel electrophoresis was done by the method of O'Farrell (1975) with the modifications of Vasilov et al. (in press). These modifications are briefly: the casting of 14 mm focusing gels in 200 μl capillaries and the use of a Studier type apparatus (Studier, 1973) to which the capillaries are attached with a glass plate and modelling clay, for the first dimension. After equilibration in the appropriate buffer, focusing gels were sealed to 7.5-15% linear gradient gels using a 1% agarose solution in sample buffer. Gradient gels were run as described above. Gels were fluorographed on Kodak XAR 5 film (Bonner and Laskey, 1974).
Most of the experiments described were performed at the Institute of Genetics, University of Cologne, F.R.G. with the invaluable assistance of H.L. Ploegh, A. Hahn and H. Mölders.

VII.3 **Results**

It has been shown (chapter VI) that both glycosylated and nonglycosylated DLA antigens can display heterogeneity in the heavy chain when analysed on 7.5-15% linear gradient gels. Therefore a preliminary screening of DLA antigens from three (incomplete) dog families immunoprecipitated by the anti-β_2m serum was done on a linear gradient gel. The method used is summarized in Fig. 1 (left column).

Figure 1. Summary of the immunoprecipitation schedules used.

VII.3.1 *One-dimensional gel electrophoresis of glycosylated and nonglycosylated DLA antigens*

Typing results for the families described in this chapter are given in Table 1. The cells of families 1-3 were labelled in the presence or absence of 5 μg/ml tunicamycin. In Fig. 2 immunoprecipitates of cells grown without tunicamycin are shown. A band consisting of actin, a common contaminant of immunoprecipitates from lymphocytes (Barber and Delovitch, 1979) is present in all precipitates, including the control precipitate with normal rabbit serum (lane a). The other major bands are precipitated from all cell lysates by the anti-β_2m serum and have apparent molecular weights of 43,000 and 12,000 daltons. Some heterogeneity of the heavy chain is observed. This could be due to different numbers of sialic acid moieties per heavy chain, as was shown to be the case for HLA antigens (Parham et al., 1974).

The same dogs from families 1-3 were used for one-dimensional gel analysis of non-glycosylated DLA antigens. The gel pattern of Fig. 3, from cells grown in the presence of tunicamycin, is evidently different from that in Fig. 2. The apparent

Table 1. Tissue typing of the dogs described in Figures 2-6.

dog			DLA typing
Family 1.	father	dog 1	A3+10*, B-, C- /A10, B6, C-
	sib	dog 2	A2, B5, C11 /A10, B6, C-
	sib	dog 3	A3+10, B-, C-, /A9, B6, C12
Family 2.	father	dog 4	A3+10, B-, C- /A9, B6, C12
	sib	dog 5	A1, B13, C- /A9, B6, C12
	sib	dog 6	A9, B6, C12 /A9, B6, C12
Family 3.	father	dog 7	A9, B6, C12 /A9, B6, C12
	mother	dog 8	A2, B4, C11 /A9, B6, C12
	sib	dog 9	A2, B4, C11 /A9, B6, C12
Family 4.	mother		A7, B-, C- /A7, B-, C-
	father		A3+10, B-, C- /A9, B6, C12
	sib 1		A3+10, B-, C- /A7, B-, C-
	sib 2		A3+10, B-, C- /A7, B-, C-
	sib 3		A7, B-, C- /A9, B6, C12
Family 5.	mother		A9, B4, C12 /A9, B6, C12
	father		A9, B4, C12 /A2, B4, C11
	sib 1		A9, B4, C12 /A9, B4, C12
	sib 2		A9, B4, C12 /A2, B4, C11
	sib 3		A9, B4, C12 /A9, B6, C12

*DLA-A3 and DLA-A10 are difficult to separate by serology.

Families 1-3 were used for the experiments described in Figs. 2 and 3. Family 4 is further described in Fig. 5 and family 5 in Fig. 6.

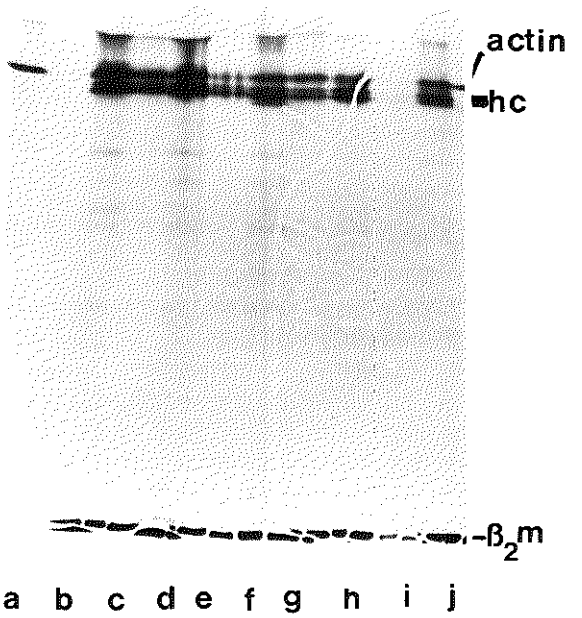


Figure 2. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after immunoprecipitation with: normal rabbit serum (lane a) or rabbit anti-dog-β_2m serum (lanes b-j).
lane a: dog 6 (Table 1) (control).
lanes b-j: dogs 1-9 (Table 1).
hc: heavy chain, β_2m: β_2-microglobulin.

MW of the DLA heavy chain is reduced to 40,000 daltons. The concentration of tunicamycin used (5 μg/ml) is such that complete inhibition of N-linked glycosylation occurred. Not even a faint band at the original MW can be found, even after long exposure times.
The amount of radioactivity recovered in β_2m is in some cases (lanes b,c,g) clearly greater than the amount in the heavy chain. As an antiserum against β_2m was used, free β_2m has probably been precipitated in addition to DLA-bound β_2m. Polymorphism as observed for mouse β_2m (Michaelson et al., 1980), was not evident in dog β_2m (Fig. 3). Although no N-linked sugar residues are present in DLA heavy chains from tunicamycin treated cells, heterogeneity of the heavy chain persists.

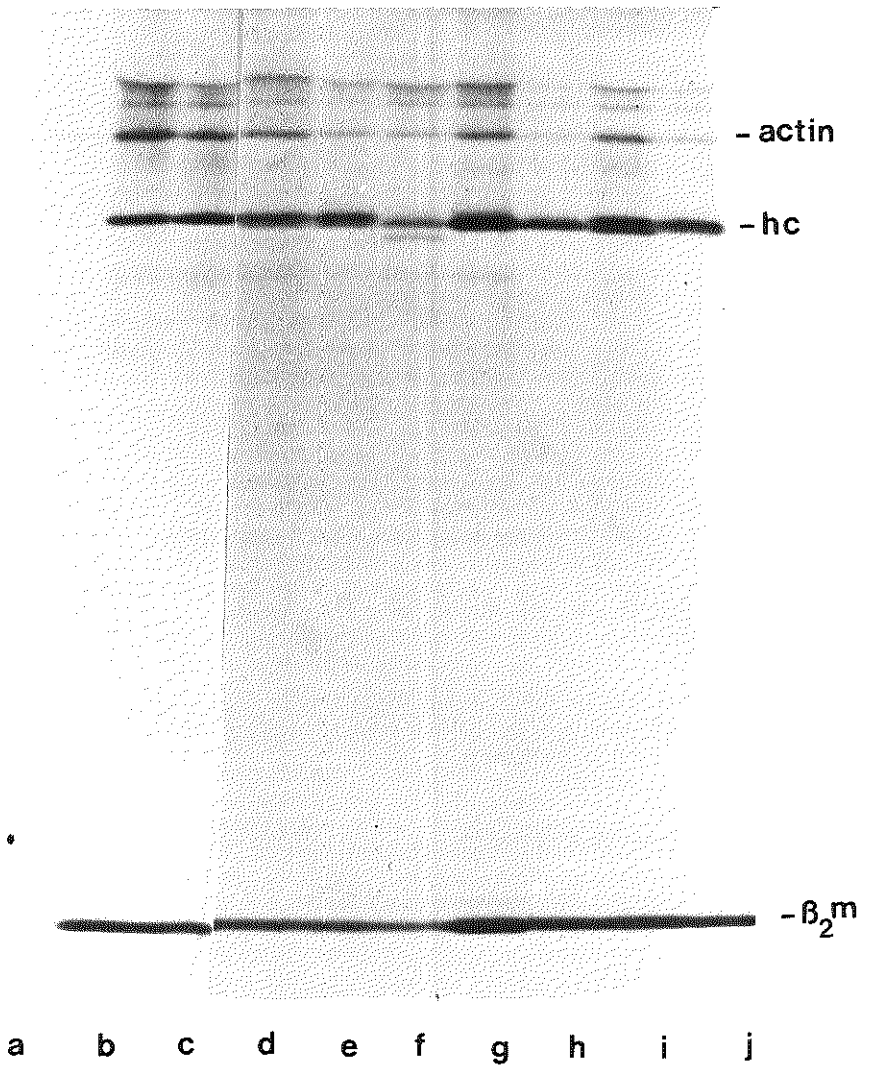


Figure 3. SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) of ^{35}S-methionine labelled, detergent solubilized DLA antigens after immunoprecipitation with: normal rabbit serum (lane a) or rabbit anti-dog-β_2m serum (lanes b-j).
lane a: dog 6 (Table 1) (control).
lanes b-j: dogs 1-9 (Table 1).
All cells were radiolabelled in the presence of 5 μg/ml tunicamycin.
hc: heavy chain, β_2m: β_2-microglobulin.

We analysed the possibility of a correlation between the gel pattern of the heavy chain and the tissue-type of the dog (Table 1). Dog 1 and 2 (Fig. 3, lanes b and c) have an identical pattern, although they have only one haplotype in common. Dog 3 (lane d) has two heavy chain bands, one in the same position as dog 1 and 2, and the other of a slightly lower MW. This band could therefore correlate with DLA-A9 and/or DLA-C12. Dog 4 (lane e) has a pattern identical to that of dog 3. They are DLA identical, but not related. The upper heavy chain band might contain DLA-A3 and/or DLA-A10. Dog 5 (lane f), a sib of dog 4, shows the greatest separation of the two heavy chain bands. The top one is of the same MW as the bottom one of dog 4, which contains DLA-A9 and/or DLA-C12. If this is so, the lower band of dog 5 contains DLA-A1 and/or DLA-B13. The one band of dog 6 (lane g), homozygous for DLA-A9, B6 and C12, is in the same position as the DLA-A9, C12 band of dog 4 and 5 (lower band, lane e and upper band, lane f). Dog 7 (lane h) is identical to dog 6, both in tissue-type and gel pattern, although they are not related. Dog 8 (lane i) has heavy chains of two different mobilities, both different from those of dog 7. This must mean that the DLA-A9, B6, C12 haplotypes of father and mother are different. Although dog 9, their sib (lane j) is identical in tissue-type to dog 8, here the difference between the DLA-A9, B6, C12 haplotypes of the parents is visible again. Dog 9 has inherited DLA-A9, B6, C12 from his father (lane h) and DLA-A2, B4, C11 from his mother (lane i, upper band). In conclusion one can say that there is heterogeneity in both the sugar and the protein part of the heavy chain of DLA class I antigens, and that it may be possible with more refined techniques to correlate heterogeneity of the heavy chain with tissue-type.

VII.3.2 *Two-dimensional gel electrophoresis of glycosylated and nonglycosylated DLA antigens*

Unfortunately the sibs used in the experments under VII.3.1 were sacrificed for other scientific purposes by the time the two-dimensional gel electrophoresis technique was set up. Two other families were therefore used (Table 1, family 4 and 5). DLA antigens were immunoprecipitated with anti-β_2m serum (Fig. 1, left column) and a representative example of a 2D pattern obtained is given in Fig. 4. The separation in the first dimension was done by isoelectric focusing, based on the charge of the molecules. A spot detected in all 2D gels at a MW similar to that of the heavy chain, but slightly more acidic, represents actin, here on the left of the picture (denoted by "A"). The second dimension brought about a separation according to MW. The spot at the bottom of the picture, corresponding to β_2m, has moved to the basic side of the pH gradient in the first dimension, and to a low MW (12,000) in the second dimension. The range of spots of about the same MW as actin, but more basic, represents the heavy chain. When certain samples had been focused in the first dimension in parallel, their second dimension gels were generally superimposable. The actin, β_2m and minor constant spots can be aligned, after which the heavy chain spots can be compared. Fig. 4 is representative for all 2D gels analysed with respect to both the specific pattern and the background, which is generally

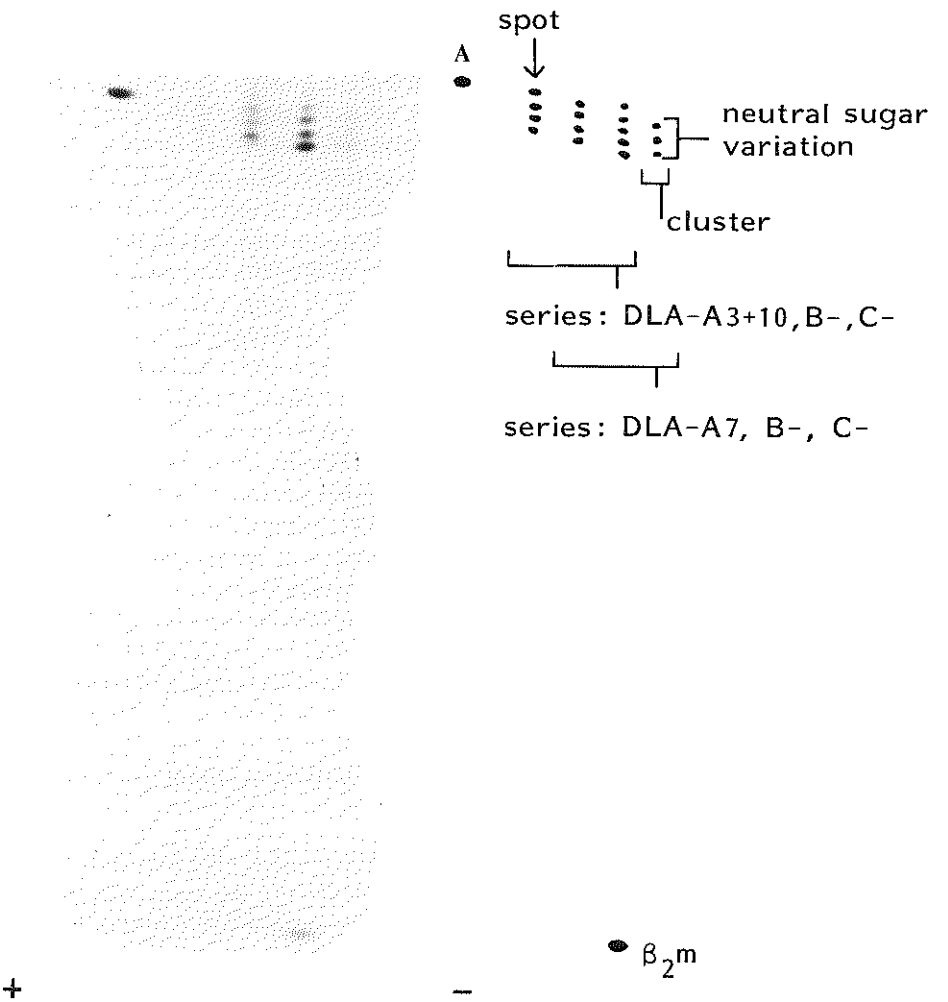


Figure 4. Two-dimensional gel electrophoresis of ^{35}S-methionine labelled, detergent solubilized DLA antigens from sib 2, family 4 (Table 1), after immunoprecipitation with anti-dog-β_2m serum. Isoelectric focusing was performed in the first dimension. The basic end of the isolelectric focusing gel is on the right. Perpendicular to the first dimension SDS-polyacrylamide gel electrophoresis (7.5-15% linear gradient gel) was carried out. No tunicamycin was used. "A" denotes actin, "β_2m" denotes β_2-microglobulin.

low. In all individuals tested only one spot in the MW 12,000 region was found. In second dimension gels of samples which have undergone isoelectric focusing in parallel, the relative position of β_2m to actin is constant. In accordance with the results from one-dimensional gels (Figs. 2 and 3), no heterogeneity of the light chain, β_2m, was found. The heavy chain, on the other hand, does vary between dogs with different tissue-types (Figs. 5 and 6). Apart from variation between individuals, there is also heterogeneity within the individual. The sample shown in Fig. 4 was radiolabelled in the absence of tunicamycin, which implies that the heavy chain should be fully glycosylated. The slight heterogeneity in charge, ranging from a position close to actin towards a more basic pI, can be partly ascribed to varying amounts of sialic acid (Parham et al., 1974). The slight heterogeneity in MW (per cluster) may be due to neutral sugars. This will become clear when patterns of glycosylated and nonglycosylated DLA antigens are compared.

For the moment, the most important conclusion is that polymorphism in DLA antigens is only detected in the heavy chain. For this reason, only the heavy chain pattern is shown in Figs. 5 and 6, where the two families are depicted. Tracings of all photographs have been made to clarify the correlation between spots and tissue-type.

Family 4 DLA antigens were radiolabelled both in the presence and in the absence of tunicamycin. Fig. 5a gives the patterns of glycosylated molecules and Fig. 5b those of nonglycosylated molecules. The concentration of tunicamycin, 3 μg/ml, was such that only partial deglycosylation occurred. Therefore it is possible to superimpose the patterns of glycosylated heavy chains of each individual on the pattern of the partially deglycosylated heavy chains and to see new, nonglycosylated spots appear. These new spots are approximately 3000 daltons lower in molecular weight than the glycosylated spots, as shown before in the one-dimensional gel analysis (Figs. 2 and 3), and they are found at a more basic position, as would be expected for molecules lacking the acidic neuraminic acid residues.

When the heavy chains were glycosylated (Fig. 5a) 3-4 clusters of different charge were found, each showing slight heterogeneity in molecular weight. Comparing Fig. 5a with Fig. 5b, where the nonglycosylated heavy chains are shown in addition to residual glycosylated chains we find 1-2 new spots in Fig. 5b which do not display any MW heterogeneity. They are found at a MW of about 3000 daltons lower than the clusters in Fig. 5a. As the only difference between the DLA antigens of the individuals in Fig. 5a and 5b is the presence or absence of sugar residues, we can conclude that the MW heterogeneity per cluster in Fig. 5a is due to neutral sugars. The charge heterogeneity is partly due to sugar residues, because 3-4 clusters from Fig. 5a are reduced to 1-2 spots in Fig. 5b. Differences in the amount of sialic acid could account for the charge heterogeneity.

Taking the intensity of the spots as a guideline, we compared the most intense spots of DLA antigens labelled in the presence (3 μg/ml) or absence of tunicamycin. It is clear that in all individuals tested, the nonglycosylated spots are more basic than the corresponding glycosylated spots. For example, the most intense glycosylated spot

Figure 5a

Figure 5b (+ tunicamycin)

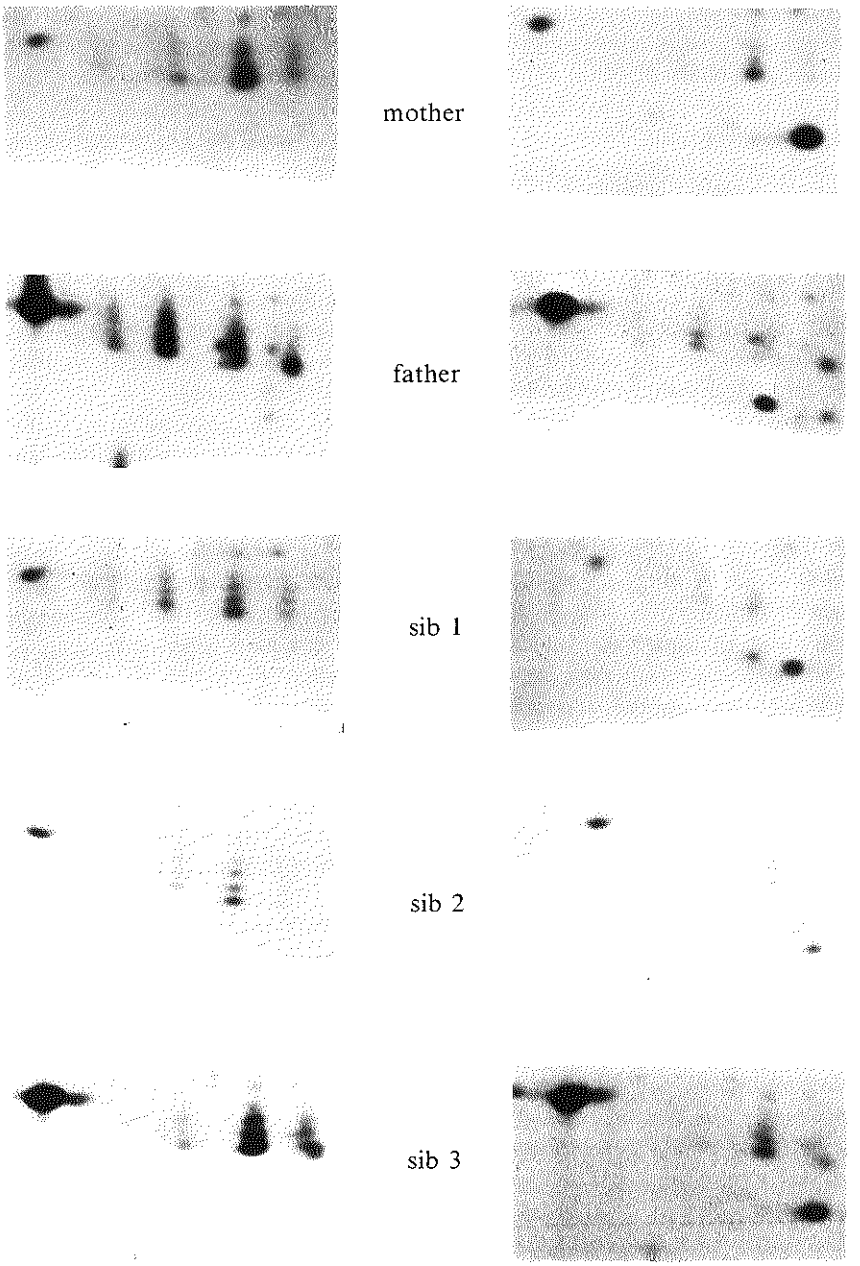


Figure 5a

Figure 5b

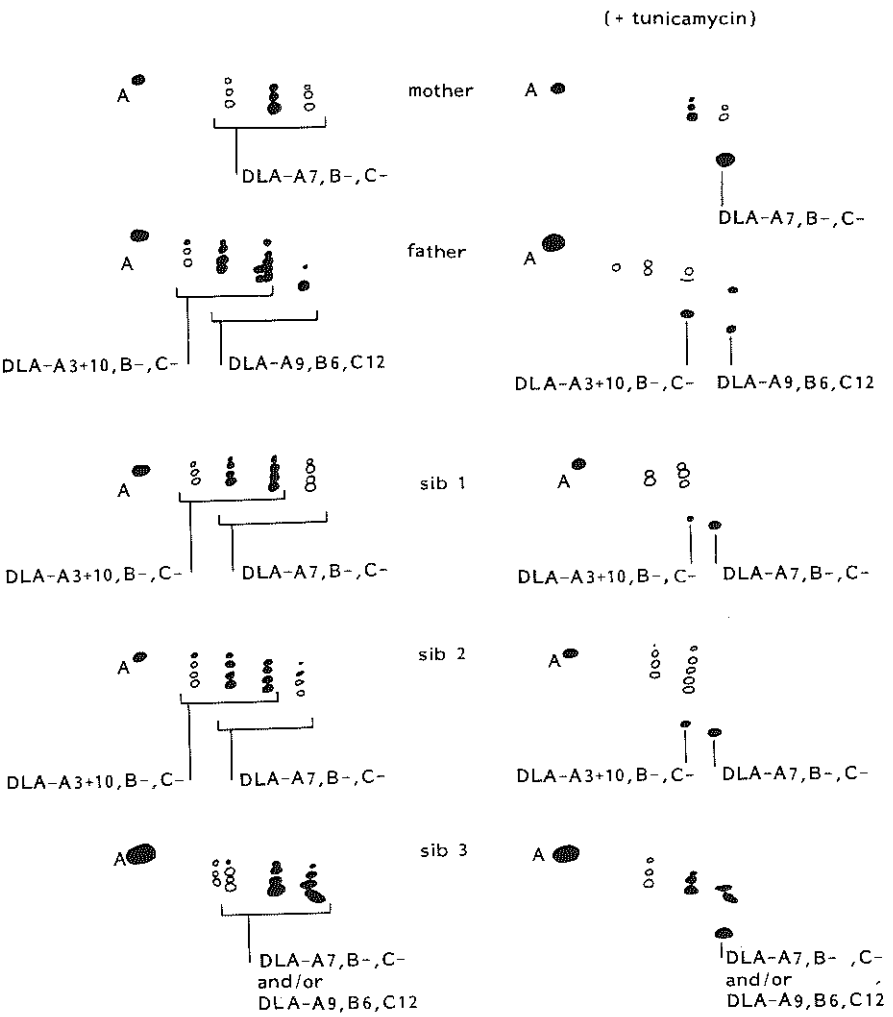
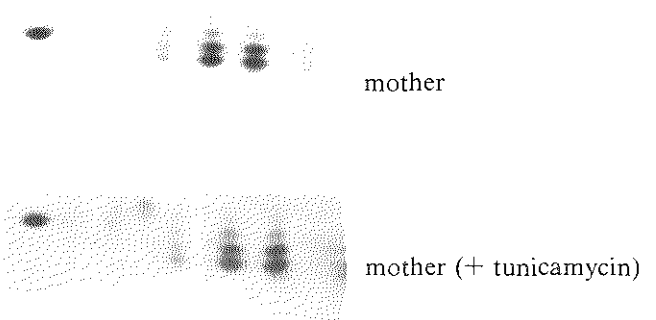


Figure 5. Two-dimensional gel analysis of DLA antigens from family 4 (Table 1) after immunoprecipitation with anti-β_2m serum.
Figure 5a: ^{35}S-methionine labelling in the absence of tunicamycin.
Figure 5b: ^{35}S-methionine labelling in the presence of tunicamycin.
Only the heavy chain region is shown. The basic end of the isoelectric focusing gels is to the right. The samples of the mother (± tunicamycin), the father (± tunicamycin), sib 1 (– tunicamycin), and sib 3 (± tunicamycin) were subjected to isoelectric focusing in parallel. Sib 1 (+ tunicamycin) and sib 2 (± tunicamycin) were analysed separately.
Further legends in Fig. 4.

of the mother (Fig. 5a) corresponds to a nonglycosylated spot located to the right (more basic) side of the original one. In sib 1, the two most intense spots of the glycosylated DLA heavy chains are turned into two nonglycosylated spots to the right of the original location. It is striking that all clusters of spots are at equal distance from each other. After tunicamycin treatment, new spots are found of a lower MW, but also each time at the most basic postion of each series of clusters (i.e. containing no sialic acid). The charge difference is not only caused by sugar heterogeneity, but also by protein differences, as the nonglycosylated heavy chains usually produce more than one spot. In the 2D system used, each spot corresponds to one polypeptide chain (or, rarely, multiple overlapping polypeptide chains of exactly equal charge and apparent size). The recurring charge differences in the heavy chain after tunicamycin treatment found in the 2D analysis support the findings of the one-dimensional gel analysis.

The assignment of haplotypes or alleles to the spots can only be tentative. The high percentage of "blanks" in tissue typing for the B and C loci (chapter I.2) is unfortunate. If all products of all loci were present, one would expect three series of glycosylated spots in homozygotes and six in total heterozygotes. One can see at a glance that this is not the case for the present individuals. Which spots are shared by dogs with overlapping tissue-types? An answer to this question might provide a way out. In family 4, the mother is homozygous for DLA-A7, B-, C-, so the one spot found after tunicamycin treatment and the series of glycosylated spots can be ascribed to DLA-A7, B-, C-. These spots are shared by sib 1, 2 and 3. The father and sib 1 and 2 share the haplotype DLA-A3+10, B-, C- which must be the spot (Fig. 5b) or series of spots (Fig. 5a) slightly more acidic than DLA-A7, B-, C-. By exclusion, the DLA-A9, B6, C12 haplotype of the father is attributed to the most basic spot after tunicamycin treatment and the series to the right of the DLA-A3+10 series in Fig. 5a. Sib 3 should share DLA-A9,B6,C12 with the father but the charge difference between DLA-A9,B6,C12 and DLA-A7,B-,C- is so small that it is difficult to distinguish them. In fact, one might as well call the DLA-A7,B-,C- of sib 3 a DLA-A9,B6,C12.

Figure 6



In family 5 (Fig. 6) the situation is less complicated. The father and mother were studied in the presence or absence of tunicamycin; the sibs were only tested for their glycosylated DLA antigens. When we compare the glycosylated heavy chains, strong resemblances are found between the mother and sib 3, and the father and sib 2. Sib 1 is homozygous for DLA-A9,B4,C12. The most intense cluster is therefore attributed to DLA-A9,B4,C12. This cluster is shared among the whole family. The difference between homozygosity and heterozygosity for DLA-A9,B4,C12 is reflected in the intensity of the most basic cluster. Sib 1's most basic cluster is about twice as intense as the most basic cluster of the rest of the family. Haplotype DLA-A9,B6,C12 is shared by the mother and sib 3 and is somewhat more acidic than DLA-A9,B4,C12. DLA-A2,B4,C11, very close to actin, is shared between sib 2 and the father. Tunicamycin treatment of the PBL of the mother caused a shift of the two most intense clusters to one position more basic and of a lower MW (denoted by the arrows). The same is true for the father. Once again, MW heterogeneity of glycosylated molecules within a cluster is due to the presence of neutral sugars and charge heterogeneity is due partly to sialic acid, partly to protein differences.

Figure 6. Two-dimensional gel analysis of DLA antigens from family 5 (Table 1) after immunoprecipitation with anti-β_2m serum. Only the heavy chain region is shown. The basic end of the isoelectric focusing gels is to the right. All glycosylated samples were run in parallel. The two nonglycosylated samples were analysed in a different experiment. The arrows denote the nonglycosylated heavy chains. Further legends in Fig. 4.

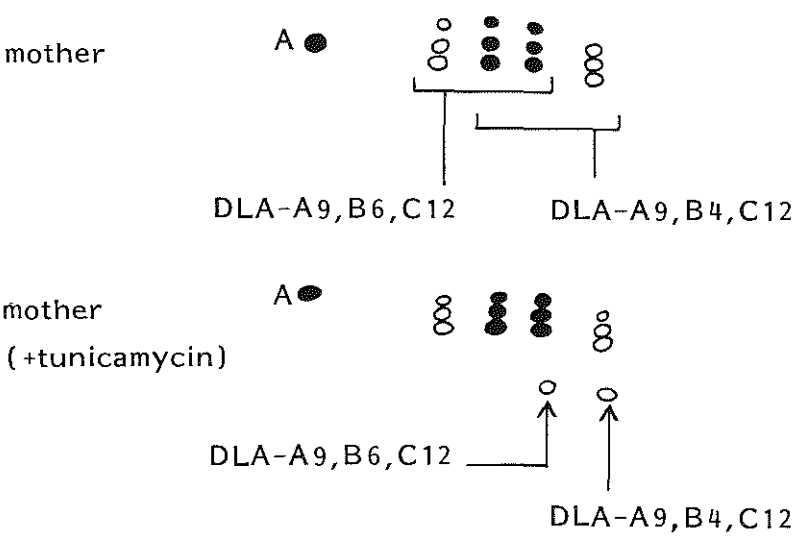
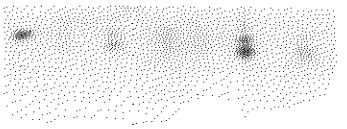
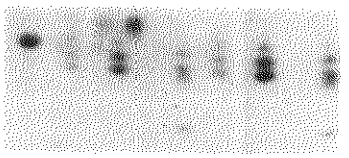


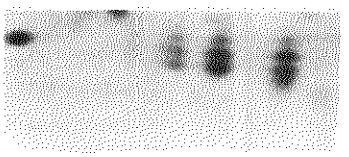
Figure 6 (continued)



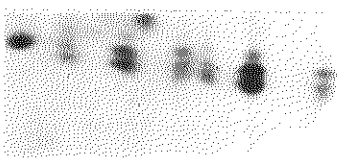
father



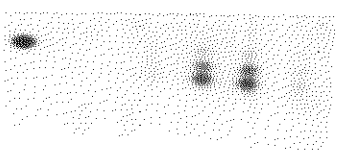
father (+ tunicamycin)



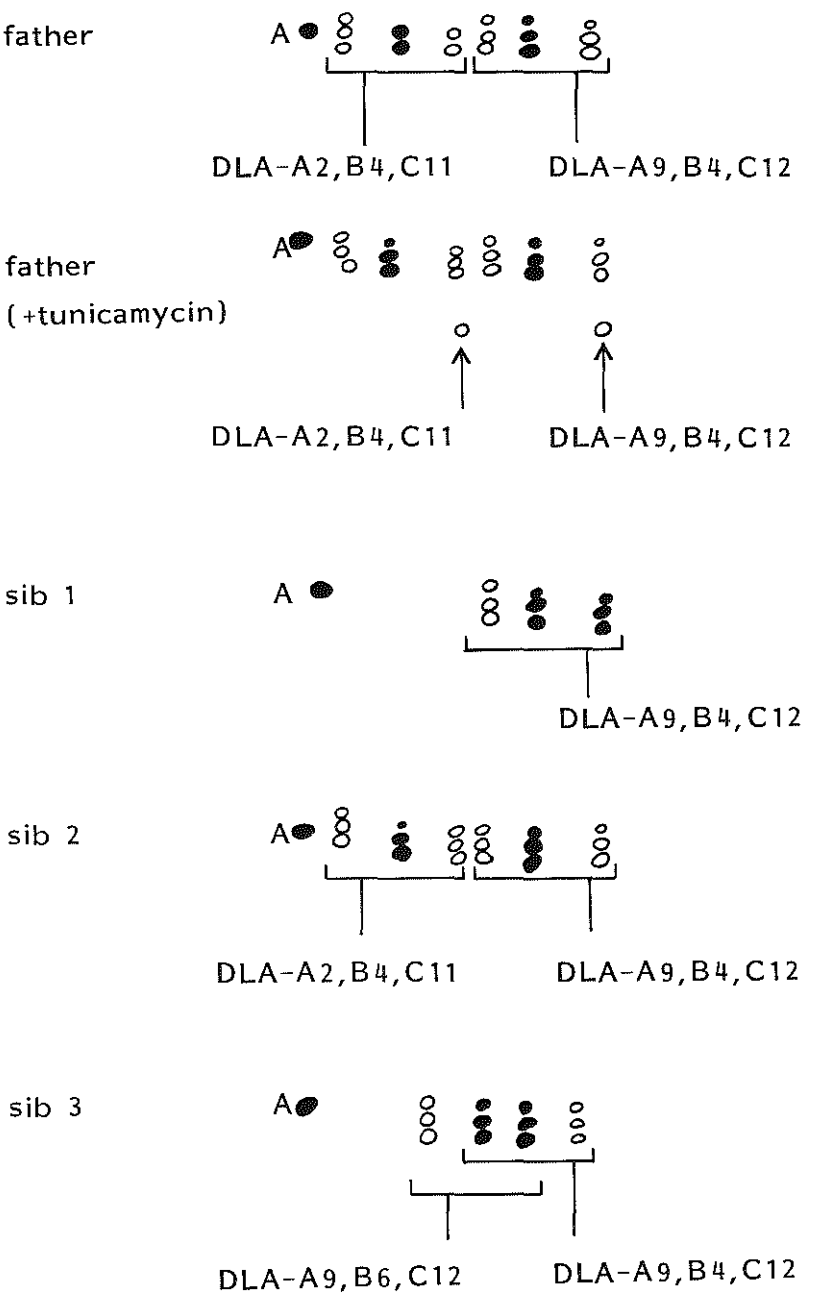
sib 1



sib 2



sib 3


father
A
DLA-A2,B4,C11
DLA-A9,B4,C12
father
(+tunicamycin)
A
DLA-A2,B4,C11
DLA-A9,B4,C12
sib 1
A
DLA-A9,B4,C12
sib 2
A
DLA-A2,B4,C11
DLA-A9,B4,C12
sib 3
A
DLA-A9,B6,C12
DLA-A9,B4,C12

One alloantiserum, serum 1302, has been shown to immunoprecipitate DLA antigens (chapter V). The anti-β_2m serum reacts with all DLA class I antigens and serum 1302 with only part of them. This cannot be visualized on a one-dimensional gel, but a two-dimensional gel analysis enables one to distinguish products of similar size and different charge. Figure 7 shows the patterns obtained on two-dimensional gels after immunoprecipitation of DLA antigens from a dog which was typed as DLA-A9,B6,C12/A2,B4,C11. The experiment was only done in the absence of tunicamycin. It is clear that the anti-β_2m serum precipitated heavy chains comprising four clusters of spots whereas the heavy chain precipitated by serum 1302 consists of only two clusters of spots. Neutral sugar variation is seen with both antisera. The dog used for this experiment was neither related to family 4 nor to family 5. As the antiserum cross-reacts with DLA-A2 and DLA-B5, it is likely that the two clusters precipitated by serum 1302 represent haplotype DLA-A2,B4,C11. The additional clusters found in the anti-β_2m serum pattern then belong to haplotype DLA-A9,B6,C12.

Instead of ascribing spots or clusters to products of different loci we suggest that the clusters correspond to whole haplotypes. Firm evidence for this is lacking, but this gives the best fit between typing data and two-dimensional gel analysis results. For example, when only the A locus products would be visible, the two DLA-A9's of DLA-A9,B4,C12 and DLA-A9,B6,C12 (mother and sib 3, Fig. 6) would have to be different to account for the two series of clusters. The same can be argued for the B locus (the two DLA-B4's of DLA-A2,B4,C11 and DLA-A9,B4,C12 would have to be different in the father and sib 2, Fig. 6). If both A and B loci are expressed, the correlation between spots and tissue-type proposed above is the most logical one. The one-dimensional gel electrophoresis (Figs. 2 and 3) is also most easily explained

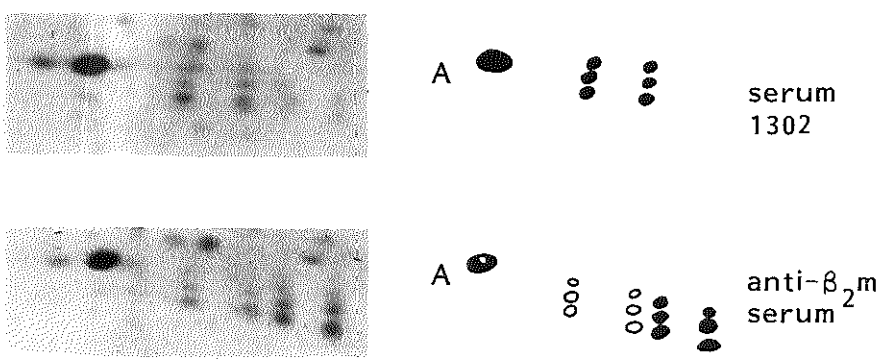


Figure 7. Two-dimensional gel analysis of DLA antigens from a dog of tissue-type DLA-A9, B6, C12/A2, B4, C11 after immunoprecipitation with alloantiserum 1302 (anti-DLA-C11) and anti-β_2m serum. No tunicamycin was used. Only the heavy chain region is shown. The basic end of the isoelectric focusing gels is to the right. The samples were run in parallel. Further legends in Fig. 4.

by attributing bands to haplotypes. The one haplotype which is shared between both families, DLA-A9,B6,C12, is found in similar positions (compare the pattern of family 4, the father, with family 5, the mother and sib 3).
In fact, the two-dimensional gel anlaysis has not revealed anything which we had not yet deduced from the one-dimensional gels. Surprisingly few spots are found for the nonglycosylated DLA heavy chains compared to what is found in the case of HLA class I heavy chains (Vasilov et al., in press). Nonetheless the same technique was applied to investigate DLA antigens on tumour cells. As all tumour material was obtained from mongrel dogs, which are certain to be less inbred than beagles, we hoped to detect more variety in these two-dimensional gels.

VII.3.3 *Two-dimensional gel electrophoresis of nonglycosylated DLA antigens from tumour cells and tumour bearer peripheral blood lymphocytes*

As there had been ample indication for protein heterogeneity in the DLA heavy chain (Figs. 3, 5, 6), probably corresponding to different haplotypes, we decided to examine only nonglycosylated DLA antigens on tumour cells and tumour bearer peripheral blood lymphocytes. Figure 1 (p. 105, middle and right column) summarizes the methods used. Unfortunately, the cryopreserved tumour bearer peripheral blood lymphocytes were difficult to stimulate with PHA. ^{35}S-methionine incorporation was low and the films had to be exposed to the gels for 8 weeks. The background then developed renders it difficult to reproduce the photographs. Therefore only tracings are shown in Fig. 8. Samples of lymphocytes and tumour cells from one dog were always run in parallel. Tunicamycin (5 μg/ml) seems to have inhibited protein synthesis in addition to glycosylation in certain cases, as the heavy chain spots are far too weak in comparison with the actin spot. The gradient gels for the second dimension in Fig. 8 had a much smaller area between actin and β_2m than those of Figs. 4-7. For this reason, the nonglycosylated heavy chains of Fig. 8 are much closer to actin in MW than those of Figs. 4-7. The spots below the heavy chain spots in Fig. 8 may be constant minor spots, usually of less intensity than the heavy chain. In the tumour cells, the maximum amount of nonglycosylated heavy chain spots is two, like in Figs. 5 and 6. One exception is found in the peripheral blood lymphocytes of HT70 where three nonglycosylated heavy chain spots of about equal intensity are visible.
No great diversity of MHC products is found when one compares the patterns of the tumour cells with each other. When we compare tumour cells with peripheral blood lymphocytes of the same dog, only HT70 is a possible candidate for a difference in MHC antigens between tumour cells and PBL, as the lymphocytes have one spot more than the corresponding tumour cells. This spot probably corresponds to a DLA product. So instead of finding an alien histocompatibility antigen on a tumour we found the loss of a DLA class I antigen. HT43 lymphocytes and tumour cells have similar two-dimensional gel patterns. The CR lymphocyte pattern is difficult to interpret, as the only spots visible are much lower in MW than one would expect for DLA class I antigens. The lymphocytes of the last dog (DG) did not give a visible pattern even after 8 weeks exposure.

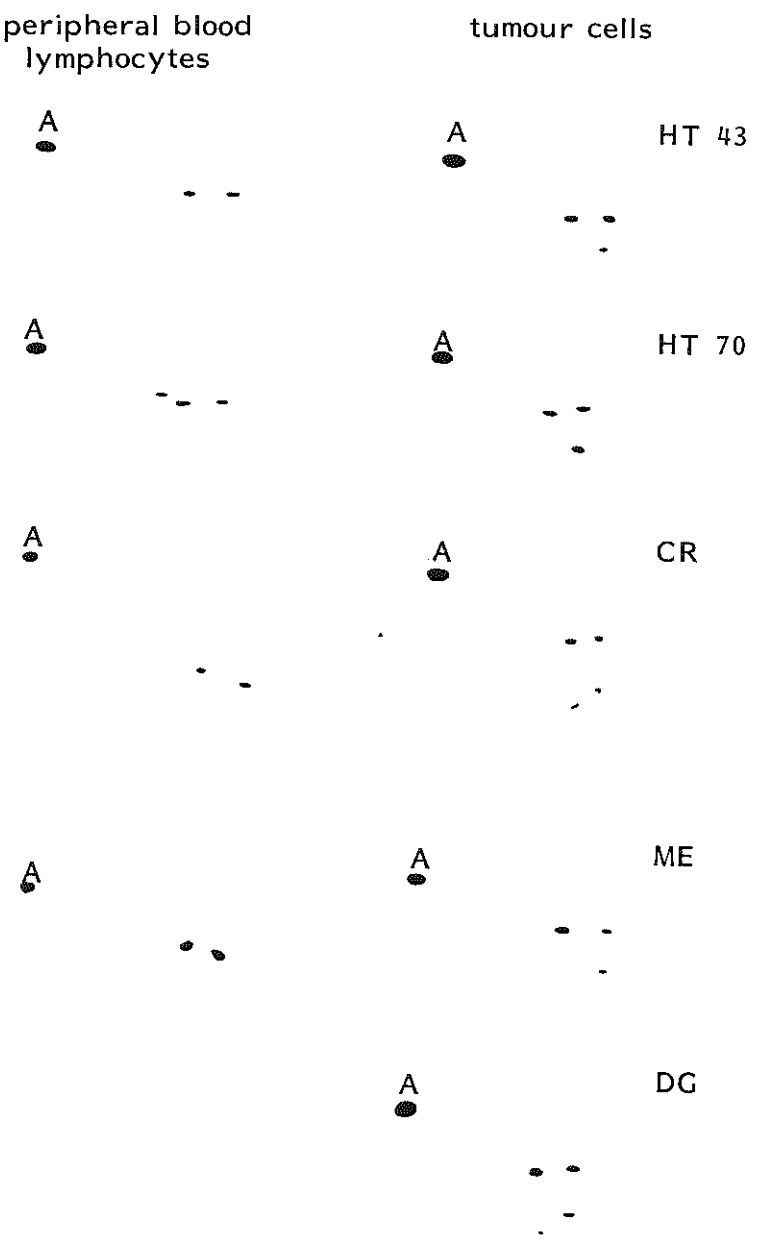


Figure 8. Two-dimensional gel analysis of DLA antigens from tumour cells and peripheral blood lymphocytes of the tumour bearers after immunoprecipitation with anti-β_2m serum. All samples were labelled in the presence of tunicamycin and they were all subjected to isoelectric focusing in parallel. No typing assignment is given. Abbreviations stand for the various tumours. Further legends in Fig. 4.

VII.4 Discussion

The investigation into the DLA class I antigenic determinant has revealed some interesting aspects. Like in most other species, the light chain of DLA class I antigens is invariant and polymorphism resides in the heavy chain. When fully glycosylated, the heavy chain shows heterogeneity even in one-dimensional gels (Fig. 2). Nonglycosylated heavy chains still show variation in mobility on one-dimensional gradient gels (Fig. 3), as has been shown in human and primate MHC antigens (Parham and Ploegh, 1980). Different HLA-A and -B, and H-2K and -D antigens can be distinguished on the basis of their mobility in SDS-polyacrylamide gel electrophoresis (Dobberstein et al., 1979, Krangel et al., 1979, Ploegh et al., 1981). The variation in mobility of DLA heavy chains in Fig. 2 is probably of a similar kind.

The two-dimensional gel analyses confirm the finding that the heavy chains display both protein and sugar heterogeneity (Figs. 4-7). The sugar heterogeneity is caused by varying amounts of neutral and charged sugar residues. Neutral sugar variation, visible as slight MW differences and constant charge, was also demonstrated in two-dimensional gels of Thy-1 (a T cell marker in the mouse), immunoprecipitated with a monoclonal anti-Thy-1 antibody (Ledbetter and Herzenberg, 1979). The MW heterogeneity displayed by DLA antigens is not apparent when they are synthesized in the presence of tunicamycin (Fig. 5b). Charge heterogeneity caused by sialic acid variation is described in HLA (Parham et al., 1974) and H-2 antigens (Kvist et al., 1977). HLA class I antigens can carry 0-3 sialic acid residues which results in four spots for each product analysed by two-dimensional gel electrophoresis. After neuraminidase treatment, this type of charge heterogeneity disappears. We did not check for the presence of sialic acid by use of neuraminidase. Indirect evidence that a sugar residue variation causes charge differences in DLA heavy chains is obtained by comparing the patterns of glycosylated and nonglycosylated heavy chains (Figs. 5a and 5b) where 3-4 clusters are reduced to 1-2 spots. As after tunicamycin treatment heterogeneity in charge persists, we conclude that there must be protein heterogeneity as well.

An indication of the amount of sialic acid residues in DLA heavy chains can be obtained by establishing how many glycosylated clusters correspond to one nonglycosylated spot in Figs. 5 and 6. In family 4, Fig. 5, the most intense glycosylated clusters correspond to DLA heavy chains with one sialic acid residue as in all cases the nonglycosylated spot (no sialic acid residues) appears one position to the right (basic) side. So, from what is detectable in Fig. 5, one would conclude that DLA antigens contain either two, one or no sialic acid residue. This is especially clear in the mother, who is homozygous for DLA-A7, B-, C- (Fig. 5a, topmost diagram). In family 5, Fig. 6, the same reasoning can be applied. Sib 1, homozygous for DLA-A9,B6,C12, and the father and sib 2, who seem to have non-overlapping series of spots for both haplotypes, are the clearest examples. Pulse-chase experiments would have to be performed to corroborate the hypothesis that the heterogeneity of glycosylated DLA heavy chains represents terminal

modification of a limited number of polypeptide chains. If vertical and horizontal heterogeneity appear together as a function of chase, it is likely that one non-glycosylated spot (representing one gene product) corresponds to several other glycosylated spots (representing one gene product with several terminal modifications).

Almost all experiments described in this chapter were performed with DLA antigens immunoprecipitated by anti-β_2m serum. The advantage of using this antiserum is that in principle, all class I antigens should be isolated. In the case of HLA class I antigens it was found that there are additional class I gene products which are not recognized serologically but can be found upon 2D gel analysis of HLA antigens immunoprecipitated with anti-β_2m serum (Vasilov et al., in press). The disadvantage of using anti-β_2m serum is that it becomes more difficult to correlate spots on 2D gels with tissue-type. Comparison of patterns from individuals with overlapping MHC class I specificities can solve the problem.
A better assignment of patterns to tissue-type may, however, be obtained by using alloantisera (Blankenhorn et al., 1980, Jones, 1977). Unfortunately only one dog alloantiserum is suitable for immunoprecipitation. This serum was used in the experiment in Fig. 7. It is clear that the alloantiserum pattern consists of two clusters whereas the anti-β_2m pattern contains two additional clusters. The sequential immunoprecipitation experiments referred to in chapter V also suggested that the DLA antigens reacting with alloantiserum 1302 were of a more limited specificity range than those reacting with the anti-β_2m serum. Immunoprecipitation of DLA antigens by alloantiserum 1302 and 2D gel analysis should be repeated with DLA antigens labelled in the presence of tunicamycin to confirm the assignment of clusters to haplotypes. Moreover, immunoprecipitation of non-glycosylated DLA antigens by the alloantiserum would indicate that lack of glycosylation need not have any influence on the alloantigenicity of the molecule. The protein nature of the alloantigenic determinant would then be established. For the moment, we can only say that the alloantigenic determinant is located on the heavy chain and that it is most probably protein in nature.

The assignment of spots to different haplotypes or alleles was found to be difficult. In other systems, more MHC class I products are found by 2D gel analysis than by conventional serology (Jones, 1977, Vasilov et al., in press). The dog MHC class I antigens, however, show very little variation in the heavy chain on 2D gels, especially in nonglycosylated heavy chains. Serological complexity is greater than that found in biochemical assays. The 2D gel analysis might not be the best method to study MHC antigens in the dog, although in other species it gives better resolution of different MHC products than does serology.
By attributing spots to whole haplotypes, correlation of tissue-typing data with 2D gel patterns supported this as being the best hypothesis. One would have expected, however, that at least one nonglycosylated spot would be found for each locus. It is possible that DLA-C is not detected because it is too labile. HLA-C has been reported to be labile (Snary et al., 1977) and not readily detected in 2D gels (Vasilov et al., in press). Another, rather theoretical, possibility is that there are one or two

DLA class I loci that express products that are not or only weakly associated with β_2m. These would not have been identified by the anti-β_2m serum used. A trivial explanation for the relative lack of heterogeneity could be that heavy chains from certain loci do not contain methionine and therefore cannot be labelled with ^{35}S-methionine. Another possibility may be that the pI of some heavy chains lies outside the pH range covered in the first dimension (roughly pH 4-8). It is also possible that spots are overlapping in the 2D gels. For example, in Fig. 5 DLA-A7,B-,C- is difficult to distinguish from DLA-A9,B6,C12. Peptide analysis by HPLC might reveal heterogeneity which is not detected by 2D gel analysis.
One is tempted to think that the so-called DLA class I loci may in fact represent three determinants on one molecule rather than three different molecules, comparable to immunoglobulin allotypes. However, statistical analysis of serological data argues against this possibility.
The serologically defined DLA antigens, which we have assumed to be homologous to murine class I antigens, may in fact also comprise class II antigens. It was recently suggested (Deeg et al., 1982) that Ia-like antigens are present on most dog peripheral blood lymphocytes, quite contrary to what is found in other species. As these Ia-like antigens would not react with an anti-β_2m serum, this could be another explanation for the discrepancy between our biochemical data and present-day serology. Metzger and colleagues (1981) described that they could not recognize products of different loci of SLA antigens by 2D electrophoresis. They suggest that the reason for this has to be looked for at the DNA level.
The simplest explanation remains that, in the dogs described, only one product was detected per haplotype. The issue could be investigated by analysing recombinants or by using alloantisera. Recombinants are scarce, however, and alloantisera generally do not work in immunoprecipitation (chapter V). Beagles do show a high linkage disequilibrium of certain alleles. This is supposed to be due to the founder effect (chapter I.2). The limited biochemical complexity of the DLA antigens studied here may also be caused by this founder effect.
In spite of the little variation in heavy chain spots for DLA antigens, a few things can be remarked upon. The DLA-A9,B6,C12 haplotype occurs in both families and in a very similar location to actin. Dogs that share haplotypes also share spots on the 2D gels. Homozygous dogs have simpler 2D patterns than heterozygous dogs, but the heavy chain spots are of double intensity, when compared with heterozygotes.

The investigation of the tumour cells has been difficult. More complicated patterns were expected as mongrel dogs are less inbred than beagles. Mongrels are difficult to tissue-type with the beagle typing sera (chapter I.2). No attempt therefore was made to attribute spots in Fig. 6 to typing data. Bad typing, bad ^{35}S-methionine incorporation and very uniform 2D patterns made comparison of tumours and tumour bearer lymphocytes precarious. The fact that ^{35}S-methionine incorporation was low in PHA-stimulated tumour bearer lymphocytes may have been caused by the addition of tunicamycin. Although freshly isolated peripheral blood lymphocytes can be metabolically radiolabelled in the presence of 5 μg/ml

tunicamycin (VII.3.1), this concentration may have been too high for the cryopreserved tumour bearer PBL. Tunicamycin is known to be able to decrease protein synthesis (Hickman et al., 1977).
Tumour tissue and blood samples were taken on the day of operation. As a cell line had to be prepared from the tumour material, peripheral blood lymphocytes had to be stored in liquid nitrogen in the meantime. It was difficult to obtain blood samples at a more suitable time because the dogs used for this part of our investigation were pets from all over Holland. Although alien class I histocompatibility antigens do contain β_2m, the association between β_2m and the heavy chain is not always as strong as in "normal" histocompatibility antigens (Rogers et al., 1979). The choice of this antiserum for immunoprecipitation was unavoidable, however, as alloantisera had proved to be unsuitable.
Despite the poor quality of the photographs used to draw Fig. 8, it was clear that in the case of one tumour, a melanoma (HT70), the peripheral blood lymphocytes of the tumour bearer showed one spot more than the tumour cells. This was confirmed by comparison of HT70 tumour cells and PBL in the 2D gel electrophoresis system used by Shackelford and Strominger (1980) where SDS-polyacrylamide gel electrophoresis precedes isoelectric focusing (results not shown). Because of the scarcity of material and the difficulties encountered in metabolic labelling of the cryopreserved peripheral blood lymphocytes, lactoperoxidase-catalysed iodination of both tumour cells and tumour bearer PBL was carried out. After immunoprecipitation with anti-β_2m serum, 2D gel electrophoresis was performed (VII.2.4). When 2D gel patterns of ^{125}I-labelled H-2 (Jones, 1977) and HLA-DR (Charron and McDevitt, 1980) antigens are compared with patterns obtained in short labelling experiments with ^{35}S-methionine, the ^{125}I-labelled spots correspond to the larger and more acidic ^{35}S-methionine labelled spots, i.e. the end products which have reached the cell surface. The patterns of the ^{125}I-labelled tumour cells and tumour bearer PBL were difficult to interpret (results not shown). The tumour cell line patterns looked very much alike, comprising two clusters of almost identical pI and slightly different MW. Two PBL patterns contained six spots of identical MW and different pI reminiscent of the patterns obtained with HLA class I antigens (Vasilov et al., in press).

Even if the 2D gel analysis of DLA antigens immunoprecipitated from tumour cells and tumour bearer PBL would give a clearer picture, it remains to be seen if differences in DLA antigens between these cell types can be detected by a technique in which the dog does not reveal nearly as much heterogeneity as other species studied so far. The reason for the discrepancy between the amount of DLA products defined in serology and the amount of DLA products demonstrated by 2D gel analysis has not yet been found.

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CHAPTER VIII

GENERAL DISCUSSION

VIII.1 **Introduction**

When one is faced with the problem of biochemically characterizing MHC antigens of a species that has not been investigated in this way before, the first thing to do is to find out which techniques have been applied by others in this type of research. In the general introduction to this thesis a brief summary has been given of the most successful experimental protocols for the isolation of MHC antigens (chapter I.4). Prerequisites are: 1) cells or tissue which contain sufficient MHC antigens as a starting material and 2) antisera directed against MHC antigens, either for monitoring the purity of the products during the isolation procedure or for use in the isolation procedure directly.

As a starting material, dog spleens were easily obtainable from donors for transplantation experiments carried out in our laboratory. Alternatively, peripheral blood lymphocytes could be collected from our stock of beagles. Tissue typing sera, by definition directed to the alloantigenic determinant(s) on DLA antigens, were available from planned immunizations or pregnancy sera (Vriesendorp et al., 1971). In all species investigated, the light polypeptide chain of the MHC class I antigens is identical to β_2m, a protein found in several body fluids. An antiserum against β_2m will therefore react with DLA antigens of different specificities. Consequently, we thought it necessary to raise an antiserum against dog β_2m. This was not an insuperable problem, however, as dogs who had received a kidney transplant were at hand. The level of urinary β_2m is usually raised after kidney transplantation. β_2m was isolated by biochemical techniques as described in chapter III and the purification was monitored with a sample of a rabbit anti-dog-β_2m antiserum given to us by Dr. Poulik (Smithies and Poulik, 1972a). Subsequently, rabbit antisera were prepared against the purified β_2m.
Appropriately equipped, we set out to investigate DLA antigens, bearing in mind the model of the HLA-B7 molecule as it has been summarized in chapter I.3.

In order to obtain DLA antigens, two approaches were taken: 1) the isolation of DLA antigens on a preparative scale by papain digestion and biochemical purification (chapter II) and 2) the isolation on an analytical scale by detergent solubilization and immunoprecipitation (chapters IV and V). In chapter I.9 some questions were formulated which were approached in the chapters II-VII. We gained knowledge about the chemical composition of the DLA antigens during the large scale isolation procedure (chapter II) and by radiolabelling them with different tracers (chapters IV and VI). The role of β_2m was investigated in immunoprecipitation studies (chapter IV) using the rabbit anti-dog-β_2m serum described in chapter

III. SDS-polyacrylamide gel patterns of DLA antigens immunoprecipitated with anti-β_2m serum were compared to patterns obtained after immunoprecipitation with antisera directed against the heavy chain (chapter V). The localization and the nature of the alloantigenic site was examined by 2D gel analysis of radiolabelled DLA antigens after reaction with anti-β_2m serum (chapter VII). In this same chapter, DLA antigens of tumour cells were investigated.
In the following paragraphs the results will be discussed and brought into perspective. For clarity's sake this is done mostly following the sequence of the chapters. Some of the issues, however, are only resolved by taking together the results described in several chapters.

VIII.2 Large scale isolation of DLA class I antigens

DLA class I antigens have originally been defined by alloantisera in the microlymphocytotoxicity test. We used an entirely different method for the isolation of these molecules independent of antisera (chapter II). Once they were isolated, they were tested for biological activity in the system in which they were originally defined. DLA class I antigens behaved like glycoproteins in the isolation procedure. They are susceptible to papain cleavage, their elution from a Sephadex G-150 column can be followed by measuring the absorbance at 280 nm, and they have affinity for lentil lectin. Lentil lectin affinity chromatography was the last purification step. When DEAE ion exchange chromatography was used as the final purification method, no useful material was obtained. In the HLA system, DEAE chromatography is an excellent way of separating different class I antigens (Turner et al., 1975). R. Robb et al. (1976) introduced the anti-β_2m immunoadsorbent column for efficient purification of HLA antigens. In our hands an anti-β_2m immunoadsorbent column was difficult to elute (chapter III.4).
In the case of HLA, both class I and class II antigens have affinity for lentil lectin. Separation according to molecular weight is required after lentil lectin chromatography (Springer et al., 1977). Using the parameters for class II polypeptide chains of man and mice (29,000 and 34,000 daltons, 28,000 and 33,000 daltons), we decided that in our preparations the amount of putative class II antigens was negligible as judged by SDS-polyacrylamide electrophoresis gels stained for protein. When the papain digested molecules were radioiodinated, however, and immunoprecipitated by the anti-HC serum, extra bands of 19,000, 30,000 and 39,000 daltons of respectable intensity were visible on SDS-polyacrylamide gels (chapter V, Fig. 2). This means that amongst others molecules of an approximate molecular weight of 30,000 daltons (putative DLA class II antigens) were present in the lentil lectin eluates. The reason that they were detected better after radioiodination may be that their tyrosine residues (which are labelled by lactoperoxidase-catalysed iodination) are more easily accessible than those of DLA class I antigens, although on a molar basis they were in a minority.
Unfortunately no dog MHC monoclonal antibodies were available; otherwise a

series of monoclonal antibody columns would have been used for the final purification step as described for HLA (Parham, 1979). Whatever the optimal method for purification of DLA antigens may be, the purity and the yield that we have obtained so far can be improved upon.

In all species studied so far, the major part of the extracellular portion of the MHC class I antigens is cleaved off the membrane by papain with the alloantigenic site intact. The same is true in the dog. The molecules isolated by papain digestion reacted with specific alloantisera which could then no longer lyse lymphocytes in the cytotoxicity test (chapter II). Therefore the alloantigenic site was preserved. The major part of the heavy chain was recovered; the difference in molecular weight between the heavy chain of the papain digested (37,000) and the detergent solubilized (41,700-43,000) DLA heavy chains (chapters II, IV) is 4,500 to 6,000 daltons. This difference in MW between papain digested and detergent solubilized DLA antigens is comparable to the MW difference found in HLA antigens isolated in these two ways (Cresswell et al., 1973, Springer et al., 1974). Papain digestion of the detergent solubilized HLA molecules yielded an intermediate product of MW 39,000 and an end product of MW 34,000 (Springer et al., 1974). The NH_2-terminal amino acid sequences of papain digested and detergent solubilized HLA heavy chains were identical, which indicates that the papain cleavage site is close to the carboxyl-terminus (Springer and Strominger, 1976).

Although spleen-derived MHC antigens comprise several allelic forms, in the case of HLA class I antigens from three loci, amino acid sequencing is possible. As HLA-A and -B products display long stretches of homology, mixed specificities obtained from spleens are well suited to investigate the variable regions between differing loci and allelic products (Trägårdh et al. 1979a,c).

As mentioned before, it was difficult to determine the NH_2-terminal amino acids from the isolated DLA heavy chains. Nevertheless, a few amino acids could be identified which correspond with HLA and H-2 terminal amino acids (chapter IV, Fig. 7b).

The DLA light chain had an NH_2-terminal sequence in agreement with the sequence published for dog urinary β_2m (Smithies and Poulik, 1972a and Chapter IV, Fig. 7a). In all MHC class I antigens studied, the light chain is identical to β_2m (Björck et al., 1977, Chardon et al., 1978, Grey et al., 1973, Katagiri et al., 1975, Rask et al., 1974, Zaalberg et al., 1976). Only in the case of HLA is this formally established by amino acid sequencing of both the light chain and urinary β_2m (Bridgen et al., 1976, Peterson et al., 1972).

Because of the relationship between β_2m and the heavy chain, attempts were made to immunoprecipitate the papain digested DLA class I antigens with the rabbit anti-dog urinary β_2m serum described in chapter III. SDS-polyacrylamide gel electrophoresis profiles of the immunoprecipitates after fluorography (chapter IV, Fig. 2) were identical to the gel patterns of the purified antigens after staining for protein with Coomassie Brilliant Blue (chapter II, Fig. 4). This is additional evidence for the association of β_2m and the DLA heavy chain.

An advantage of large scale isolation by papain digestion is the availability of

sufficient material for crystallization experiments. Crystallization is only possible with very pure material, so additional immunoadsorbents would be necessary to obtain DLA antigens pure enough for this purpose. The shape of the crystals gives information about the three-dimensional structure of the molecule, which is important in view of the site of the alloantigenic determinant(s) and the possible evolutionary relationship between immunoglobulins and MHC class I antigens. References are made to attempts to crystallize HLA (Korman et al., 1982, Ploegh et al., 1981) and SLA (Metzger et al., 1981) antigens in order to see if the hypervariable spots in the amino acid sequence form one or more hypervariable site(s).
MHC antigen molecules of one specificity can be used for immunization in order to raise monoclonal antibodies against polymorphic MHC determinants (Brodsky et al., 1979, Parham and Bodmer, 1978, Radka et al., 1982). Our material will be used for that purpose, but in addition to that we decided to raise a conventional heterologous antiserum in rabbits which would be able to react with all DLA heavy chains (chapter V). The serum obtained gave a high background in immunoprecipitation experiments with papain and detergent solubilized DLA antigens (chapter V, Figs. 2, 3). This background could be reduced by absorption of the serum onto and elution from thrombocytes (chapter V, Fig. 5). Alternatively, the unabsorbed antiserum gave a fairly clean two chain precipitate when intact cells were used instead of solubilized antigen (chapter V, Fig. 4). The reaction between the anti-heavy chain (anti-HC) serum and intact cells could only be partly inhibited by purified β_2m, so the antiserum definitely contains antibodies recognizing heavy chain determinants.

VIII.3 **Small scale isolation of DLA class I antigens**

VIII.3.1 *The anti-dog urinary β_2m serum*

Some of the questions regarding the biochemical composition of DLA class I antigens have been answered in chapter II, and were discussed in the previous section. Other problems, however, such as the nature of the DLA alloantigenic site, could be approached more accurately by immunochemical techniques.
An immunoprecipitation assay was set up (chapter IV) with the use of the rabbit anti-dog-β_2m serum described in chapter III. Preliminary evidence for the DLA light chain being identical to β_2m has already been discussed in the previous section. In certain cases, antisera against β_2m from closely related species can be used for immunoprecipitation of MHC antigens (Giphart et al., 1978, Kvist et al., 1977, Rask et al., 1974 and Vitetta et al., 1976). As for the dog, immunoprecipitation with a rabbit anti-human-β_2m serum did not result in a typical DLA pattern (unpublished observation). Smithies and Poulik (1972a) did not observe cross-reactivity of an anti-human-β_2m serum with dog β_2m, and their rabbit anti-dog-β_2m sera did not react with human β_2m either.
Our rabbit anti-dog-β_2m serum proved to be suitable for our purpose. It was a fairly strong antiserum as is to be expected from a heterologous antiserum. In theory,

DLA heavy chains of all specificities should be co-precipitated with β_2m. In one case, anti-β_2m antiserum was said to dissociate MHC heavy chains from β_2m (Nakamuro et al., 1975). This generally happens at longer incubation times than those required for immunoprecipitation. Although our rabbit anti-dog-β_2m serum contains impurities (chapter III.3), the immunoprecipitation patterns of this serum with membrane preparations are not affected by this.
In the mouse β_2m on the membrane is not only associated with MHC class I heavy chains but also with H-Y, Qa-2 and TL (other membrane proteins) (Fellous et al., 1978, Michaelson et al., 1977, and Vitetta et al., 1976); in man with possible TL homologues (van Agthoven and Terhorst, 1982, Gazit et al., 1980, Tada et al., 1978). Both TL and Qa-2 heavy chains have molecular weights very close to MHC class I heavy chains. On peripheral blood lymphocytes, however, the MHC antigens are much greater in number than TL or Qa-2 antigens. TL antigens are found predominantly on thymocytes and leukaemia cells. Qa-2 antigens are found on mature T cells (Michaelson et al., 1977). We used PHA-stimulated peripheral blood lymphocytes for most experiments. PHA is a T cell mitogen. As MHC antigens outnumber Qa-2 and TL antigens on T cells, it is likely that the molecules immunoprecipitated by the anti-β_2m serum from PHA-stimulated PBL are mainly DLA antigens.

β_2m is a highly conserved molecule amongst all species investigated. There is considerable sequence homology between β_2m and the C_H3 domain of immunoglobulin (Peterson et al., 1972, Smithies and Poulik, 1972b). Based on the chemical and physical-chemical properties of HLA and H-2 antigens, a common evolutionary origin for these molecules has been suggested (Peterson et al., 1975, Terhorst et al., 1977). When more information on the amino acid sequence of HLA class I heavy chains became available, statistically significant homology was found between the second disulfide loop of HLA heavy chains and constant region domains of immunoglobulin (Orr et al., 1979, Trägårdh et al., 1979b). Recently, significant sequence homology was reported between immunoglobulin constant region domains and the carboxyl-terminal domain of the HLA-DR light (Kaufman and Strominger, 1982) and heavy (Korman et al., 1982) chain. The fact that dog MHC class I antigens contain β_2m as the light chain extends the amino acid sequence homology between certain domains of MHC antigens and immunoglobulin to yet another species.
Interaction between β_2m from several species and RT-1 (rat MHC) antigen has been shown to be possible by Lögdberg and colleagues (1980). This was taken as evidence for the evolutionary conservation of the β_2m binding site on the MHC heavy chain. Exchange between human β_2m and mouse H-2 antigens on the cell surface or in solution was achieved by Schmidt et al. (1981). These techniques could be valuable in the elucidation of the structure of histocompatibility antigens in species where β_2m or anti-β_2m sera are not available.

The role of β_2m in the structure of histocompatibility antigens is only tentative. β_2m is supposed to play a part in the transport of HLA antigens to the cell membrane. Daudi cells, which lack β_2m, have been shown to posses intracellular

HLA heavy chains (Ploegh et al., 1979) while they do not express them on the membrane. After fusion with other human cell lines or mouse cells, HLA specificities have been detected on the hybrid cell membrane which could not be ascribed to the fusion partner (Arce-Gomez et al., 1978, Fellous et al., 1977). The Daudi HLA heavy chain had apparently been rescued by the β_2m from the fusion partner. D. Lancet and colleagues (1979) have suggested that β_2m might stabilize the heavy chain conformation for antigenic activity. One of the most widely used HLA monoclonal antibodies, W6/32, which is directed against a heavy chain determinant, reacts only with β_2m associated heavy chains, so β_2m influences the conformation of the heavy chain (Parham et al., 1979). Nevertheless, the H-2 heavy chain can still react with H-2 alloantisera after careful separation from β_2m (Kvist et al., 1977).
The function of β_2m in the dog has not been analysed. It was used as a starting point for the immunochemical isolation of DLA antigens.

VIII.3.2 *Immunoprecipitation of DLA antigens by the anti-β_2m serum*

In our studies we have analysed DLA antigens from several sources. The immunoprecipitation of DLA antigens isolated from spleens has been discussed in section VIII.2. DLA antigens have also been immunoprecipitated after incubation of intact PBL or membranes with the antiserum. PBL were labelled by lactoperoxidase-catalysed radioiodination or metabolically by incorporation of ^{35}S-methionine (chapter IV) or ^{3}H-fucose (chapter VI). The non-ionic detergents NNP10 or NP-40 were used to solubilize the DLA antigens. In all cases, a heavy chain of 41,700-43,000 daltons was precipitated together, in the case of ^{125}I- and ^{35}S-labelled cells, with the light chain of 12,000 daltons. This indicates that DLA antigens are glycoproteins, that they can be solubilized by detergent and that they consist of two polypeptide chains of MW 41,700-43,000 and 12,000. As observed previously, the antiserum directed against urinary β_2m of MW 12,000 co-precipitates another polypeptide chain, as in the immunoprecipitation of papain solubilized DLA antigens (chapter IV, VIII.2). The molecular weight of the detergent solubilized heavy chain is somewhat larger than that of the papain digested heavy chain, which is to be expected, as detergent solubilizes the whole molecule and papain the major part. As all gels used for analysis of immunoprecipitation patterns were run under reducing circumstances, no evidence could be found for a structure consisting of four chains or more. Four chain structures have been found in preparations of detergent solubilized HLA antigens but were later shown to be artefacts (Stromihger et al., 1977). At any rate, the two chain structure of DLA antigens after detergent solubilization shows that the two chain structure of papain digested DLA antigens was not caused by papain cleavage of one large chain.
DLA antigens behaved like glycoproteins in the large scale isolation procedure, and their ability to incorporate sugar and amino acid confirms this. The ^{3}H-fucose labelled DLA antigens (chapter VI) provide an interesting case; immunoprecipitation with an antiserum directed against the light chain resulted in a gel pattern of the heavy chain only. Like in other species (Nathenson and Muramatsu, 1971, Parham

et al., 1977) the heavy chain carries a sugar side chain whereas β_2m is devoid of carbohydrate.
The anti-β_2m serum has been further characterized in an experiment where immunoprecipitation of DLA antigens by the anti-β_2m serum was inhibited by purified urinary β_2m. The anti-β_2m serum could also react with denatured light chains from a DLA preparation that had been boiled in 1% SDS (chapter IV).

The anti-β_2m serum has been used to corroborate the location of the DLA antigens. From the microlymphocytotoxicity test it is already clear that the antigenic determinant(s) must be on the outside of the cells. Lactoperoxidase-catalysed iodination only labels surface proteins with accessible tyrosine residues. ^{125}I-labelled DLA antigens can be precipitated with the anti-β_2m serum from lactoperoxidase-catalysed radioiodinated cells. When intact, ^{35}S-methionine labelled cells were incubated with the anti-β_2m serum before solubilization, the anti-β_2m serum could also react with DLA molecules. Therefore the location of those determinants which could react with alloantisera (in the microlymphocytotoxicity test) and with the anti-β_2m serum (in immunoprecipitation) is on the outside of the cell membrane.
More elegant experiments have been performed with HLA and H-2 antigens to show that they were located on the cell membrane (Henning et al., 1976, Snary et al., 1974, Springer and Strominger, 1976). The transmembrane location of the HLA heavy chain was demonstrated by lactoperoxidase-catalysed iodination of inside-out membrane vesicles (Walsh and Crumpton, 1977). HLA antigens were labelled whereas membrane immunoglobulins were not.
The advantage of using detergent over papain for the isolation of MHC antigens is that the whole molecule is recovered, so that insertion into the plasma membrane can be studied. Functional characteristics of membrane molecules can be investigated by incorporating them into liposomes. Both HLA (Turner and Sanderson, 1978) and RT-1 (Willoughby et al., 1978) antigens have been incorporated. Some experiments on the function of HLA antigens inserted into liposomes have been summarized in chapter I.4.2. Dog MHC antigens have not been studied in this way yet.

VIII.3.3 *Immunoprecipitation of DLA antigens by sera directed against the heavy chain*

As mentioned in section VIII.2, an antiserum was raised in rabbits by immunization with the DLA heavy chain (described in chapter V.2.1). This serum gave a high background when it was reacted with DLA antigens in solution. Therefore intact cells were reacted with the serum before detergent solubilization and in this way the antiserum turned out to be useful. The gel patterns obtained were similar to those seen with the anti-β_2m serum (chapter V, Fig. 4).
Since the anti-HC serum could react with DLA antigens on intact cells, the location of the DLA antigens on the cell membrane was corroborated. Whereas purified urinary β_2m could completely inhibit the reaction between DLA antigens and the

anti-β_2m serum, it could only partially inhibit the reaction between DLA antigens and the anti-HC serum. This is evidence that the two antisera react with different determinants. As both sera react with non-polymorphic "backbone" structures, they should react with DLA antigens of all specificities. The anti-HC serum did not react with DLA antigens in solution which were denatured by boiling in 1% SDS. Hence the serum may not be able to detect in vitro translated DLA products which have not as yet assumed their native three-dimensional conformation.

Most alloantisera did not react readily with DLA antigens in the immunoprecipitation technique (chapter V). Other investigators have experienced the same difficulties (Allison et al., 1978). One of the reasons could be that alloantisera generally have a much lower titre than heterologous antisera. One alloantiserum, however, did react with DLA antigens in solution (chapter V), just like the anti-β_2m serum (chapter IV). This alloantiserum is directed against DLA-C11 but it cross-reacts with several specificities (A.M. Dekkers-Bijma, personal communication). Whatever the specificity, the determinant for this serum is located on the heavy chain, because the serum cannot be inhibited by purified urinary β_2m. The finding that both the anti-β_2m serum (anti-light chain) and the alloantiserum (anti-heavy chain) yielded the same precipitation pattern consisting of a light chain of 12,000 daltons and a heavy chain of 43,000 daltons (chapter V, Fig. 6) confirms the association of β_2m with the DLA heavy chain. Sequential immunoprecipitation studies verified that the anti-β_2m serum reacts with cells of all specificities whereas the alloantiserum is more limited in specificity.

In summary, small scale isolation of DLA antigens has provided information concerning the chemical composition of DLA antigens, the two chain structure, the susceptibility to detergent solubilization and the MW of the component chains then obtained. The association of β_2m and heavy chain and the location on the outside of the cell surface were also established for DLA antigens. The alloantigenic site was investigated with the techniques described above together with one- and two-dimensional gel electrophoresis.

VIII.3.4 *The alloantigenic site*

The investigation of the DLA alloantigenic site was concentrated on two issues: 1) the location on either heavy or light chain, 2) the chemical composition; sugar and/or protein. The presence of a sugar moiety on the DLA heavy chain was demonstrated in the experiments described in chapter VI. Some heterogeneity in the heavy chains from dogs of different tissue-types was observed on SDS-polyacrylamide gradient gels, whereas β_2m was invariant (chapter VII, Fig. 2). Even nonglycosylated DLA heavy chains showed microheterogeneity on SDS-polyacrylamide gradient gels (chapter VII, Fig. 3). On two-dimensional gels it became even clearer that the DLA heavy chain is polymorphic, whereas the light chain does not vary between dogs (chapter VII, Figs. 4-7). Thus the DLA alloantigenic site is located on the heavy chain.

The question of the nature of the alloantigenic specificities is more difficult to answer. On the two-dimensional gels, nonglycosylated DLA heavy chains from

dogs of different tissue-type still displayed different spots, confirming what was observed previously on one-dimensional gels. There was extensive sugar heterogeneity in fully glycosylated DLA heavy chains in addition to the protein heterogeneity. Therefore, the alloantigenic site is certainly protein in nature, but we cannot as yet make a definite statement about the role of the sugar moiety. The neutral sugar variation observed (chapter VII, Figs. 4-7) is not a general feature of MHC antigens.

The importance of the sugar chain in the alloantigenic site should be studied by immunoprecipitation of nonglycosylated versus glycosylated DLA antigens with an array of alloantisera. Unfortunately, up till now, only one alloantiserum has been found to be suitable for immunoprecipitation (chapter V). The origin of the charge heterogeneity found in DLA heavy chains upon 2D gel analysis has been discussed in chapter VII.4. It was suggested that this is partly due to protein differences, and partly to differences in the amount of sialic acid. In spite of the uncertainty regarding the chemical nature of the alloantigenic determinant, it was possible to tentatively assign 2D gel patterns to haplotypes.

Two-dimensional gel electrophoresis of MHC antigens can be employed as a way of "biochemical tissue-typing" (Vasilov et al., in press), also called "molecular genotyping" (Charron and McDevitt, 1980) or "haplotype-specific molecular fingerprinting" (Jones, 1977). Jones used alloantisera for immunoprecipitation and she could demonstrate that identical haplotypes from different mouse strains were identical in 2D gel pattern. Different haplotypes appeared different on 2D gels. She noticed the molecular complexity of the 2D gel patterns and suggested that most of the complexity is due to different glycosylation stages. Due to the labelling procedure employed (4-5 hours of ^{35}S-methionine incorporation), precursors as well as end products were found. When lactoperoxidase-catalysed radioiodinated molecules (end products) were used for immunoprecipitation, some heterogeneity was left. She suggests that the spots may represent multiple gene products.

It is also possible to assign 2D patterns to haplotypes by comparing immunoprecipitates of MHC antigens from various homozygous typing cells using anti-backbone sera (Charron and McDevitt, 1980, Vasilov et al., in press). An indication for the existence of the second HLA-DR locus was obtained in this way (Charron and McDevitt, 1980, Shackelford and Strominger, 1980). In homozygous typing cells, evidence was found for up to three additional HLA class I gene products apart from HLA-A, -B, and -C (Vasilov et al., in press). In general, more products are found on 2D gels after immunoprecipitation of H-2 or HLA class I or class II antigens than would be expected from serology.

In the dog, however, the situation is quite different. Neutral and charged sugar variation contribute to the complexity of the 2D gel patterns of fully glycosylated DLA heavy chains. However, nonglycosylated DLA molecules obtained after radiolabelling in the presence of tunicamycin, show surprisingly little heterogeneity. Some possible causes have already been mentioned in chapter VII.4.

With respect to MHC polymorphism, there is one species that has attracted attention because of its lack of serologically detectable MHC class I antigens. The

Syrian hamster alloantisera only detect class II-like molecules; class I polymorphism is deficient even between wild and inbred hamsters (Phillips et al., 1981). A trivial explanation for this is that all domestic inbred hamsters are derived from an original trio caught in 1930 (Duncan and Streilein, 1977). This does not explain, however, why alloantisera produced between "wild" and inbred hamsters do not detect class I products. The arguments developed by Duncan and Streilein (1977) to account for the lack of class I alloantigenicity (class I identity, antigenic determinants not detected by B cells, lack of a class I locus) do not hold true in the case of the dog. In the dog, alloantisera directed against class I antigens are found, but biochemically the polymorphism seems to be smaller than would be predicted from serology. The most logical correlation between 2D gel patterns and tissue-type was found when spots were assigned to haplotypes rather than individual locus products (chapter VII.3.2). As in the Syrian hamster, inbreeding probably plays a role, but in the Syrian hamster there is a straightforward lack of class I antigens, whereas in the dog there is a discrepancy between serological and biochemical data. This issue has already been discussed in chapter VII.4. One possible explanation must be stressed again. That is that the number of DLA class I loci is in fact smaller than assumed. The evidence for a DLA-A and -B locus is indisputable (Joint report of the 2nd international workshop on canine immunogenetics, 1976; J. d'Amaro, personal communication). The existence of DLA-C is not well-founded, statistically speaking. The possibility that one of the "class I" loci is in fact a class II locus cannot be ruled out in view of recent data on the distribution of Ia-like antigens on dog peripheral blood lymphocytes (Deeg et al., 1982).
Another problem is the alloantisera which are selected for beagle tissue typing and which are unsuitable for tissue typing mongrel dogs (chapter I.2). Although the beagle used to be considered as an outbred preclinical model, our colony does tend to accumulate certain DLA specificities in preference to others. If the investigation into the DLA alloantigenic determinants could have been performed in tissue-typed mongrel dogs, more variety might have been detected. It is very likely that mongrel dogs will have combinations of DLA-A, -B and -C products different from those present in the beagle, which are very much influenced by the linkage disequilibrium (chapter I.2). The mongrel dogs used for the examination of DLA antigens on tumour cells were, as was to be expected, also difficult to tissue-type.

VIII.3.5 *DLA antigens on tumour cells*

After the characterization of DLA antigens on peripheral blood lymphocytes, we decided to examine DLA antigens on tumour cells. Originally we planned to use alloantisera for immunoprecipitation of DLA antigens from tumour cells. Unfortunately the alloantisera, which are difficult to work with in mongrel dogs anyway (chapter I.2) did not give interpretable immunoprecipitation patterns, with one exception (chapter V). Another fundamental problem was that we worked with tumour cell lines, which represent inevitably a selection of cells from the original tumour. The in vivo relevance of our data would therefore have to be considered carefully. PBL were chosen as a control because DLA antigens are defined by their

presence on PBL. One has also to consider using the normal tissue equivalent of the tumour studied. As most of the tumours we obtained were melanoma or mammary carcinoma cells, we tried to establish cultures of melanocytes and mammary epithelial cells. This turned out to be very difficult. Therefore we chose for the approach described in chapter VII.3.3, VII.4, using anti-β_2m serum for immunoprecipitation of DLA antigens from tumour cells and PBL of the tumour bearer. Analysis was done by 2D gel electrophoresis. In one dog, a difference between tumour cells and PBL was demonstrated (chapter VII.3.3). It is questionable, in retrospect, whether the 2D gel analysis of anti-β_2m precipitated DLA antigens on tumour cells and PBL was the best way to detect possible differences in DLA content on both types of cells. Given the failure of other methods (described briefly above), it was the most promising approach.
It is a certainty that, by establishing conditions for the biochemical investigation of DLA antigens on tumour cells, we have learned a lot about the biochemistry of DLA antigens on peripheral blood lymphocytes. A beginning has been made with the "detailed biochemical characterization" necessary to establish the "true and final picture of the detailed structure of the SD part of the (dog) MHC" as suggested by Vriesendorp in 1973 (chapter I.1).

VIII.4 References

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CHAPTER IX

SUMMARY

In this thesis, an immunochemical analysis of dog Major Histocompatibility Complex (MHC) antigens, also called DLA antigens, is described. MHC antigens play a prominent role in the immune system, particularly in the recognition of foreign material. They can be divided into four classes. As only DLA class I antigens have been defined by well characterized reagents (antisera), they were chosen as the object of the investigation.

In chapter I, a short historical survey of research into histocompatibility antigens in general, and DLA antigens in particular is given. Some fundamental methods for the isolation of MHC antigens as well as a model of the structure of a human MHC antigen, HLA-B7, are described. These have served as a guideline for our experiments. More recent developments in the investigation of the MHC antigens are mentioned to show the scope of present-day MHC research. The importance of the function of the MHC antigens in relation to immunological reactions and (malignant) disease is indicated.

For a thorough investigation of DLA class I antigens some basic knowledge of their biochemical nature is indispensable. The first questions we tried to answer regarded the isolation procedure of DLA class I antigens, their chemical composition and their molecular weight. Subsequently we investigated the light and the heavy chain, the localization of the alloantigenic site and the presence of DLA antigens on tumour cells.

Chapter II deals with large scale isolation of DLA antigens from spleens by papain digestion of crude membrane preparations. The DLA antigens are susceptible to cleavage by papain and behave like proteins in the rest of the isolation procedure. After papain digestion, they consist of two polypeptide chains of 37,000 and 12,000 daltons. They bind spontaneously to a Sepharose-lentil lectin column and must therefore contain a carbohydrate residue. The isolated products can specifically inhibit microlymphocytotoxicity tests using dog peripheral blood lymphocytes and DLA typing serum.

The next step of our investigation is described in chapter III. In order to assess whether the DLA light chain is identical to β_2m, as is the case in other species, it was necessary to raise an antiserum against dog β_2m. The isolation of β_2m from urine of a dog kidney transplant recipient is described. Two rabbits were immunized with purified dog β_2m. This yielded antisera which reacted with dog β_2m in Ouchterlony tests and on immuno-electrophoresis. The antisera are cytotoxic for dog peripheral blood lymphocytes.

In chapter IV it is demonstrated that the DLA light chain is identical to β_2m. Radiolabelled papain solubilized DLA molecules reacted with anti-β_2m serum in the immunoprecipitation assay. They are shown to consist of two polypeptide chains of 37,000 and 12,000 daltons. Detergent solubilization of peripheral blood lymphocytes or PBL membranes also yields molecules reactive with the anti-β_2m serum. After detergent solubilization, the MW of the component chains is 41,700 and 12,000 daltons. It was shown that the reaction of the anti-β_2m serum with DLA molecules can be inhibited by purified β_2m.
The limited NH_2-terminal amino acid sequence of the heavy chain from papain solubilized DLA antigens shows homology with corresponding chains of MHC products of other species. The NH_2-terminal amino acid sequence of the DLA light chain points to identity with dog urinary β_2m.
The protein nature of the DLA antigens is confirmed by the fact that they can be radiolabelled by lactoperoxidase-catalysed iodination and incorporation of ^{35}S-methionine. DLA antigens can be immunoprecipitated after incubation of intact cells with antiserum, which means that they are located on the outside of the plasma membrane.

Most experiments from chapter IV have been repeated using antisera against the heavy chain instead of antiserum against the light chain (β_2m). This is described in chapter V. Both an anti-heavy chain backbone (anti-HC) serum and an alloantiserum can immunoprecipitate DLA antigens from different sources, isolated either by papain digestion or by detergent solubilization. In addition to DLA antigens, the anti-HC serum precipitates polypeptide chains of 19,000, 30,000, and 39,000 daltons. It is possible that there are class II antigens amongst these. This was not tested any further due to lack of class II specific antisera. Taken together the results of chapter IV and V demonstrate the two chain structure of DLA class I antigens.

The presence of a carbohydrate chain on the heavy chain of DLA class I antigens was established by the experiments described in chapter VI. Metabolic labelling of PHA-stimulated dog peripheral blood lymphocytes with ^{3}H-fucose and subsequent immunoprecipitation with anti-β_2m serum yields a gel pattern of the DLA heavy chain only. Metabolic labelling of PHA-stimulated PBL with ^{35}S-methionine in the presence of tunicamycin, an inhibitor of N-linked glycosylation, results in a DLA heavy chain of 40,000 daltons, about 3000 daltons lower than the MW of the glycosylated heavy chains analysed in the same gel system. The light chain remains unaltered in the presence of tunicamycin. The nonglycosylated DLA antigens can be immunoprecipitated with anti-β_2m serum as easily as their glycosylated counterparts. This shows that N-linked glycosylation is not necessary for the association of the DLA heavy chain with β_2m.

Chapter VII is devoted to the alloantigenic site. For that purpose DLA antigens from the members of several dog families were isolated from radiolabelled peripheral blood lymphocytes by immunoprecipitation with anti-β_2m serum. They were analysed by one- and two-dimensional gel electrophoresis. Heterogeneity is found in the heavy chain, whereas the light chain from different dogs is constant. Therefore the alloantigenic site is located on the heavy chain. Comparison of

glycosylated and nonglycosylated DLA antigens shows that there is heterogeneity in the heavy chain even when there is no N-linked sugar chain present. The chemical nature of the alloantigenic determinant could be sugar and/or protein according to the above experiments. A definite answer cannot be given at present.

Dogs of differing tissue-types have DLA antigens showing different two-dimensional gel patterns. A tentative assignment of patterns to DLA haplotype is presented. When an alloantiserum is used in parallel with anti-β_2m serum against DLA antigens of one dog, the two-dimensional gel pattern of the anti-β_2m serum contains more spots in the heavy chain area than the pattern of the alloantiserum. This confirms that the alloantiserum reacts with the DLA antigens from a limited number of tissue-types. The data of the biochemical analysis of DLA antigens, immunoprecipitated by anti-β_2m serum, after two-dimensional gel electrophoresis suggest that DLA antigens are much more limited than HLA antigens and that there may be only one DLA class I locus. Finally, DLA antigens on tumour cells were studied and compared with DLA antigens on peripheral blood lymphocytes of the tumour bearer. In one case out of four there may have been a loss of DLA on the tumour cell. The difficulties encountered in the study of DLA antigens on tumour cells are discussed in chapter VII.

CHAPTER X

SAMENVATTING

In dit proefschrift wordt een immunochemische analyse van de antigenen van het belangrijkste histocompatibiliteitscomplex (MHC) van de hond beschreven. Bij de hond heet het MHC DLA complex; wij spreken daarom van DLA antigenen. MHC antigenen spelen een centrale rol in het immuunsysteem, in het bijzonder bij de herkenning van microörganismen en lichaamsvreemde stoffen. Zij kunnen worden verdeeld in vier klassen. Omdat alleen DLA klasse I antigenen gedefinieerd zijn door goed gekarakteriseerde reagentia (antisera), werden zij gekozen als voorwerp van onderzoek.

In hoofdstuk I wordt een kort historisch overzicht gegeven van het onderzoek naar MHC antigenen in het algemeen, en DLA antigenen in het bijzonder. Beschreven worden enkele fundamentele methoden voor de isolering van MHC antigenen, alsmede een model voor de structuur van een humaan MHC antigeen, namelijk HLA-B7. Deze methoden en dit model hebben als leidraad gediend voor ons onderzoek. Voorts worden recente ontwikkelingen in het onderzoek van de MHC antigenen vermeld om de reikwijdte van het MHC onderzoek van de laatste tijd te illustreren. Het belang van de functie van de MHC antigenen in verband met immunologische reakties en met het vóórkomen van (kwaadaardige) ziekten wordt aangegeven.

Voor een grondig onderzoek van DLA klasse I antigenen is enige basale kennis van hun biochemische eigenschappen onontbeerlijk. De eerste vragen die wij trachtten te beantwoorden betroffen de methode van isoleren van DLA klasse I antigenen, hun chemische samenstelling en hun molecuulgewicht. Vervolgens onderzochten wij de lichte en de zware keten; wij bepaalden de plaats van de alloantigene determinant(en) en wij bestudeerden DLA antigenen op tumorcellen.

Hoofdstuk II behandelt de isolering van DLA antigenen op grote schaal. Hiervoor werden ruwe membraanextracten van milten geïncubeerd met het enzym papaïne. Het blijkt dat de DLA antigenen door papaïne afgesplitst worden en dat zij zich als eiwitten gedragen tijdens de verdere zuivering. Na de papaïne behandeling bestaan zij uit twee polypeptideketens van 37.000 en 12.000 dalton. Zij hebben de eigenschap spontaan te binden aan een Sepharose-linzen-lectine kolom, waaruit blijkt dat zij een suikerketen bevatten. De geïsoleerde produkten kunnen in de microlymfocytotoxiciteitstest de reaktie tussen hondelymfocyten en DLA typeerserum specifiek remmen.

De volgende stap in ons onderzoek wordt beschreven in hoofdstuk III. Het was noodzakelijk een antiserum op te wekken tegen β_2m van de hond, om te onderzoeken of de lichte DLA keten identiek is aan β_2m, zoals dat bij andere

soorten het geval is. Daartoe werd β_2m uit urine van een hond met een getransplanteerde nier geïsoleerd. Twee konijnen werden geïmmuniseerd met gezuiverd honde β_2m. Dat leverde antisera op die met honde β_2m reageerden in de Ouchterlony test en in immuno-electrophorese proeven. Deze antisera zijn cytotoxisch voor honde-perifere-bloed-lymfocyten.

In hoofdstuk IV wordt aangetoond dat de lichte DLA keten identiek is aan β_2m. Radioactief gemerkte DLA moleculen die met behulp van papaïne gezuiverd waren reageerden met anti-β_2m serum in de immunoprecipitatie-techniek. Ook met behulp van deze methode werd aangetoond dat zij bestaan uit twee polypeptideketens van 37.000 en 12.000 dalton. Behandeling van perifere blood lymfocyten of hun membranen met detergens levert ook moleculen die met het anti-β_2m serum kunnen reageren. Na behandeling met detergens is het molecuulgewicht van de beide ketens respectievelijk 41.700 en 12.000 dalton. De reaktie van het anti-β_2m serum met DLA moleculen bleek te kunnen worden geremd door gezuiverd β_2m. De beperkte informatie over de aminozuurvolgorde aan het NH_2-uiteinde van de zware DLA keten na isolering door middel van papaïne laat zien dat er homologie bestaat met overeenkomstige ketens van MHC producten bij andere soorten. De aminozuurvolgorde aan het NH_2-uiteinde van de lichte DLA keten wijst erop dat deze keten identiek is aan β_2m uit honde-urine.
Het feit dat DLA antigenen radioactief gemerkt kunnen worden met ^{125}I door middel van lactoperoxidase of door inbouw van ^{35}S-methionine tijdens het kweken van de cellen in vitro bevestigt dat DLA antigenen eiwitten zijn. Na incubatie van intacte cellen met antiserum is immuunprecipitatie van DLA antigenen mogelijk, hetgeen inhoudt dat zij zich aan de buitenkant van de plasmamembraan bevinden.

De meeste proeven uit hoofdstuk IV zijn herhaald met antisera die gericht zijn tegen de zware keten in plaats van de lichte keten (β_2m). Zij worden beschreven in hoofdstuk V. Zowel een antiserum gericht tegen het constante deel van de zware keten (anti-HC) als een alloantiserum kan reageren met DLA antigenen, die door middel van papaïne of detergens uit respectievelijk milten en lymfocyten geïsoleerd werden. Het anti-HC serum reageert niet alleen met DLA antigenen maar ook met polypeptide ketens van 19.000, 30.000 en 39.000 dalton. Het is mogelijk dat hier klasse II antigenen bij zijn. Dit werd niet verder onderzocht omdat er geen klasse II specifieke antisera beschikbaar waren. Het onderzoek dat beschreven wordt in de hoofdstukken IV en V toont aan dat DLA klasse I antigenen uit twee ketens bestaan.

De aanwezigheid van een suikerketen aan de zware keten van DLA klasse I antigenen werd vastgesteld door middel van proeven die beschreven zijn in hoofdstuk VI. Na radioactief merken van PHA gestimuleerde honde-perifere-bloed-lymfocyten met ^{3}H-fucose geeft immuunprecipitatie daarvan met anti-β_2m serum een gelpatroon van uitsluitend zware DLA ketens. Merken van PHA gestimuleerde lymfocyten met ^{35}S-methionine in aanwezigheid van tunicamycine, een remmer van N-gebonden glycosylering, geeft als resultaat een zware DLA keten van 40.000 dalton, ongeveer 3000 dalton minder dan het molecuulgewicht van geglycosyleerde zware ketens in hetzelfde gelsysteem. De lichte keten verandert niet

in aanwezigheid van tunicamycine. De niet geglycosyleerde DLA antigenen reageren even goed met het anti-β_2m serum in immuunprecipitaties als de geglycosyleerde. Dit toont aan dat N-gebonden glycosylering niet noodzakelijk is voor de associatie van de zware DLA keten met β_2m.

Hoofdstuk VII is gewijd aan de localisering van de alloantigene determinant(en). Daartoe werden DLA antigenen geïsoleerd uit radioactief gemerkte perifere-bloed-lymfocyten van verschillende hondefamilies door middel van immuunprecipitatie met anti-β_2m serum. Deze DLA antigenen werden geanalyseerd door middel van één- en twee-dimensionale gelelectroforese. In de zware keten werd heterogeniteit vastgesteld, terwijl de lichte keten van verschillende honden constant bleek te zijn. Daarom kunnen verschillen in weefseltypering herleid worden tot verschillen in de zware keten. Vergelijking van geglycosyleerde met niet-geglycosyleerde DLA antigenen laat zien, dat er zelfs nog heterogeniteit in de zware keten gevonden wordt, wanneer er geen N-gebonden suikerketen is. De alloantigene determinant zou volgens bovenvermelde experimenten een suiker- en/of eiwitgroep kunnen zijn. Op dit moment kan daarover geen definitieve uitspraak worden gedaan.

Honden met verschillende weefseltypering hebben DLA antigenen met verschillende twee-dimensionale gelpatronen. Een voorlopige identificatie van patronen volgens DLA haplotype wordt voorgesteld. Bij vergelijking van het gebruik van een alloantiserum met dat van het anti-β_2m serum tegen DLA antigenen van één hond, blijkt het twee-dimensionale gelpatroon van het anti-β_2m serum meer stippen in het zware keten gebied te bevatten dan het patroon van het alloantiserum. Dit bevestigt dat het alloantiserum alleen reageert met DLA antigenen van een beperkt aantal typeringen. De gegevens van de biochemische analyse van DLA antigenen, na immuunprecipitatie met anti-β_2m serum, gevolgd door twee-dimensionale gelelectroforese, suggereren dat DLA antigenen minder heterogeen zijn dan HLA antigenen en dat er misschien maar één DLA klasse I locus is.
Tenslotte werden DLA antigenen op tumorcellen bestudeerd en vergeleken met de DLA antigenen op de perifere-bloed-lymfocyten van de tumordrager. In één geval (van de vier) was er misschien verlies van DLA op de tumorcel. De moeilijkheden die werden ondervonden bij het bestuderen van DLA antigenen op tumorcellen, worden besproken in hoofdstuk VII.

NAWOORD

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Bep Smit, Anne Hagemeijer-Hausman, An Langeveld en Elly Roza-de Jong (afdeling Celbiologie en Genetica) maakten en bekeken de chromosoompreparaten. Arthur van der Kamp en Andries Westerveld (afdeling Celbiologie en Genetica) gaven waardevolle adviezen. Anton Grootegoed (afdeling Biochemie 2), Hugo de Jonge (afdeling Biochemie 1) en Wim van Noort (afdeling Chemische Pathologie) hielpen bij het opzetten van verschillende technieken.

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CURRICULUM VITAE

In 1970 eindexamen aan het Stedelijk Gymnasium te Leeuwarden.
Studie biologie van 1971-1973 aan de Rijks Universiteit te Groningen en van 1973 tot december 1977 aan de Rijks Universiteit te Leiden.
Doktoraalexamen met als hoofdvak biochemie (tumorvirologie bij Prof. dr A.J. van der Eb en dr P.J. Abrahams, Laboratorium voor Fysiologische Scheikunde). Bijvakken: stralengenetica bij dr J.W.I.M. Simons, Laboratorium voor Stralengenetica en Chemische Mutagenese, en tumorimmunologie bij Prof. dr D.L. Westbroek en dr J.G. Bowen, Laboratorium voor Experimentele Chirurgie van de Erasmus Universiteit te Rotterdam.
Van 1 januari 1978 tot 1 januari 1982 werkzaam op het Laboratorium voor Experimentele Chirurgie van de Erasmus Universiteit te Rotterdam waar het in dit proefschrift beschreven onderzoek werd verricht in het kader van een onderzoeksproject van het Koningin Wilhelmina Fonds.
Vanaf september 1982 verbonden aan de afdeling Pathologische Anatomie I van de Erasmus Universiteit te Rotterdam.