FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF THE ANTIGEN RECEPTORS OF CYTOTOXIC T LYMPHOCYTES

FUNCTIONELE EN MOLECULAIRE KARAKTERISERING VAN DE ANTIGEEN RECEPTOREN VAN CYTOTOXISCHE T LYMFOCYTEN

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Chapters 2 - 6 represent the appendix papers of this thesis.

CHAPTER 1

1. GENERAL INTRODUCTION

Most higher organisms possess a complex system of cells to protect them from infections. This defense system consists of a non-specific part and a specific one. The non-specific cells are polymorphonuclear granulocytes and cells of the mononuclear phagocytic system. They react non-specifically to invading microorganisms. In this manner a beginning infection is delayed so that the specific cells, constituting the immune system, have time and opportunity to mount a specific response. This specific response is usually followed by a state of memory. Later infections with the same pathogen will elicit faster and more efficient responses.

The immune system itself is a dual system, that is able to mount two different responses, the humoral and the cellular immune response. The humoral immune response is mediated by B cells. On encountering antigen, B cells start producing antibody molecules and the antibodies will circulate through the circulation. As a substantial part of the antibodies can leave the vascular system, antibodies occur in almost all extracellular compartments of the body so that they can be effective against all antigens that are present in these fluids.

The cellular immune response is mediated by T lymphocytes and is mainly directed against micro-organisms, that replicate intracellularly and will not be exposed to the circulating antibodies. The response against cancer cells and foreign tissues is also mediated by T lymphocytes. T cells respond to antigenic challenge by either the production of lymphokines, produced by helper T cells, or lysis of infected target cells by cytotoxic T cells. These two main populations of T lymphocytes are distinguishable from each other by the presence of typical surface markers. Cytotoxic T cells carry the CD8 antigen, while helper T cells possess the CD4 surface antigen. Indispensable to the function of T lymphocytes are the molecules encoded by the Major Histocompatibility Complex (MHC). These molecules are expressed primarily on cells of the immune system but also on other cells. They are crucial for the recognition of antigen by T cells and regulate the immune response.

Recognition of foreign precludes the recognition of self. It is typical for the immune system that, under normal conditions, immune responses against antigenic determinants that belong to the normal components of an organism are minimal. The thymus has an important function in establishing and maintaining so-called self tolerance.

The receptor repertoire of both T and B cells is practically sufficient to enable the immune system to respond to virtually all antigens. A repertoire of that size implies a large number of antigen receptors (in the order of 10×10^6). The antigen

receptor of B cells is the surface-bound immunoglobulin. The antigen receptor of T cells (TcR) is a similar dimeric molecule that consists of two polypeptide chains, the α and β chain. The general structure of immunoglobulin molecules and T cell receptors is analogous. They both contain constant and variable domains and homologous mechanisms are employed to create diversity. Instead of coding 10 x 10^6 complete receptors in the genome (which would occupy a major portion of the available mammalian genome), different receptors are generated by random combinations of several gene segments. In each individual B or T cell precursor, DNA of various variable, diversity, joining and constant genes is joined to form a unique functional transcriptional unit, giving rise to a functional antigen receptor on the cell surface.

2. THE MAJOR HISTOCOMPATIBILITY COMPLEX

The function of T cells cannot be discussed without the biology of the major histocompatibility complex (MHC). T cells recognize antigens in association with molecules that are encoded by genes of the major histocompatibility complex. This phenomenon is called MHC restriction. Therefore these molecules and the genes coding for them are discussed in detail below. The major histocompatibility gene complex is a genetic locus, that has different names in different species. In the mouse the MHC is called the H-2 locus and is located on chromosome 17 [Klein, 1975; Klein et al. 1981]. The MHC of man is named HLA (Human Leukocyte Antigen) and is located on chromosome 6 [Jongsma et al, 1973; Bach & van Rood, 1976].
The MHC antigens have originally been discovered as major transplantation antigens. Successful transplantation of tumors from one animal to the other was only possible in inbred mouse strains. Gorer postulated for the first time that differences between donor tissue and tissue of the host could account for graft rejection [Gorer, 1938]. This phenomenon is called alloreactivity and is the basis for rejection of grafts when transplanted from one individual to another within the same species. In mice the H(istocompatibility)-2 complex (figure 1) appeared to be the major genetic factor influencing transplant surival, but several non-H-2 transplantation antigens have also been described. These antigens have been called minor transplantation antigens [Klein, 1975].
The size of the H-2 complex is about 1-2 cM (recombination analysis) or 2000-4000 kb (DNA analysis) [Hood et al, 1983]. Three classes of genes, I, II and III are located within this complex of loci (figure 1). Class I gene products K, D and L are encoded in the K and D region. These molecules function as restriction elements for cytotoxic T cells [Zinkernagel & Doherty, 1974]. Class I molecules are expressed on most cells in varying densities. Many more class I genes are found in the Tla complex, which is located directly 3' to the H-2 complex. The class II genes are encoded in the I-region of the H-2 complex and in most strains of mice two gene products are derived from this region, (I-)A and (I-)E antigens. These

molecules serve as restricting elements for $CD4^+$ helper T cells and are expressed mainly on antigen presenting cells (APC) and B cells. The S-region (i.e. Slp, Ss, Bf, C2) encodes class III gene products. These molecules are components of the complement system. The complement system consists of a series of serum proteins that can be sequentially activated and functions as a non-specific effector mechanism of the immune system.
Probably the most striking feature of the MHC genes is the extreme degree of polymorphism. The K and D regions in mice are known to have at least 50 different alleles in inbred and wild mouse strains [Klein, 1979; Klein et al, 1983b; Klein & Figueroa, 1986]. Class II genes are also polymorphic [Figueroa & Klein, 1986]. The combination of different alleles on the various loci of a single chromosome is called: a haplotype. A mouse with the $H\text{-}2^k$ haplotype for example is homozygous for all alleles and possesses the "k" allele at the K, I and D region. The chance that two unrelated individuals possess the same haplotype on both chromosomes is extremely small (10^{-4}-10^{-5}) and therefore transplantation of organs or tissues between individuals of the same species usually results in rejection.

2.1. Class I major histocompatibility antigens

Class I molecules are encoded by the K- and D-region in the mouse (figure 1) and by HLA-A, -B and -C in man. The typical structure of a class I molecule is a glycoprotein with a molecular weight of 44 kD [Klein et al, 1981, 1983a; Hood et al, 1983; Flavell et al, 1985]. This molecule consists of three domains of about 90 amino acid residues each ($\alpha1$,$\alpha2$,$\alpha3$, figure 2). The chain is anchored in the membrane with a transmembrane portion of 25 residues [Ploegh et al, 1981; Coligan et al, 1981]. The COOH terminus of about 30 residues protrudes into the cytoplasm. Class I antigens, when expressed on the surface

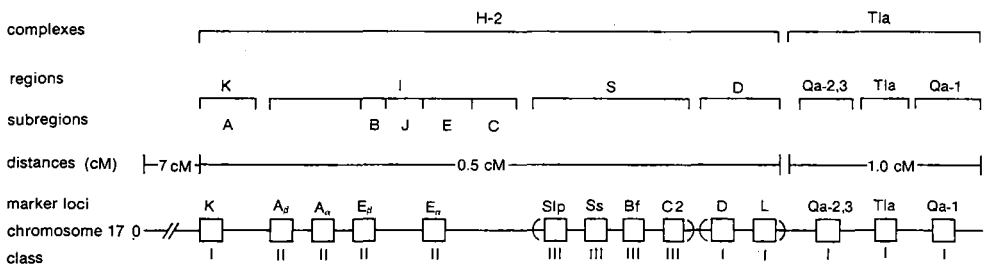


Figure 1. Genetic map of the murine major histocompatibility complex, H-2, on chromosome 17. Indicated are the regions, subregions, classes and marker loci. 0 represents the centromere. The order of loci within parentheses is not known. Adapted from Hood et al. 1984.

of cells, are linked to a non-polymorphic molecule, β2-microglobulin [Grey et al, 1973]. The molecular weight of β2-microglobulin is 12 kD and its structure resembles that of the third domain (α3) of the longer polypeptide chain [Orr et al, 1979]. The structure of β2-microglobulin and of the α3 domain of the class I molecule are homologous to the structure of the individual domains of immunoglobulin molecules (figure 2) [Peterson et al, 1972; Orr et al, 1979; Strominger et al, 1980]. Each domain consists of 80 - 90 amino acid residues with an internal disulfide bridge which spans about 60 residues. These domains are characteristic of many molecules that have a function in the immune system, Thy-1, β2-microglobulin, MHC, immunoglobulin, T cell receptor, CD4 and CD8 antigens. Therefore, this gene family is often called the immunoglobulin gene superfamily (figure 3) [Hunkapiller & Hood, 1986].
The α1 and α2 domains have a distinct structure, different from that of immunoglobulin but similar to each other [Orr et al, 1979].
The three dimensional structure of a human class I molecule has recently been published [Bjorkman et al, 1987a]. The X-ray cristallographic structure determination revealed that the α3 and β2-microglobulin domains are associated and each maintain the typical strucure of an immunoglobulin-like domain. The α1 and α2 domains form a symmetrical structural unit that consists of a platform composed of a single β-pleated sheet topped by two α-helices with a long groove between the helices [Bjorkman et al, 1987a].
After initial isolation of a cDNA clone coding for the COOH-terminal part of an HLA molecule [Ploegh et al, 1980], the organization of MHC genes was rapidly clarified [Hood et al, 1983]. A typical class I gene consists of 8 exons (figure 4) [Steinmetz et al, 1981; Evans et al, 1982; Moore et al, 1982]. The first exon codes for the 5' untranslated sequence and the leader peptide of 21 hydrophobic or uncharged residues. The second, third and fourth exon code for the α1, α2 and α3 domain, respectively. The fifth exon encodes the transmembrane domain and the sixth, seventh and eighth the cytoplasmic portion and 3' untranslated region.
When the amino acid sequences of the various class I genes (K, D and L) of various haplotypes are compared [Maloy & Coligan, 1982; Klein & Figueroa, 1986], it is striking that alleles of the same locus (K) are not more homologous to each other than to alleles of other loci (D and L). There are only a few "K-specific" residues and most of them are in the cytoplasmic portion of the molecule. It can also be noted that most allele specific residues are found in the α1 and α2 domain and that the α3 domain is relatively conserved. Most of these polymorphic residues seem to be located in the antigen binding site of the class I molecule [Bjorkman et al, 1987b]. Thus, they could influence binding of antigenic peptides that are presented to T cells.
By genetic recombination, hybrid H-2 molecules have been constructed that contain one or more domains derived from one allele and the remaining domains from another allele or locus. From these data it can be concluded that antibodies as well as

alloreactive and antigen specific, restricted CTL recognize determinants on the H-2 class I molecule that are formed by a combination of the α1 and α2 domain [Allen et al, 1984; Arnold et al, 1984].
The availability of probes for class I MHC genes has led to another discovery. When genomic libraries of mouse DNA were screened with a class I probe, a great number (±30) of class I-like genes were isolated. These genes are primarily localized in the Qa and Tla regions [Steinmetz et al, 1982b], which are directly 3' to the H-2 complex (figure 1). Many of these genes are in fact pseudogenes and cannot be functionally expressed. It has been demonstrated in at least one example that these genes can function as donor genes for mutation events. They can provide sequences for mutant class I genes that are generated by gene conversion events [Pease et al, 1983], and thus may be important for the generation of polymorphism. The tissue distribution of Qa and Tla antigens, which is different from that of K and D antigens, and the limited degree of polymorphism of Qa and Tla antigens, suggest that the function is different from that of the classical class I antigens. Tla antigens are only expressed during particular stages of thymic differentiation and on some leukemic cells. Qa antigens are expressed on subpopulations of hemopoietic cells and in liver cells. Recently it has been shown that both soluble and membrane bound forms of class I molecules can be synthesized from one gene [Stroynowski et al, 1987]. The function of the

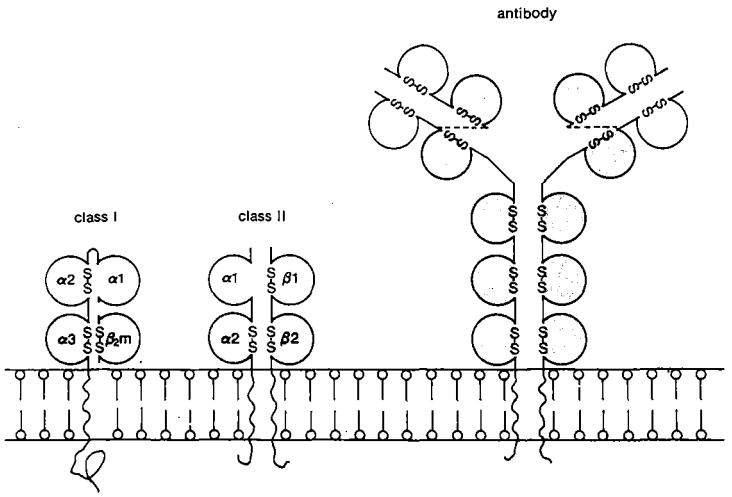


Figure 2. A schematic representation of the domain structure of a class I, class II and an antibody molecule inserted in the membrane. The shaded domains are homologous to each other and suggest common evolutionary ancestry (immunoglobulin gene superfamily, figure 3). Adapted from Hood et al. 1984).

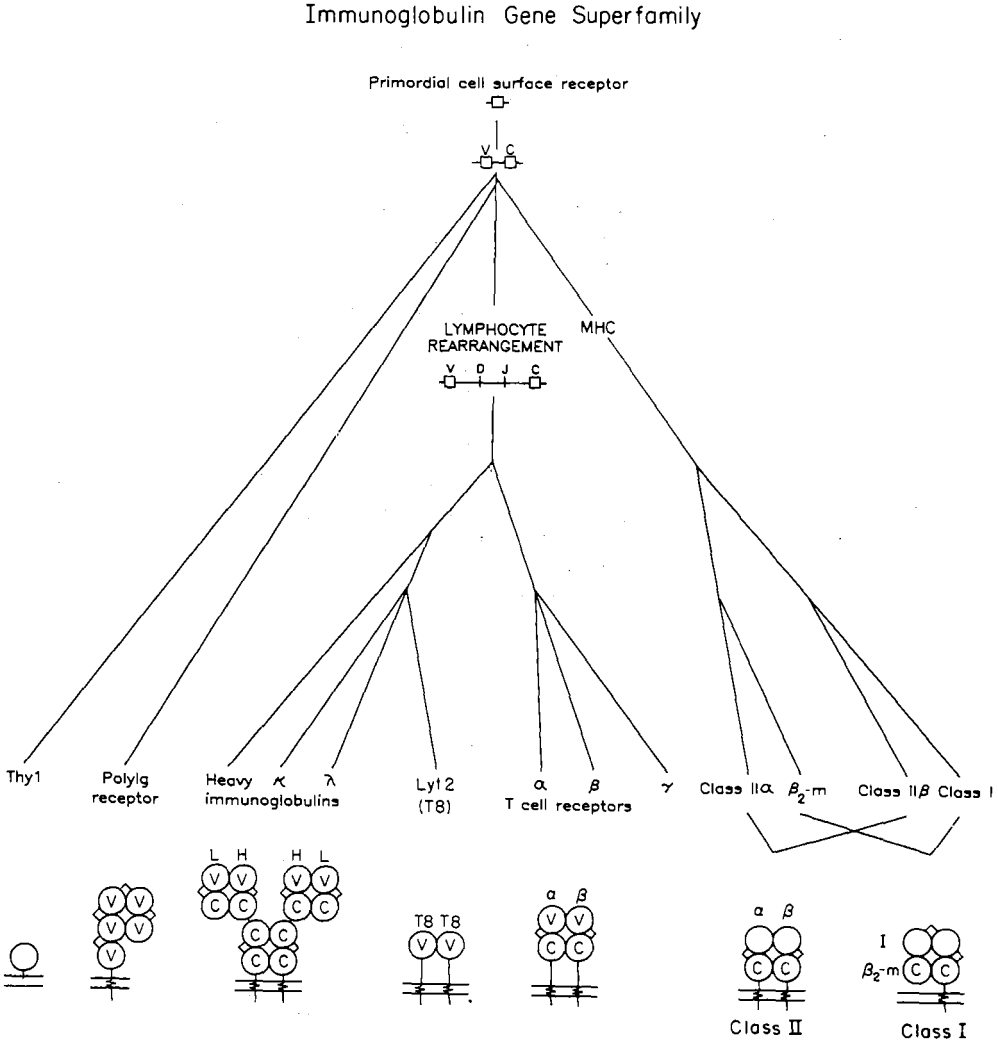


Figure 3. A proposed model for the evolution of the genes of immunoglobulin gene superfamily. There are variable (V) and constant (C) homology units. The open circles of the MHC molecules are not sufficiently homologous to the immunoglobulin domains. Thy-1 may have diverged prior to the V-C divergence and is not easily classified as V or C. Adapted from Kronenberg et al. 1986.

gene products of the Tla complex remains unclear [Klein et al, 1983a].

2.2. Class II major histocompatibility antigens

The class II genes are encoded by the genes in the I-region (figure 1) of the H-2 complex (Ia or Ir genes) [Klein et al, 1983a; Hood et al, 1983; Bell et al, 1985]. The membrane molecules encoded by this region (I-A and I-E in mice, HLA-DP, DQ, DR in man) consist of two non-covalently linked polypeptide chains (figure 2). The α chain has a molecular weight of 30-33 kD and the β chain of 27-29 kD. These differences are primarily due to differences in glycosylations [Hood et al, 1983; Klein et al, 1983a]. Both the α and the β chain consist of two domains (like the domains found in class I molecules) of about 90 amino acid residues, a transmembrane portion of 25 amino acids and a short (10-15) cytoplasmic region [Kaufman et al, 1984]. The $\alpha 2$ and $\beta 2$ domains, which are most proximal to the membrane, are homologous to immunoglobulin constant regions and have intradomain disulfide bridges. The $\beta 1$, but not the $\alpha 1$, domain also has a disulfide loop. Thus, although class II molecules are constructed differently than class I molecules, the final structure on the cell membrane may be quite similar

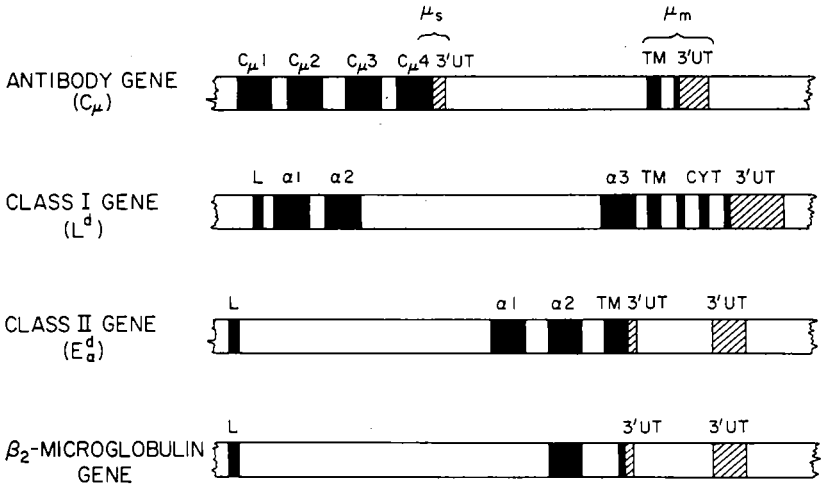


Figure 4. A schematic diagram of the intron-exon organization of the antibody, class I, class II and $\beta 2$-microglobulin genes. Dark boxes represent coding regions and hathed boxes are untranslated sequences. L indicates the exon that codes for leader or signal peptide; $\alpha 1$, $\alpha 2$ and $\alpha 3$ represent the exons encoding the external domains; TM designates the exon where the transmembrane portion is encoded; CYT exons code for the cytoplasmic domains; 3'UT is the 3' untranslated region. Adapted from Hood et al. 1983.

[Bjorkman et al, 1987a].
The intron-exon organization of class II genes is similar to that of class I genes (figure 4) [Hood et al, 1983; Flavell et al, 1985]. There are separate exons for leader, first and second external domains, transmembrane, cytoplasmic and 3' untranslated regions. The only variation between the various chains is in the number of exons that code the 3' portion of the molecule (transmembrane, cytoplasmic, 3'untranslated). The genomic organization of the genes coding for the murine class II antigens A and E is as follows, $A_{\beta 3}$, $A_{\beta 2}$, $A_{\beta 1}$, A_{α}, $E_{\beta 1}$, $E_{\beta 2}$, E_{α}, $E_{\beta 3}$ [Steinmetz et al, 1982a; Bell et al, 1985; Flavell et al, 1985]. The A and E antigens are encoded by $A_{\beta 1}/A_{\alpha}$ and $E_{\beta 1}/E_{\alpha}$, respectively. The other genes probably do not give rise to functional products. However, they provide information on the evolutionary origin of these genes when they are compared with the human class II sequences [Figueroa & Klein, 1986].
The α chain of murine class II molecules is relatively monomorphic but the β chain is very polymorphic [Figueroa & Klein, 1986]. Most of the polymorphic residues are located in the two distal ($\alpha 1$ and $\beta 1$) domains. It has been suggested [Bjorkman et al, 1987a] that the three dimensional structure of class II antigens may resemble that of class I antigens. That would imply that in class II molecules, as in class I molecules, polymorphic residues are involved in antigen binding and presentation.

3. T LYMPHOCYTES

When antigen enters the body of a vertebrate, a complex defense mechanism is triggered. Antigen is phagocytosed and degraded by antigen presenting cells and presented to T and B cells. The T

Figure 5. Two models for MHC restricted recognition of antigen by the T cell receptor. In the left model two receptors, R1 and R2, (or two receptor sites) are present, one recognizing MHC, the other recognizing antigen. The alternative model (right) proposes one receptor molecule (or receptor site) recognizing a newly formed complex of antigen and MHC.

cells can either directly exert their influence on the infected cells (cytotoxic T cells) or they can support activation and differentiation of B cells and macrophages (helper T cells). The molecules encoded by the MHC are crucial to the interactions that are required between antigen presenting cells, T and B cells. Class I antigens are the restricting elements for $CD8^+$ (cytotoxic) T cells. In vivo activated cytotoxic T cells only lyse infected target cells, if they share class I antigens [Zinkernagel & Doherty, 1974]. In a similar way class II molecules are the restricting elements for $CD4^+$ (helper) T cells. T cells can only provide help to B cells if the antigens encoded by the I-region of the MHC are the same on both cells [Katz et al, 1975].
These findings have usually been interpreted to imply that T cells recognize antigens (or fragments) as well as certain polymorphic determinants of MHC molecules simultaneously. Two models for the interaction between T cells and target cells have been proposed (figure 5) [Zinkernagel & Doherty, 1979]:
1. Two receptor recognition sites. One receptor binds to self MHC and a second receptor binds to a foreign antigenic determinant.
2. One receptor recognition site. A single receptor recognizes a determinant that is composed of an MHC encoded molecule plus antigen.
Over the last few years evidence has accumulated in favour of the second model. Fusion of two T cells with different MHC and antigen specificities did not yield hybrids with new combinations of specificities [Kappler et al, 1981]. Furthermore, when the genes coding for the T cell antigen receptor were isolated, it was shown that transfection of the two chains of the T cell receptor is sufficient to transfer specificity for antigen plus self MHC to a recipient cell [Dembic et al, 1986; Saito et al, 1987].
Originally, the antigen receptors of T lymphocytes have been postulated to be encoded by the Immune response (Ir) genes, which were closely linked to the H-2 transplantation antigen genes [Benacerraf & McDevitt, 1972]. In 1981 it became clear that the immune response (Ir) genes, which determine the magnitude of the immune response to certain antigens, function through the same antigens, Ia, which are serologically detectable on the cell membrane [Klein et al, 1981]. The observation that antigen binds directly to the Ia (class II) molecule, although with a low affinity [Babbitt et al, 1985], explains the Ir phenomena. Instead of the originally proposed specific receptors for antigen of T lymphocytes, the Ir genes encode molecules, that present antigenic fragments to the T cell receptors. Class II molecules of one haplotype (high responder) bind certain peptide antigens well, while class II molecules of a second haplotype (low responder) bind the same peptide with a lower affinity. The function of class I and class II molecules is in fact very similar and is described in more detail below (6. Antigen Presentation).

4. SURFACE MARKERS OF T LYMPHOCYTES

Since the production of monoclonal antibodies, a great number of antibodies against membrane antigens of lymphocytes have been generated. These antibodies distinguish certain subpopulations of lymphocytes. The antigens that are recognized also involve surface molecules that are involved in specific functions of lymphocytes.

4.1. Thy-1

In mice, T cells have long been defined using the Thy-1 antigen [Reif & Allen, 1966]. This antigen is found on T cells, although not exclusively (cf. neurones). The Thy-1 antigen is also a member of the immunoglobulin gene superfamily (figure 3). Mouse strains differing only at the Thy-1 locus are available and are often used for repopulation, homing and cell kinetic experiments. Donor and recipient cells can easily be distinguished on the basis of expression of either Thy-1.1 or Thy-1.2.

4.2. CD4 and CD8

Subpopulations of murine T cells were first distinguished with two rat-anti-mouse monoclonal antibodies [Cantor & Boyse, 1975; Ledbetter & Herzenberg, 1979]. Lyt-1 and Lyt-2 are markers that distinguish T helper cells (class II restricted) and cytotoxic T cells (class I restricted), respectively. The Lyt-1 marker has proved not so useful, because it is expressed on most T cells at a higher (Th) or lower (Tc) level and it is occasionally found on B cells as well [Herzenberg et al, 1986]. A less ambiguous marker of helper T cells is the L3T4 antigen [Dialynas et al, 1983]. This antigen is expressed only on $Lyt\text{-}2^-$ T cells and double positive cells are hardly observed, except in the thymus. The antigens that are defined with the anti-L3T4 and anti-Lyt-2 antibodies in the mouse are homologous with similar molecules, T4 and T8, respectively, in man. L3T4 in the mouse and T4 in man are collectively described as C(luster)D(eterminant) 4, while Lyt-2 and T8 are called CD8 antigens (Workshop on Human Leukocyte Differentiation, Oxford, UK 1986 [McMichael, 1987]). The genes that encode the CD4 and CD8 antigens [Littman, 1987] have been isolated and sequence comparison reveals homology with immunoglobulin genes. Therefore, these surface markers have also been classified as members of the immunoglobulin gene super family (figure 3) [Hunkapiller & Hood, 1986].
Because the CD4 and CD8 expression correlates so well with class II and class I recognition, respectively [Swain, 1983], it has been proposed that direct interactions between CD4/8 and class II/I molecules exist that increase the avidity between effector and target cell [MacDonald et al, 1982].
T cells that did not respond to certain antigens have been made responsive by transfection of CD4/CD8 antigens into them [Dembic et al, 1987; Gay et al, 1987]. On the other hand, inhibition of T cell activation by anti-L3T4 antibodies in the

absence of class II recognition [Owens & Fazekas de St.Groth, 1987], suggests that some direct interaction between T cell receptor and L3T4 antigen may also exist [Saizawa et al, 1987; Takada & Engleman, 1987]. The role of the CD4 and CD8 antigen and their ligands under in vivo conditions has to be further investigated [MacDonald et al, 1982].

4.3. CD3

A surface marker that is expressed on virtually all T cells is the CD3 antigen (T3) [McMichael, 1987]. Murine antibodies against the human CD3 antigen have been produced first [Kung et al, 1979]. Monoclonal antibodies specific for the murine CD3 antigen have been produced only recently by immunizing Armenian hamsters [Leo et al, 1987]. Under mild conditions anti-CD3 antibodies precipitate a molecular complex from the membrane of T cells that represents the T cell receptor [Oettgen & Terhorst, 1987]. Besides the α and β chain, which mediate specific antigen recognition, at least three other proteins are precipitated, T3γ, T3δ and T3ϵ. These molecules have been biochemically characterized and the corresponding genes have been cloned [Oettgen & Terhorst, 1987]. The function of these molecules is not yet resolved, but it is generally assumed that they have a function in signal transduction after the α/β chains have recognized their ligand [Oettgen & Terhorst, 1987].

5. THE THYMUS

The importance of the thymus for adequate immunological function has been acknowledged for a long time [Miller, 1961]. After the discovery of MHC restriction [Zinkernagel & Doherty, 1974], it became clear that the thymus plays a role in the development of this restriction [Zinkernagel et al, 1978; Zinkernagel & Doherty, 1979].
The thymus is an epithelial organ, derived from the third and fourth pharyngeal pouches. It contains endo- and ectodermal tissues (epithelium) as well as bone marrow-derived cells (macrophages and dendritic cells). During embryogenesis prothymocytes enter the thymus from the blood. The thymus has its largest size during early life and is reduced significantly when puberty is reached.
Anatomically, the thymus can be divided in cortex and medulla. Most of the thymocytes are found in the cortex, where also most of the proliferative activity is found. The medulla contains lymphocytes with a mature phenotype. Four subpopulations of thymocytes are usually distinguished on the basis of the two surface markers, CD4 (L3T4) and CD8 (Lyt-2) [Scollay et al, 1984]. The double negative population contains the precursor cells that are capable of repopulating the thymus in vivo [Fowlkes et al, 1985] or in vitro [Kingston et al, 1985]. A few progenitors seem to suffice to populate the whole thymus [Wallis et al, 1975; Ezine et al, 1984]. The majority of thymocytes belongs to the double positive population and these cells have been described to represent an intermediate stage of

thymocyte development [Kyewski et al, 1987; MacDonald et al, 1988]. Single positive ($CD4^+$ or $CD8^+$) cells resemble peripheral T cells and are thought to be mature cells, about to leave the thymus.
One of the most intriguing questions in immunology has always been: How does the immune system distinguish self from non-self? The antigen receptors of T lymphocytes are generated by rearrangement of various germline gene segments, to form a unique receptor in each individual T lymphocyte. It is assumed that unrearranged cells enter the thymus and are induced to proliferate and differentiate. Rearrangement of T cell receptor genes will take place at this stage of development [Williams et al, 1986; Lindsten et al, 1987]. At some time during rearrangement of T cell receptor genes, receptors will originate with a specificity for self antigens. The thymus has been postulated to eliminate the self-reactive cells [Kappler et al, 1987].
In addition, the receptors seem to be positively selected to recognize antigens preferentially in the context of self MHC antigens that are expressed in the thymus [Zinkernagel et al, 1978; Marrack & Kappler, 1987].
Many experiments have been devoted to discover which cell types of the thymus, epithelial cells or bone marrow derived cells, are responsible for induction of tolerance and education of T cells to recognize antigen preferentially in the context of self MHC antigens. Thymic epithelium has been shown to induce a state of tolerance in birds [Ohki et al, 1987] and mice [Owen et al, 1986]. In some studies, tolerance has not been demonstrated in <u>in vitro</u> assays but the thymus epithelium itself as well as subsequent organ grafts were not rejected [von Boehmer & Schubiger, 1984; Ready et al, 1984]. It seems that thymic epithelium alone is not sufficient to induce complete tolerance and this suggests a role for bone marrow derived cells in tolerance induction. Epithelial cells have been shown to dictate the restriction elements recognized by T cells [Zinkernagel, 1982; Lo & Sprent, 1986], although one study has suggested that bone marrow cells were responsible [Longo & Schwartz, 1980]. A model for thymic differentiation of T lymphocytes could be that, as T cells start to express an antigen receptor at low level, they interact with Ia^+ epithelial cells to be selected for restriction for self MHC. Subsequently, the receptors would be expressed at higher levels and self-reactive T cells are eliminated when they recognize self antigens on bone marrow derived cells. Histological analysis of thymocytes in situ supports this concept [Kyewski, 1987].

<u>6. ANTIGEN PRESENTATION</u>

Since it has been realized that recognition of antigen by T cells involves the simultaneous recognition of molecules encoded by the MHC [Zinkernagel & Doherty, 1974; Katz et al, 1975], an interaction between antigen and MHC has been hypothesized [Rosenthal, 1978; Benacerraf, 1978]. Macrophages

were shown to be actively involved in the presentation of antigen in the context of MHC [Unanue, 1984]. Instead of presenting native antigen, small fragments of larger antigens are presented to T cells [Grey & Chesnut, 1985; Schwartz, 1985]. It has since long been postulated that these fragments bind to MHC molecules but such an interaction was first demonstrated in 1985 [Babbitt et al, 1985].
Detergent solubilized purified class II antigen was shown to bind an immunogenic peptide of hen-egg lysozyme with an apparent equilibrium constant (K_D) of 2 μM. This peptide was not bound by I-A molecules of a non-responder mouse strain. The formation of the I-A peptide complexes was shown to be slow, but once formed, they appeared to be rather stable [Buus et al, 1986].
If antigen binds to MHC molecules the question can be raised whether there is only one binding site for antigen on MHC, or whether more distinct sites exist. Indications for the existence of only one binding site have been described. Different antigens have been shown to inhibit each others presentation by accessory cells to specific helper T cells [Werdelin, 1982; Guillet et al, 1987], indicating that they are competing for one and the same binding site on class II molecules. The binding of peptides to I-A molecules can be inhibited by related, slightly modified peptides [Babbitt et al, 1986] but also by unrelated peptides [Guillet et al, 1986]. The interaction between peptides and Ia has been further characterized by examining which structural properties of the antigen are important for binding. Substitutions of amino acid residues at all locations of an antigenic peptide have been made and the new peptides have been tested for their capacity of inhibiting binding of a labeled peptide to Ia. Several residues, but not all, within an antigenic peptide have been shown to be important for binding to class II molecules [Allen et al, 1987; Sette et al, 1987]. In this manner, "motives" can be defined that are shared between various peptides binding to the same Ia molecule. [Guillet et al, 1987; Sette et al, 1987]. Motives are general structural characteristics that a peptide should possess in order to bind to an Ia molecule.
The same peptides with substituted amino acid residues have been used to evaluate which residues are important for stimulation of T cells [Sette et al, 1987; Allen et al, 1987]. Replacements that resulted in a lack of stimulation, but no lack of binding to MHC molecules, were interpreted to indicate residues that were actually recognized by the T cell receptor. It is noteworthy that T cell recognition is more sensitive to substitutions than the interaction with Ia. [Sette et al, 1987]. This reflects the flexibility of the binding site of Ia molecules, which is able to present many different antigenic peptides, and the high degree of specificity displayed by T cell receptors.
For class I restricted, cytotoxic T cells it has long been assumed that the viral glycoproteins expressed on the cell surface represent the antigenic determinants recognized by CTL [Zinkernagel & Doherty, 1979]. It has, however, been difficult to demonstrate inhibition of antigen specific cytotoxicity with

antibodies directed at antigen. Recently, it has been shown that influenza specific CTL recognize internal viral proteins (nucleoproteins) [Townsend et al, 1984; Townsend, 1987]. CTL specific for determinants of the surface glycoprotein of influenza virus, hemagglutinin (HA), did also lyse cells that were transfected with the cDNA encoding the hemagglutinin molecule without the signal peptide [Townsend et al, 1986b]. This implies that expression of native viral proteins on the cell membrane is not necessary for T cell targeting. In addition, target cells incubated with the appropriate peptides were shown to be recognized by specific CTL [Maryanski et al, 1986; Townsend et al, 1986a]. Therefore, recognition of antigen by class I restricted T cells seems to be similar to class II restricted antigen recognition. Important in this respect is the three dimensional structure of a class I molecule, HLA-A2 [Bjorkman et al, 1987a, 1987b]. A class I molecule seems to possess a single antigen binding site which can accommodate a peptide of 8 to 20 amino acid residues, although different positions of antigen peptides within this binding site may be possible. It has been suggested that the three dimensional structure of class II molecules would be similar to that of class I molecules [Bjorkman et al, 1987a].

Class I as well as class II MHC molecules present peptide antigens to T lymphocytes. In both cases there seem to be general properties that a peptide should possess, in order to be able to bind to MHC [Guillet et al, 1987; Sette et al, 1987]. A small antigen may not have suitable peptide fragments that can be bound by MHC [Babbitt et al, 1985]. This might explain why mice with some MHC haplotypes are responders while others are non-responders, if one looks at the response against a relatively small antigen [Benacerraf & McDevitt, 1972; Shevach & Rosenthal, 1973; Zinkernagel et al, 1985; Kast et al, 1986; Pala & Askonas, 1986]. However, most physiological antigens are complex antigens. Organisms will therefore always have at least one MHC molecule, either at the level of an individual (different alleles and different loci) or at the level of the species (extreme degree of polymorphism) that is capable of presenting one of the degraded fragments of an antigen. This will ensure that a functional immune response will be mounted.

An immune response is not always a necessary consequence of peptide-Ia binding. In at least one example, binding of a peptide to I-E^d has been described to be even stronger than binding of the same peptide to I-A^d. Yet, when BALB/c (H-2^d) mice are immunized with this peptide, the T cell response is exclusively restricted to I-A^d [Guillet et al, 1987]. A likely explanation of this phenomenon would be that the T cells, specific for this antigen MHC combination have been deleted or inactivated (a hole in the repertoire), e.g. because the antigenic epitope would be too similar to an endogenous (self) peptide.

7. ALLOREACTIVITY

The MHC molecules have been discovered because they are highly polymorphic and, generally, do not allow transplantation of tissues from one individual to another. This phenomenon is called alloreactivity and seems to be mediated primarily by T lymphocytes. Although alloantigens do not seem to form a threat to the survival of an individual organism, the frequency of alloreactive cells in an unprimed animal is unexpectedly high [Simonsen, 1967; Nisbet et al, 1969; Wilson et al, 1970]. The number of distinct T lymphocyte clones that are activated in response to a single alloantigen is large [Sherman, 1980], suggesting that lymphocytes with many different receptors are involved in an allogeneic response.
Until the demonstration that MHC restricted, antigen specific T cell clones cross-react with alloantigens [von Boehmer et al, 1979; Sredni & Schwartz, 1980] it has been unclear whether the same subset of lymphoid cells is responsible for both specificities. Evidence that the same T cell receptor molecule is involved in both interactions, was provided by the fact that a monoclonal antibody specific for the receptor of a T cell clone blocked antigen specific activation as well as activation by an alloantigen [Kaye & Janeway, 1984].
The fact that the recognition of allogeneic MHC determinants seems to be unrestricted is unique. This means that self MHC molecules need not be recognized when a cell responds to an allogeneic MHC antigen. For example, cytotoxic T cell clones raised against products encoded by the I region are not restricted by polymorphic determinants of K or D region products [Klein et al, 1977]. This observation illustrates another peculiarity of alloreactive cells. In general, it can be said that $CD8^+$, cytotoxic T cells recognize class I alloantigens and $CD4^+$, helper T cells recognize class II alloantigens [Swain, 1983]. But in contrast to the situation with antigen specific, MHC restricted T cells, the correlation between phenotype (CD4 or CD8) and specificity (class I or class II) is weaker for alloreactive T cells [Vidovic et al, 1981; Miller & Stutman, 1982; Flomenberg et al, 1983; Spits et al, 1983; Haas & von Boehmer, 1984; Pierres et al, 1984; Shinohara & Kojima, 1984].
The unrestricted recognition of alloantigen mentioned above has mainly been demonstrated using cultured, clonal populations of T lymphocytes. *In vivo* experiments, however, suggest that the expression of a cellular immune response against an alloantigen may be a restricted phenomenon [Bianchi et al, 1984]. Two models that might explain the puzzling phenomenon of alloreactivity have been proposed. Alloreactivity has been described as a special case of antigen recognition, in which alloantigen substitutes for self MHC and antigen.
Alternatively, alloreactivity might be recognition of self determinants restricted by a foreign MHC molecule [Matzinger & Bevan, 1977; Zinkernagel & Doherty, 1979].
In the first case, the binding site (or part of it) on the T cell receptor that normally binds to antigen, would be occupied by a part of the allogeneic MHC molecule that differs from the

self MHC molecule. The second hypothesis assumes that a multitude of self determinants (peptides) are shared between individuals that have different MHC haplotypes. Although an animal is tolerant to its own tissue constituents in the context of its own MHC, it may well be that the same constituents in the context of another MHC may be seen as 'foreign'. It has indeed been demonstrated that tolerance for minor histocompatibility antigens is H-2 restricted [Matzinger et al, 1984; Rammensee & Bevan, 1984].

8. THE T CELL ANTIGEN RECEPTOR

Each individual T lymphocyte expresses a unique receptor molecule. To obtain sufficient homogeneous material for the characterization and isolation of the receptor, cloned T lymphocyte populations should be used. This can be achieved by using either transformed or long term cultured and subsequently cloned T cells.
The discovery that medium conditioned by phytohemagglutinin stimulated lymphocytes is capable of selectively stimulating the growth of T lymphocytes [Morgan et al, 1976] has offered the possibility of cloning functional T lymphocytes [Fathman & Hengartner, 1984; von Boehmer et al, 1979; Hengartner & Fathman, 1981; von Boehmer & Haas, 1981; Schreier et al, 1982]. Helper and cytotoxic T lymphocyte clones of murine or human origin have been produced with specificities for soluble antigen, viral antigens, or alloantigens. The growth factor that is responsible for this effect is interleukin 2 (IL-2). This molecule and the receptor for it on the cell membrane have now been extensively characterized and the respective genes have been cloned [Smith, 1984; Shimizu et al, 1986].

8.1. Characterization of the protein

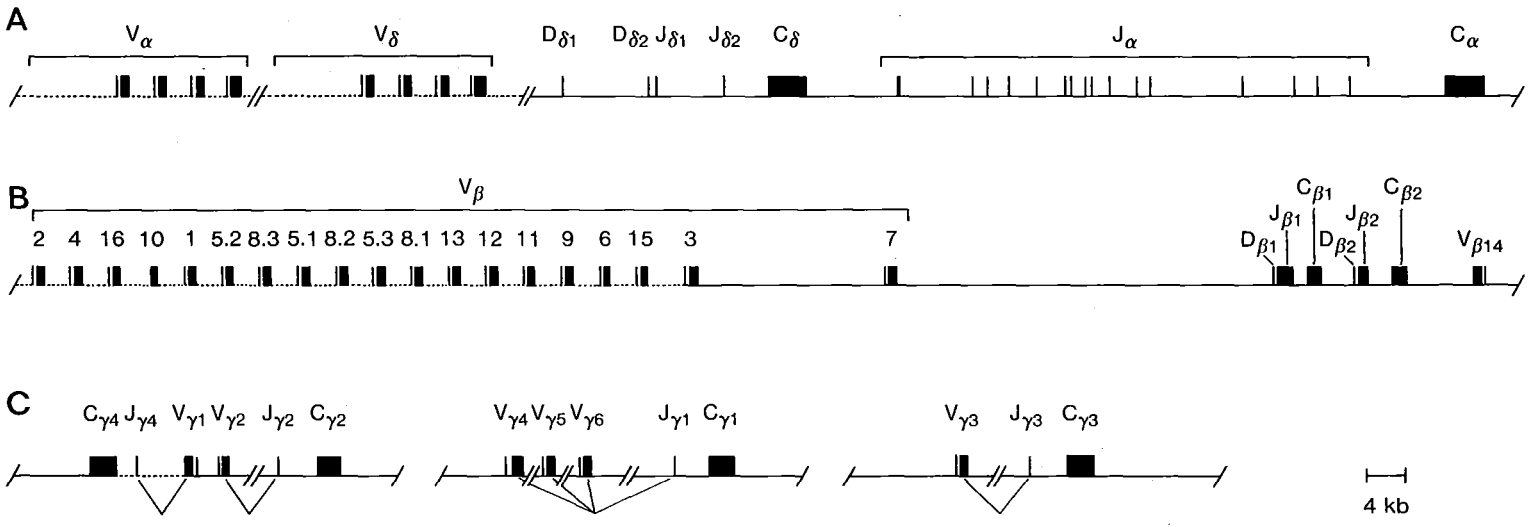
In order to identify the surface structure (Ti) that is responsible for antigen recognition, monoclonal populations of T cells have been used to generate antibodies against clone specific structures. The first report [Allison et al, 1982] describes the isolation of a T cell tumor specific monoclonal antibody which precipitates a disulfide-bound heterodimer composed of subunits of 39 kD and 41 kD. The presence of this antigen was not demonstrated in thymus, spleen or lymph node cells. Subsequently, a monoclonal antibody has been produced that specifically blocked the antigen specific, MHC restricted activation of a particular T cell hybridoma [Haskins et al, 1983], but not the activation of a number of closely related hybridomas. This antibody, as well as others, also recognized a 90 kD dimer on the surface of T cells [Haskins et al, 1983; Meuer et al, 1983a; Kaye et al, 1983; Samelson et al, 1983]. Precipitation of the 90 kD heterodimer from different T cell clones and subsequent peptide mapping revealed that, although certain peptides were shared, variable peptides existed as well [Acuto et al, 1983; Kappler et al, 1983b; McIntyre & Allison, 1983]. These data suggested that the T cell receptor contains

constant and variable parts and resembles the immunoglobulin molecule.
Another observation was that antibodies specific for T3 inhibit cell mediated cytotoxicity [Reinherz et al, 1982]. In humans, the T3 antigen [Kung et al, 1979; Oettgen & Terhorst, 1987] is present on peripheral T cells, and is acquired during thymic differentiation. The 90 kD clonotypic heterodimer, Ti, has been demonstrated to modulate with the T3 antigen [Meuer et al, 1983a]. Also, T3 and Ti antigens are expressed simultaneously and at equal density on the cell surface (30.000-40.000 molecules per cell) [Meuer et al, 1983b].
These observations led to the hypothesis that the 90 kD heterodimer, Ti, together with the T3 antigen represent the T cell antigen receptor. The two chains composing Ti have been designated α and β chain. The α and β chain are bound together by an extracellular disulfide bridge that is in close proximity to the membrane [Kronenberg et al, 1986]. Each of the two chains consists of two domains that resemble the structure of the immunoglobulin domains. Therefore, the T cell receptor genes belong to the immunoglobulin gene superfamily (figure 3). The distal domain is encoded by variable gene segments and the proximal one is encoded by a constant gene segment. Each domain has an intradomain disulfide bridge [Kronenberg et al, 1986]. While in humans the two chains have a distinct molecular weight (α:43-49 kD, β:38-44 kD,[Meuer et al, 1983a]) in mice both chains have a molecular weight of about 40-44 and can only be distinguished by isoelectric focussing [Kappler et al, 1983a; McIntyre & Allison, 1983; Samelson & Schwartz, 1984].
The α chain has an isoelectric point of about 5.5 and the β chain is less acidic (pI$\approx$7.0) [Samelson & Schwartz, 1984]. Both chains contain several N-linked carbohydrate chains that are attached to a peptide backbone of 28-32 kD [McIntyre & Allison, 1984; Samelson & Schwartz, 1984].

8.2. Isolation of the genes

Two different approaches have been followed to isolate the genes that code for the two chains of the T cell receptor.
For the first approach, it was assumed that DNA coding for the T cell receptor, which had been shown to contain constant and variable peptides [Acuto et al, 1983; Kappler et al, 1983b; McIntyre & Allison, 1983], would rearrange to generate its diversity just like immunoglobulin genes in B cells [Tonegawa, 1983]. A library of T cell specific cDNA clones was produced by subtracting T and B cell mRNA [Sim et al, 1984; Saito et al, 1984a; Hedrick et al, 1984a; Yanagi et al, 1984]. Some of the clones revealed rearrangements of DNA in functional T cell clones [Hedrick et al, 1984a; Toyonaga et al, 1984] and showed extensive homology to immunoglobulin genes [Yanagi et al, 1984; Hedrick et al, 1984b].
The second method that has been used, was to determine the amino acid sequence of the proteins that were precipitated with clone-specific monoclonal antibodies. NH2-terminal sequence data [Acuto et al, 1984] as well as sequence data of tryptic peptides [Hannum et al, 1984; Sim et al, 1984] have been

GENOMIC ORGANIZATION OF MURINE T CELL RECEPTOR GENES



collected. These peptide data confirmed that the genes isolated by the first approach [Hedrick et al, 1984a; Yanagi et al, 1984; Sim et al, 1984; Saito et al, 1984a] were indeed coding for the 90 kD α/β heterodimer. Independently, oligonucleotide families derived from these peptide sequences have been used to screen cDNA libraries and to isolate full length clones that code for the α chain [Sim et al, 1984].
The initial isolation of the genes coding for the T cell receptor has led to an overwhelming quantity of data. The genomic organization of the T cell receptor genes, shown in figure 6, and the mechanism of rearrangement (figure 7) has proved to be very similar to that of the immunoglobulin genes [Tonegawa, 1983; Yancopoulos & Alt, 1986]. One variable, diversity and joining gene segment are joined during differentiation of immature thymocytes. This will result in a unique variable gene in each T cell (figure 7). As the mRNA is transcribed and processed, the introns between joining gene segment and the constant gene will be removed by splicing and a functional mRNA will result. This RNA will be translated to give a protein consisting of a variable and a constant part.

8.3. The alpha chain

The genes encoding the α-chain of the T cell receptor are located on chromosome 14 in mouse [Dembic et al, 1985] and man [Collins et al, 1985]. The locus consists of one constant (C) gene segment consisting of 4 exons (figure 5) [Hayday et al, 1985a; Yoshikai et al, 1985]. The joining (J) gene segments are encoded by the DNA that is located 5' to the constant region. An estimated number of 50 [Kronenberg et al, 1986] J segments is spread over 50-60 kb [Hayday et al, 1985a; Yoshikai et al, 1985; Winoto et al, 1985]. At a distance of 70 kb 5' to the C_α gene, the genes coding for the δ protein are located (see below). The variable (V) genes are located at an unknown distance to the J-C cluster (figure 5). Estimations about the

Figure 6. The genomic organization of the murine T cell receptor genes α, β, γ and δ.
A. The α and δ genes. Immediately 5' to the constant gene (C_α) of the α chain are 50-100 joining (J_α) gene segments (not all are indicated). The variable gene segments are located at an unknown distance. In between the V_α and J_α genes, the δ genes are located. Several V_δ, 2 J_δ, $2D_\delta$ and 1 C_δ gene segments have been identified. **B.** The β genes have two clusters, each containing 1 D_β, 7 J_β and 1 C_β genes. Most V_β genes are located upstream to the constant genes and have been mapped. In this figure only relative positions are indicated. $V_{\beta 5.3}$ is a pseudogene. The $V_{\beta 14}$ gene is located 3' to the constant genes. **C.** Four constant γ genes have been identified each closely linked with a joining gene segment. Only 6 variable gene segments have been found until to date and rearrangements that have been detected are indicated. The $C_{\gamma 3}$ is a pseudogene and not present in C57BL/10 mice.

number of V_α genes are in the order of one hundred or more [Becker et al, 1985; Kronenberg et al, 1986]. The variable gene segments are grouped in gene families according to their degree of homology [Becker et al, 1985; Arden et al, 1985]. Each family contains 1-10 members. Numbers of one family are more than 75% homologous to each other at the nucleic acid level. Differences between various V_α families are greater than the differences observed between various V gene families of the immunoglobulin genes.
The variable gene segments of the α and the β chain genes are encoded in two exons. The first exon encodes 5' untranslated sequence and the larger part of the leader peptide [Siu et al, 1984b; Chien et al, 1984]. The second exon codes for the rest of the leader plus the complete variable gene segment. The variable gene segments have some residues that are conserved in all V genes including immunoglobulin genes [Hedrick et al, 1984b; Bougueleret & Claverie, 1987]. For example, there are two cysteine residues (60 - 70 residues apart) that are supposed to form an intradomain disulfide bridge.

8.4. The beta chain

There are two constant β-chain genes. They are located on chromosome 6 in the mouse [Caccia et al, 1984] and on chromosome 7 in humans [Morton et al, 1985]. The two genes are

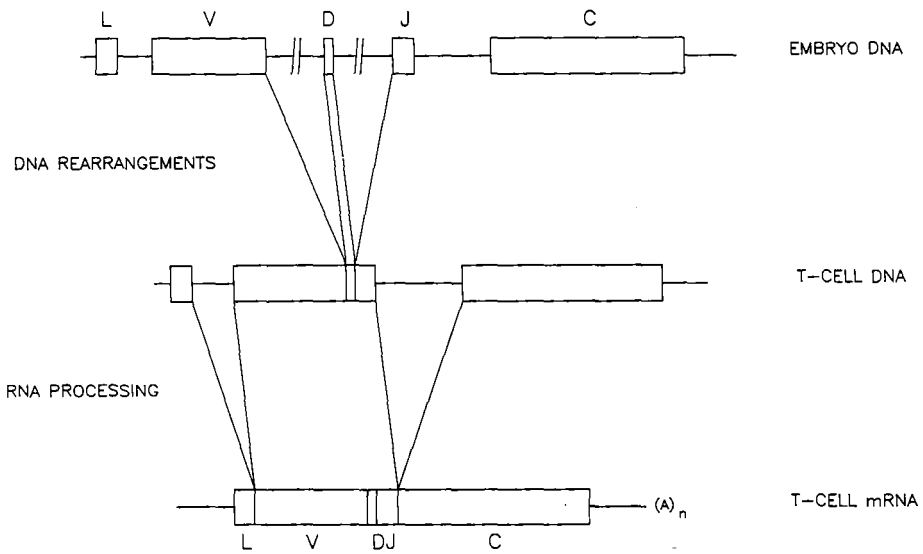


Figure 7. The DNA rearrangements and subsequent mRNA processing events necessary for the expression of one of the chains of a functional T cell receptor protein. L is leader, V is variable gene segment, D is diversity and J is joining gene segment. C is the constant gene segment. Adapted from Hood et al. 1984.

organized in 4 exons and very similar, except in the 3' portion that encodes the transmembrane and the cytoplasmic portions of the molecule [Chien et al, 1984; Malissen et al, 1984; Gascoigne et al, 1984]. There are 16 nucleotide changes which only result in 4 amino acid changes [Gascoigne et al, 1984]. The human C_β genes are similarly organized and this indicates that gene duplication probably has occurred before the divergence of the species [Gascoigne et al, 1984; Tunnacliffe et al, 1985; Marrack & Kappler, 1986]. Both C_β genes are preceded at the 5' side by a cluster of seven J gene segments (figure 5) [Gascoigne et al, 1984]. The J_β gene segments are grouped within a few hundred nucleotides and each cluster has one pseudogene. In contrast to the α chain, the β chain can use a D gene segment to enlarge its diversity [Siu et al, 1984a]. At present, two D_β gene segments have been identified, each located 5' to a J_β gene cluster.
In mice, the variable gene segments of the β-chain are not as numerous ($\approx$ 20) as the V_α gene segments [Patten et al, 1984; Barth et al, 1985]. Most of them are organized in one-member families, that are less than 50% homologous to each other at the protein level [Barth et al, 1985].
Comparison of 60 murine and human V_β sequences [Bougueleret & Claverie, 1987] revealed a major site of variability at the V-D junction, which suggests that this is a site of special importance for the recognition of antigen. Recently, an almost complete physical map of the β locus has been published, that links variable and constant genes [Lai et al, 1987].

8.5. Generation of a functional T cell receptor gene

The diversity of T cell receptors is a consequence of somatic recombination of variable, diversity, joining and constant gene segments (figure 7) [Chien et al, 1984; Kronenberg et al, 1986] and is similar to somatic recombination as described for immunoglobulin genes [Tonegawa, 1983; Yancopoulos & Alt, 1986]. The gene segments that are involved in the rearrangement process are flanked by specific recognition sequences (figure 8). These recognition sequences consist of a palindromic heptamer sequence 5'-CACAGTG-3' and a nonamer sequence that is relatively A/T rich. These two sequences are separated by 12 or 23 non-conserved nucleotides.
According to the rules for recombination [Tonegawa, 1983; Yancopoulos & Alt, 1986] two gene segments can be joined if the spacer sequence in one recognition sequence is 12 nucleotides and that of the second 23. In the recognition sequence directly 3' to the variable gene segments the spacer sequence is 23 nucleotides long (about 2 turns of the DNA helix) and 5' to the J segments the spacer is only 12 nucleotides in length (1 turn of the helix) [Chien et al, 1984; Siu et al, 1984b]. Thus, V and J gene segments can be joined directly. For the β-chain genes there is the optional use of the D gene segments, that can be included in the rearrangement [Siu et al, 1984b; Kavaler et al, 1984; Siu et al, 1984a]. The D gene segment has a one-turn spacer on its 5' side and a two-turn sequence on its 3' side. Theoretically, this offers the possibility of

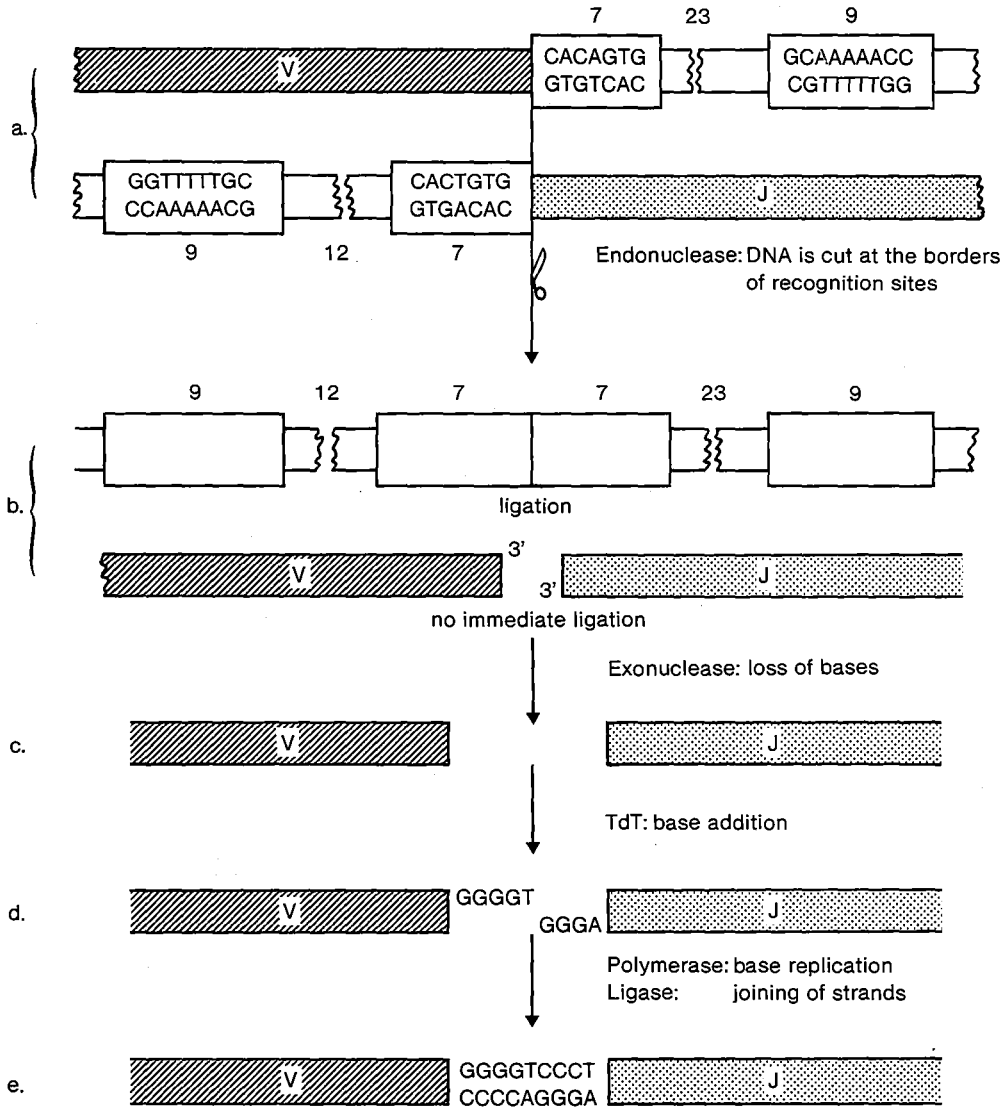


Figure 8. A model for the rearrangement events that are involved in the joining of a variable (V) and a joining (J) gene segment. The model is analogous to the model described for the immunoglobulin genes [Yancopoulos & Alt, 1986]. See text for explanation.

including 0, 1 or 2 D gene segments in a functional receptor. The model for joining coding sequences of a variable and a joining gene segment is shown in figure 8. Two heptamer-spacer-nonamer sequences of the V and the J will combine and this will bring the V and the J immediately adjacent to each other. Both DNA strands will now be cut and religated to form a V-J joining product and a reciprocal heptamer-heptamer joining product. The validity of this model has been supported by the finding that circular DNA has been isolated from thymocyte nuclei, that contained such heptamer-heptamer joining sequences [Okazaki et al, 1987]. When the DNA strands are cut, exonucleases may remove some of the terminal nucleotides of either the V or the J. In addition, random nucleotides can be added to the 3' termini. This is thought to be achieved by the enzyme terminal deoxynucleotidyl transferase (TdT) [Desiderio et al, 1984]. Finally, the DNA strands are filled in by DNA polymerase and religated.
The resulting rearranged T cell receptor gene is a functional one if an open reading frame is maintained that joins V and J gene segments properly.
The model for rearrangement that has been discussed above, is the excision (deletion) model (figure 9.a). However, in some cases alternative mechanisms may be employed. One of the V_β gene segments ($V_{\beta 14}$, figure 5) is located 3' to the constant gene cluster in an opposite transcriptional orientation. A functional rearrangement can only be explained by assuming that inversion of a chromosomal segment has occured (figure 9.b) [Kronenberg et al, 1986; Malissen et al, 1986]. A third mechanism of rearrangement is sister chromatide exchange (figure 9.c) [Kronenberg et al, 1985; Kronenberg et al, 1986]. Evidence for this model comes from the observation that DNA, which should have been deleted in the case of excision, is retained in the genome of some T cell clones.
The genes coding for the T cell receptor proteins appear to be involved in chromosomal translocations [Denny et al, 1986; Reynolds et al, 1987], just like the immunoglobulin genes [Tsujimoto et al, 1984]. The finding that in some of these translocations [Denny et al, 1986; Baer et al, 1987] rearrangement of immunoglobulin V genes to J_α genes has occurred, illustrates that the mechanism of rearrangement in B and T cells is very similar. Although endogenous T cell receptor genes are in general not rearranged in B cells [Traunecker et al, 1986], T cell receptor genes do recombine, when introduced in pre-B cells [Yancopoulos et al, 1986].

8.6. Mechanisms of diversification

The molecular basis of diversification involved in the generation of the T cell receptor repertoire is summarized below,
1. The existence of different germline gene segments (α: 100 V and 50 J gene segments; β: 30 V, 2 D and 12 J gene segments)..
2. Combination of different gene segments. The same germline V gene segment can combine with different J gene segments. The β chain has the additional possibility to introduce a D gene

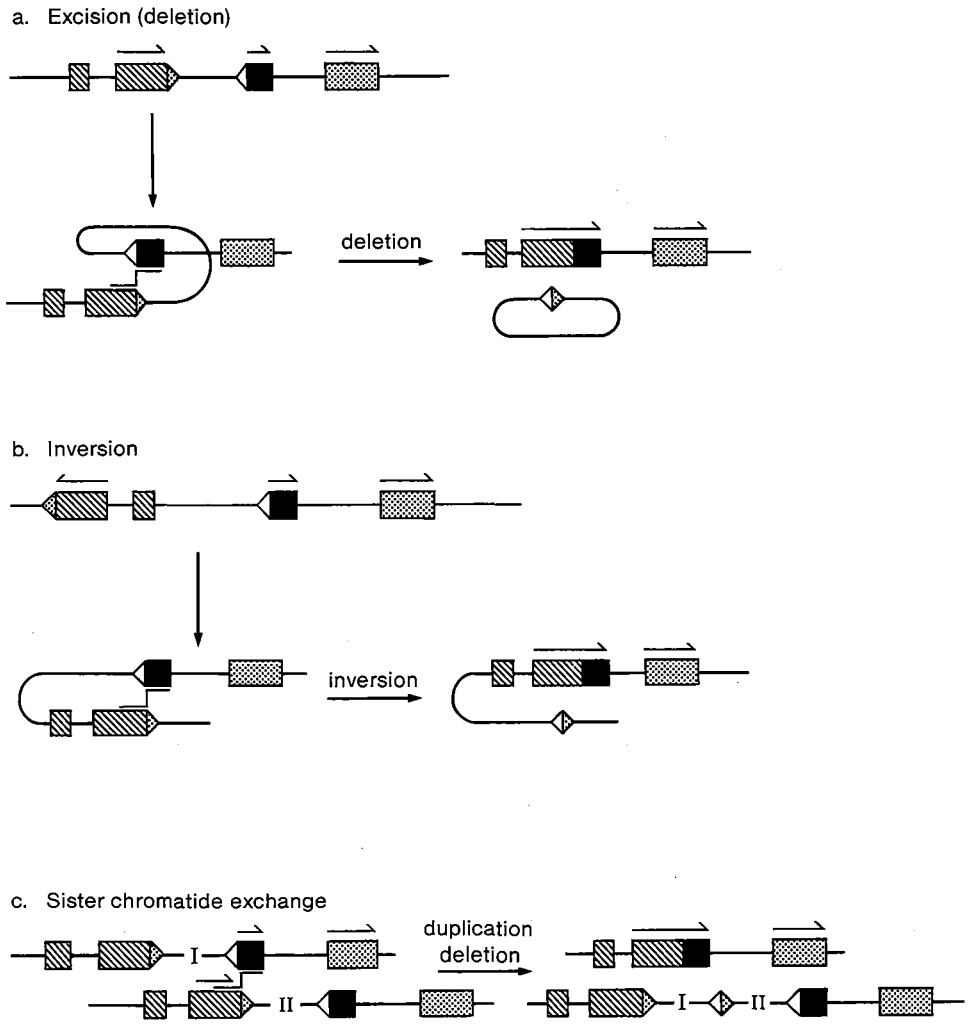


Figure 9. Three possible mechanisms for receptor gene rearrangements. Rectangular boxes are gene segments to be joined and triangles are the respective recognition sequences. Arrows indicate transcriptional orientation.
a. Deletion (excision). This mechanism is probably used in most cases. The products are a rearranged gene and an excised loop. **b.** Inversion. Gene segments in opposed transcriptional orientation are joined by inversion. In this case there is no loss of DNA from the genome. **c.** Sister chromatide exchange. A DNA fragment is excised from one chromatide and inserted in the other. The relative use of this mechanism is not clear yet. Adapted from Hood et al. 1984.

segment. In contrast to the introduction of a D gene segment in the gene coding for the heavy chain of the immunoglobulins [Tonegawa, 1983], the use of a D gene segment in the production of a functional β chain gene is optional. An important additional contribution to diversification comes from the combination of α and β chains, although it has not yet been established that every α chain can combine with every β chain.
3. Junctional variability. Imprecise joining of two (V and J) gene segments increases variability. At the site of recombination germline nucleotides may be deleted or inserted (N-region diversity). If the D gene segment is included in the β-chain, this segment may be read in three different reading frames [Barth et al, 1985; Goverman et al, 1985].
Somatic hypermutation, as it is observed in immunoglobulin genes [Tonegawa, 1983; Yancopoulos & Alt, 1986], does not occur as extensively in T cell receptor genes, if at all.

8.7. A second T cell receptor?

The search for T cell specific rearranging genes has led to the identification of a gene that did not encode any known protein. Initially mistaken for the α chain [Saito et al, 1984b], it was later named the γ chain. Like α and β chain genes, functional γ chain genes are assembled from variable, joining and constant gene segments. The diversity of receptors that can be generated is limited compared with that of α and β chains [Hayday et al, 1985b; Kranz et al, 1985], due to a smaller number of variable and joining gene segments. Variability in the receptor repertoire is largely due to N region diversity [Kranz et al, 1985]. In mice, four constant gene segments exist, although one of these genes is a pseudogene and cannot be functionally expressed (figure 6) [Hayday et al, 1985b; Iwamoto et al, 1986]. Each constant gene is accompanied by a joining gene segment and one or a few variable genes [Iwamoto et al, 1986; Garman et al, 1986; Heilig & Tonegawa, 1986]. Positive identification of the protein encoded by the γ gene has only been possible via the generation of antiserum specific for synthetic peptides predicted by the nucleotide sequence [Lew et al, 1986; Brenner et al, 1986]. The γ protein has been shown to be expressed on the surface of a subpopulation of T cells in association with the T3 antigen [Lew et al, 1986; Brenner et al, 1986; Bank et al, 1986].
Usually, γ is expressed as a heterodimer with a fourth T cell receptor protein, the δ chain [Brenner et al, 1986]. The genes coding for the δ protein have recently been cloned [Chien et al, 1987a; Born et al, 1987; Band et al, 1987; Hata et al, 1987]. The δ genes are located just 5' to the J_α genes (figure 6), and rearrange very early in ontogeny, probably around day 15 of gestation in mice [Lindsten et al, 1987; Chien et al, 1987b]. As a result of its localization, δ chain genes are generally deleted after V_α-J_α joining has occurred in mature α/β T cells [Lindsten et al, 1987]. The δ chain has its own set of D and J gene segments [Chien et al, 1987b], but until now it is unclear whether separate V_δ genes exist or V_α genes are used.

The γ/δ receptor is present on T cells that do express T3 but do not react with the WT31 monoclonal antibody, which detects α/β dimers [Tax et al, 1983; Brenner et al, 1986]. The γ/δ cells represent therefore a separate population of T cells. These cells are present in the thymus in low frequencies [Lew et al, 1986; Bank et al, 1986] and as a small subpopulation (1-5%) of peripheral T cells [Borst et al, 1987; Brenner et al, 1987]. The γ/δ cells have been suggested to be cytotoxic cells with a broad spectrum of MHC unrestricted specificity [Borst et al, 1987], but it has also been shown that some MHC linked specificity exists in the absence of exogenous IL-2 [Matis et al, 1987]. Cells with the γ/δ receptor seem to be relatively more frequent in situations of immunodeficiency, for example in nude mice [Yoshikai et al, 1986] and immunodeficiency patients [Brenner et al, 1986]. The function of γ/δ cells in the immune system has not been clarified yet. It has been postulated that these cells have a distinct function (e.g. defense against tumor cells) or represent a phylogenetically older system.

9. INTRODUCTION TO THE EXPERIMENTAL WORK

In this thesis the antigen recognition by T cells and in particular the recognition of alloantigen has been studied. The aim of the study was to gain more information about the structure of the T cell antigen receptor on one hand and its specificity on the other. In order to study T cell receptors in more detail, clonal populations of T lymphocytes have been produced and characterized.
In Chapter 2, a panel of cytotoxic T cells specific for lymphocytic choriomeningitis virus (LCMV) has been generated. The clones have been derived from H-2^b and H-2^k mice as well as from allogeneic bone marrow chimeras (H-2^k → H-2^b and H-2^b → H-2^k) and recognize LCMV in the context of H-$2D^b$ or H-$2K^k$. Differences in the fine specificities for virus determinants or MHC molecules have been investigated by using recombinant virus strains and inhibition of cytotoxicity by anti-MHC monoclonal antibodies, respectively. The specificity of the clones derived from the chimeric animals seemed to be different from that of the clones from normal mice.
In Chapter 3, the production and characterization of a panel of alloreactive cytotoxic T cell clones is described. The objective was to possess a number of distinct clones that use distinct receptors to recognize one and the same alloantigen. The alloantigen of choice was the murine class I antigen, H-$2D^b$, because several crucial reagents were available. Comparison of the receptors of these clones may reveal characteristics that are required for the recognition of H-$2D^b$. In order to establish that the different clones employ different receptors, several methods have been used to distinguish differences in fine specificity.
First, the clones have been analyzed with a monoclonal antibody, 44-22-1, specific for the receptor of the previously described H-$2D^b$ specific clone, 3F9 [Acha-Orbea et al, 1985]. Only one of the clones, clone 653, was inhibited by this

antibody.
Second, the clones have been analyzed for coincidental cross-reactivities with third party stimulator cells. When cross-reactivities are found for some of the cells but not for others, this indicates differences between the receptors. Only clone 433 has been found to cross-react with H-2^k target cells. While the original specificity of the clone is for a class I antigen (H-$2D^b$), it appeared to recognize a class II antigen (I-E^k) as well. Since this had not been reported before, the cross-reactivity has been extensively characterized and is described in Chapter 4.
Third, it was established that the clones have been derived from different precursors by Southern blot analysis of the genes coding for the β chain of the antigen receptor. This implies that the functional β chains in these clones are probably different.
The virus specific as well as the alloreactive clones that have been studied in detail, provide a source of well-defined biological material for further study.
The availability of two clones, 3F9 and 653, that are inhibited by the same anti-receptor antibody, 44-22-1, offered the possibility to characterize the determinant recognized by this antibody more precisely. The structure of the receptor of clone 3F9 has been described elsewhere [Rupp et al, 1985; Rupp et al, 1987] and the structure of the receptor of 653 is described in Chapter 5. Because the organization of the T cell receptor genes is well known, the structure of the protein can easily be deduced from the gene structure. Therefore, the genes coding for both chains of the receptor of clone 653 were isolated. For this purpose a cDNA library has been produced and the mRNA molecules coding for the α and β chain were cloned and sequenced. The primary structure of the α and β chain of clone 653 is presented in Chapter 5. The comparison of this receptor with the receptor structure of clone 3F9 revealed that antibody 44-22-1, which inhibits the activity of both clones, might recognize the $V_{\beta 6}$ gene segment. This hypothesis is further supported by the analysis of additional clones with known receptor structure (Chapter 6). It was established that T cell receptors were only recognized by the 44-22-1 antibody, if they functionally expressed the $V_{\beta 6}$ gene segment. $V_{\beta 6}$ positive clones could be stimulated or inhibited with the 44-22-1 antibody, depending on the presentation of the antibody.

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CHAPTER 2

CHARACTERIZATION OF VIRUS-SPECIFIC CYTOTOXIC T CELL CLONES FROM ALLOGENEIC BONE MARROW CHIMERAS

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We established several H-2-restricted lymphocytic choriomeningitis virus (LCMV)-specific cytotoxic T cell clones from spleens of virus-primed C57BL/6 or C57BL/10 ($H\text{-}2^b$) and B10.BR ($H\text{-}2^k$) mice and from allogeneic C57BL/10→B10.BR and B10.BR→C57BL/10 bone marrow chimeras. Two T cell clones of $H\text{-}2^b$ origin and restricted to $H\text{-}2^b$, 3 of $H\text{-}2^k$ origin and restricted to $H\text{-}2^k$ were compared with two clones each derived from the two types of chimeras. Their surface phenotype was found to be Lyt-2^+, L3/T4^- and KJ16-133^+ (2 of 9). Clones from chimeras expressed bone marrow donor H-2 and are restricted to the recipient H-2.
$H\text{-}2^k$-restricted clones were all specific for K^k whereas all $H\text{-}2^b$-restricted clones were specific for D^b. These restriction specificities could be further defined by the blocking activity of various monoclonal anti-H-2 antibodies. Interestingly the anti-H-$2D^b$ antibodies blocked the restricted virus-specific killing activity of the clones derived B10.BR→C57BL/10 chimeras much more effectively than the activity of the clones derived from conventional $H\text{-}2^b$ mice.
The various clones differed with respect to their fine specificity for LCMV strains. The 3 clones of conventional B10.BR origin only recognized LCMV-WE but not LCMV-Armstrong, Aggressive or Docile; $H\text{-}2^b$-restricted conventional clones recognized target cells infected with all LCMV strains except LCMV-UBC-Docile; the T cell clones from the bone marrow chimeras recognized with one exception all LCMV strains tested.

1 Introduction

Virus-specific cytotoxic T cells (CTL) play a crucial role in the control and elimination of viral infections [1–4] including lymphocytic choriomeningitis virus (LCMV) infection. Since LCMV a RNA virus of the Arena virus group, usually infects cells without causing cell destruction [5–7], this model of infection in mice can illustrate both the protective and also detrimental effects of T cell-mediated immuntiy. Studies have shown that LCMV-specific CTL and, more recently, cloned LCMV-specific T cells, dependent upon site of infection and time of adoptive transfer, may exert antiviral protection or, alternatively, death by causing immunopathologically mediated choriomeningitis [8–11]. CTL express a dual specificity for the virus and for self cell surface determinants encoded by the K and D loci of the H-2 [9, 12].

The corresponding receptors on T cells apparently consist of two glycoprotein chains, α and β, both with a variable and constant region [13–16]. The function of a third set of genes γ, that are rearranged in T cells is not yet understood [17]. Despite the fact that the primary structure of the α, β heterodimer from several antigen-specific T cells is known, it remains unclear how T cells recognize antigen and self.

We have attempted to establish a panel of cloned LCMV-specific T cells as starting material for the molecular or immunological analysis of the T cell receptor(s). This report describes the isolation and functional characterization of cloned virus-specific cytotoxic T cells derived from H-2-congenic B10 mice of $H\text{-}2^b$ and $H\text{-}2^k$ type and from H-2-incompatible irradiation bone marrow (BM) chimeras made with the same B10 strains.

2 Materials and methods

2.1 Mice

Mice were purchased from the Institut für Zuchthygiene, Abteilung Labortierkunde, Tierspital Zürich (C57BL/6J, C57BL/10J, B10.BR, B10.D2) and from Olac, Bicester, Oxon, GB (B10.HTG, B10.A(5R), B10.A(2R), B10.A, C3H.OH). Specific pathogen-free (SPF) mice were used to construct the BM chimeras and were bred from the colonies at the animal production facility of the National Institute of Radiological Sciences, Chiba, Japan, as described in [19]. Male or female mice were used at 2–6 months of age.

2.2 Chimeras

Allogeneic irradiation BM chimeras were prepared by lethally irradiating B10.BR/Sg Sn ($H\text{-}2^k$) mice and reconstituting them

with T cell-depleted bone marrow cells from C57BL/10Sn ($H\text{-}2^b$) ($H\text{-}2^b \rightarrow H\text{-}2^k$ chimeras) or *vice versa* ($H\text{-}2^k \rightarrow H\text{-}2^b$ chimeras). The methods employed have been described and analyzed in great detail previously [18–21]. Chimeras had no detectable lymphocytes of host origin when assayed by antibody plus complement treatment.

2.3 LCMV

The WE strain and the Armstrong strain of LCMV had been obtained from Dr. F. Lehmann-Grube, Hamburg, FRG, and Dr. M. B. A. Oldstone, Scripps Clinic and Research Foundation, La Jolla, CA, respectively. The two plaque variants of UBC strain Aggressive (LCMV-AGG) and Docile (LCMV-DOC) had been obtained from Prof. C. Pfau, Rensselaer Polytechnical Institute, Troy, NY [22]. Stocks were diluted in medium containing 5% heat-inactivated fetal calf serum (FCS) and were stored in multiple aliquots at –70 °C.

2.4 Media and incubation conditions

Spleen cells (SC) were prepared in Hanks' balanced salt solution (BSS). T cells were cultured in Iscove's modified Dulbecco's medium (IMDM; KC Biol., Lenexa, KS) supplemented with 3024 g/l $NaHCO_3$ (Merck, Darmstadt, FRG), 19 mM L-glutamine (KC), 5×10^{-5} M 2-mercaptoethanol (Merck), 10% heat-inactivated FCS and 10% rat concanavalin A (Con A) supernatant. Rat Con A supernatant was prepared as described [23].

2.5 Monoclonal antibodies (mAb)

Expression of cell surface antigens and blocking of cell-mediated cytotoxicity was studied using mAb produced by the following B cell hybridomas: B8-24-3 (anti-K^b) [24], BY-3 (anti-D^b) [25], 11-4-1 (anti-K^k) [26], 53-6-72.4 (anti-Lyt-2) [27], H129.19 (anti-L3/T4) [28], E.3.25 (anti-K^k,K^b) [29]. Hybridomas 172-93.2, H166-32.1, T21.440, T21.460, T17.433, K15-25, H141-31, H141-51, B22-249 (all anti-D^b) and H100-27/55, H100-5/28, H116-27/7 (all anti-K^k) were kindly provided by Dr. G. Hämmerling, Deutsches Krebsforschungszentrum, Heidelberg, FRG. mAb 28-8-65 (anti-D^b), 3.83, 15-3-1, 16-3-1M, 12-2-2, 15-1-5 (all anti-K^k) and 15-5-5 (anti-D^k) [30, 31] were a gift from Dr. D. Sachs, National Cancer Institute, NIH, Bethesda, MD. The mAb KJ16-133 [14] was a generous gift of Drs. Kappler and Marrack, National Jewish Hospital, Denver, CO.

2.6 Generation of primary CTL

Primary LCMV-immune CTL were induced *in vivo* by infecting mice i.v. with about 10^3 PFU LCMV-WE per animal at day –8; mice were killed and SC suspension were prepared as described in detail elsewhere [11].

2.7 LCMV-infected macrophages (MΦ) used as stimulator and target cells

Mice were injected with 1 ml thioglycolate (BBL®, Becton Dickinson, Cockeysville, MD) i.p. on day –6 and infected i.p. with 10^4 PFU of LCMV-WE on day –4. MΦ were harvested on day 0. MΦ used for *in vitro* restimulation of the cultures were irradiated with 2000 rds.

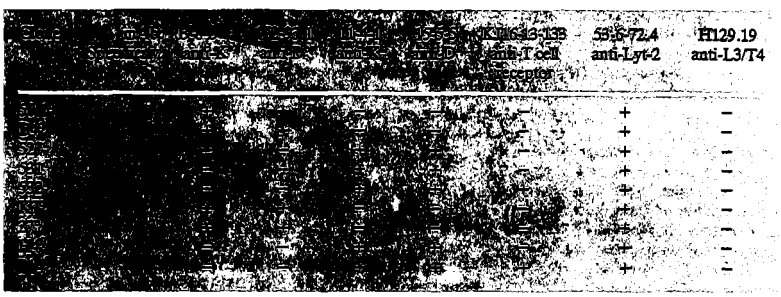
2.8 LCMV-specific CTL clones

The CTL clones studied in this report were basically generated and maintained as described previously [11, 32–34]. Briefly, responder spleen cells (2×10^6/ml) from normal or allogeneic BM chimeras, primed 2–10 weeks earlier i.v. with either LCMV-WE (about 10^5 PFU) or LCMV-Arm (about 10^5 PFU) were restimulated *in vitro* with syngeneic *in vivo* infected MΦ (1×10^5/ml, see Sect. 2.7). Responder SC from chimeras were restimulated with infected MΦ of host strain origin. Rat Con A supernatant (10%, v/v) was added to the medium after 1 week of culture. The cultures were restimulated at weekly intervals with *in vivo* infected MΦ (1×10^5–2×10^5/ml). After 1 to 3 months, cultures which showed high lytic activities were cloned under conditions of limiting dilution. The clones H4, P14, K7, 27 and 50.1 were derived from mice primed with LCMV-Arm, whereas clones B4, 527, 531 and 532 come from mice primed with LCMV-WE. All the clones were stimulated *in vitro* with LCMV-WE-infected MΦ.

2.9 Cytotoxicity assay

The following continuous cell lines maintained *in vitro* were used as target cells: L929 (fibroblasts), MC57G (methylcholantrene-induced fibrosarcoma), 4R (SV-40-transformed fibroblastic cells). The cell lines were infected with LCMV-WE (0.1 PFU/cell), LCMV-Arm (0.1 PFU/cell), UBC-DOC (0.01 PFU/cell), UBC-AGG (0.01 PFU/cell) for about 40 h. Target cells (5×10^6–10×10^6 from continuous cell lines were

Table 1. Cell surface markers of CTL clones[a)]



a) Cells were incubated with hybridoma supernatant of the indicated mAb, washed and labeled with FITC-goat anti-mouse IgG or with goat anti-rat IgG; after several washes the cells were analyzed by fluorescence microscopy.
b) C57BL/10 origin.
c) C57BL/6 origin.
d) B10.BR origin.
e) C57BL/10→B10.BR chimera origin.
f) B10.BR→C57BL/10 chimera origin.

labeled with 0.3 mCi = 11.1 MBq ^{51}Cr in 0.6 ml medium (5% FCS) at 37 °C in 15-ml plastic tubes (Falcon, Becton Dickinson, Oxnard, CA) for 1 h.

In vivo infected MΦ target cells (5×10^6–10×10^6, see Sect. 2.7) were labeled with 0.3 mCi ^{51}Cr in 0.6 ml medium (5% FCS) at 37 °C for 1 to 2 h in 50-ml plastic tubes (Falcon 2070). The labeled target cells (1×10^4) were mixed with the designated numbers of effector cells in 96-well microtiter plates. After centrifugation at 1000 rpm for 2 min the plates were incubated for 4 to 5 h at 37 °C in air containing 5% CO_2. Total ^{51}Cr release was measured by adding 0.1 ml of 1 N HCl to the target cells. In place of effectors 0.1 ml of medium were used to measure spontaneously released label. After the incubation period, supernatant was collected (75 µl) and radioactivity counted in a γ counter. The percentage of specific release was calculated by the formula:

$$\text{Specific cytotoxicity} = \frac{\text{cpm Exp. release} - \text{cpm spont. release}}{\text{cpm Total release} - \text{cpm spont. release}} \times 100.$$

Table 2. H-2 restriction of LCMV-specific CTL clones from LCMV-primed normal mice and allogeneic chimeras

Clones	% Specific ^{51}Cr release from target cells								
	Cell lines				MΦ				
	MC57G (H-2^b) LCMV[a]		L929 (H-2^k) LCMV[a]		C57BL/6 (H-2^b) LCMV[b]		B10.BR (H-2^k) LCMV[b]	B10.D2 (H-2^d) LCMV[b]	
	+	−	+	−	+	−	+	−	+
H4[c]	83[d]	70	12	nd[e]	81	0	0	1	0
	51	8	8		50	0	0	0	2
	28	4	3		28	1	1	2	3
	14	5	1		9	2	3	3	0
P14[c]	57	4	2	nd	68	2	1	1	2
	37	1	0		42	0	0	2	3
	21	2	1		20	2	0	1	0
	13	1	0		9	0	0	2	2
K7[f]	12	nd	79	11	1	2	59	0	1
	4		54	6	5	1	48	0	0
	0		37	2	0	0	35	2	1
	0		17	1	0	0	24	0	0
27[f]	4	nd	56	7	3	2	72	0	0
	2		47	5	1	0	53	0	1
	3		33	2	2	0	31	2	1
	1		9	1	1	0	16	0	2
50.1[g]	81	2	1	1	80	3	2	1	3
	78	2	0	1	62	0	0	3	2
	51	2	0	0	41	0	3	0	2
	37	3	0	1	60	2	2	1	0
B4[h]	95	9	10	nd	72	2	2	3	0
	78	5	5		41	1	3	0	1
	50	2	3		18	0	0	1	0
	23	0	0		7	0	0	0	1
527[f]	1	2	66	2	4	2	49	2	1
	0	2	54	2	3	5	42	0	6
	0	0	33	1	1	0	43	3	7
	1	0	26	0	0	1	31	0	2
531[f]	0	5	76	4	3	5	66	3	0
	0	1	70	0	2	0	52	2	0
	0	2	68	2	0	3	52	1	1
	2	0	59	3	1	0	41	0	2
532[f]	2	3	71	5	4	0	46	5	0
	1	4	70	0	2	3	51	2	0
	1	5	57	2	0	1	38	0	0
	0	2	46	0	0	0	22	0	0
8-day LCMV immune B10.D2 SC	nd	nd	nd	nd	nd	nd	10	0	20
Spontaneous release (4 h)	18	22	14	21	22	12	23	15	17

a) Infected for 2 days with LCMV-WE (0.1 PFU/cell).
b) Thioglycolate-activated *in vivo* infected peritoneal MΦ.
c) B10.BR→C57BL/10 chimera origin.
d) Effector to target ratio with clones was 1, 0.3, 0.1, 0.03:1 and with immune SC 100, 30, 10, 1:1. Results are representative of two or more similar experiments.
e) nd = Not determined.
f) C57BL/10→B10.BR chimera origin.
g) C57BL/10 origin.
h) C57BL/6 origin.
i) B10.BR origin.

2.10 Analysis of cell surface markers by immunofluorescence

Cells (2×10^5–5×10^5) in 50 µl radioimmunoassay (RIA) buffer [PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.2% (w/v) NaN_3] were mixed with an equal volume of mAb-containing culture supernatant in microtiter plates for 30 min. The cells were washed three times and resuspended in 50 µl of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgM antibodies (10 µl/ml, Tago, Burlingame, CA) or goat anti-rat IgG (50 µl/ml, E. Y., San Mateo, CA) in RIA buffer. After 30 min cells were washed twice and evaluated by fluorescence microscopy.

3 Results

3.1 Cell surface phenotypes of LCMV-specific CTL clones

CTL clones were examined for the expression of the cell surface antigens H-2K, H-2D, Lyt-2, L3/T4 and for the T cell receptor allotype defined by mAb KJ16-133. Table 1 shows that all the tested clones were Lyt-2^+ and L3/$T4^-$. The antibody against the T cell receptor allotype reacted with clones 532 and P14. As expected the clones derived from C57BL/10 and C57BL/6 mice were stained with mAb against H-$2K^b$ and H-$2D^b$ and clones derived from B10.BR were H-2^k positive. The clones H4 and P14 from the H-$2^k \rightarrow$ H-2^b chimeras were stained with mAb against H-$2K^k$ and H-$2D^k$ but not with mAb specific for H-$2K^b$ and H-$2D^b$. In analogy clone 27 and K7 derived from H-$2^b \rightarrow$ H-2^k chimeras reacted exclusively with mAb specific for H-$2K^b$ and H-$2D^b$. These findings confirmed that the latter cloned T cells were derived from donor BM cells.

3.2 Pattern of H-2 restriction of LCMV-specific CTL clones

The restriction specificity of the established clones was tested on infected LCMV-WE strain) and noninfected fibroblast and MΦ target cells. Table 2 demonstrates that clones H4 and P14 (derived from H-$2^k \rightarrow$ H-2^b chimeras) lysed infected target cells of H-2^b haplotype. Clones K7 and 27 (derived from H-$2^b \rightarrow$ H-2^k chimeras) lysed infected H-2^k target cells. All clones were specific since neither noninfected target cells nor infected target cells expressing the irrelevant H-2 type were lysed. The CTL clones 50.1 and B4 derived from C57BL/10 and C57BL/6 mice lysed LCMV-infected H-2^b target cells whereas the clones 527, 531 and 532 (all of B10.BR origin) recognized LCMV-infected H-2^k targets exclusively.

To examine the fine restriction specificity of these CTL clones LCMV-infected MΦ target cells from different recombinant mouse strains were used (Table 3). All H-2^b-restricted LCMV-specific CTL clones (from normal and chimeric mice) exclusively lysed infected target cells which carried the H-$2D^b$ allele. All the H-2^k-restricted clones (from normal and chimeric mice) lysed infected target cells from B10.A (K^k, D^d) mice but not from C3H.OH (K^d, D^k), and this mapped their restriction specificity to H-$2K^k$.

3.3 Inhibition of H-2 cytotoxicity by mAb anti-H-$2D^b$ and anti-H-$2K^k$

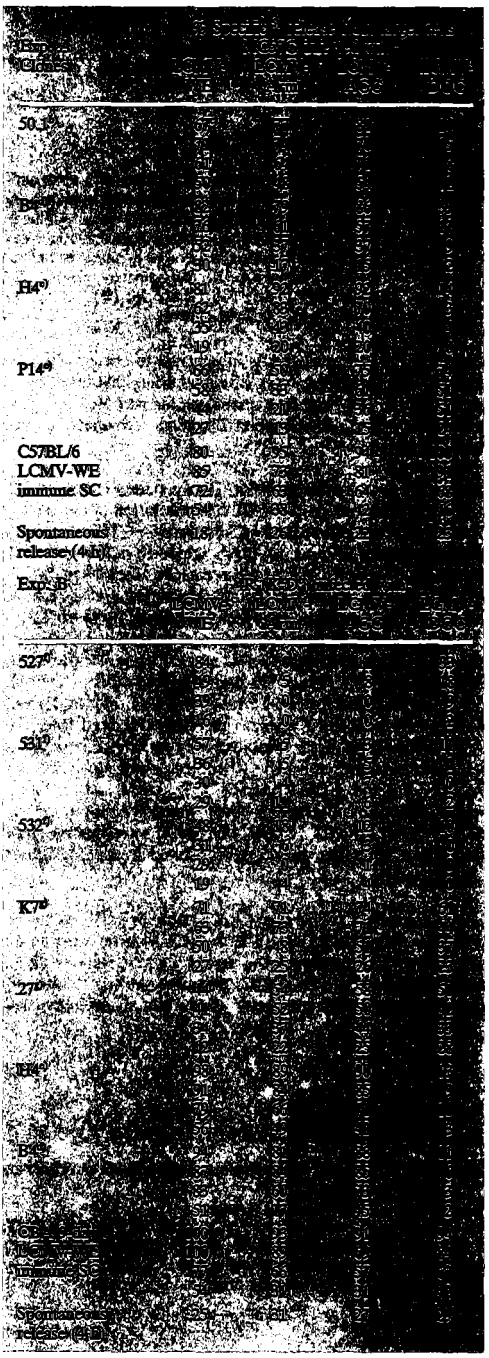
To further analyze the restriction specificity, several mAb anti-D^b and K^k were assayed for their capacity to block the

Table 3. H-2 restriction fine specificity of LCMV-specific CTL clones

Clones	% Specific ^{51}Cr-release from MΦ LCMV-WE target cells				
	B10.HTG d $b^{a)}$	B10.A(5R) b d	B10.A(2R) k b	B10.A k d	C3H.OH d k
H4[b]	58[c]	1	87	1	5
	51	3	58	0	4
	35	1	36	2	5
	21	1	19	4	4
P14[b]	59	3	66	0	2
	41	0	39	2	4
	25	0	26	1	1
	10	2	12	4	1
K7[d]	nd	nd	50	88	6
			32	55	1
			22	38	1
			10	17	1
27[d]	nd	nd	68	95	4
			50	75	3
			39	66	3
			18	29	1
50.1[e]	77	3	nd	nd	nd
	66	0			
	46	4			
	27	2			
B4[f]	61	4	80	2	1
	47	1	64	3	1
	26	1	40	4	1
	10	1	25	0	1
527[g]	nd	nd	67	85	6
			57	48	5
			56	39	6
			45	8	3
531[g]	nd	nd	92	82	9
			79	47	5
			76	30	4
			70	5	3
532[g]	nd	nd	45	81	5
			40	50	2
			31	33	7
			26	14	3
8-day C3H.OH LCMV immune SC	nd	nd	7	nd	37
			2		32
			2		19
			0		12
8-day C57BL/6 LCMV immune SC	41	51	nd	nd	nd
	40	45			
	24	28			
	16	14			
Spontaneous release (3.5 h)	19	15	17	23	22

a) Small letters indicate alleles expressed at the H-2K and H-2D loci of the target cells.
b) B10.BR→ C57BL/10 chimera origin.
c) Effector to target ratio were 1, 0.3, 0.1, 0.03 : 1 for cloned CTL. For primary immune SC 100, 30, 10, 3 : 1. Results are representative of at least two similar experiments.
d) C57BL/10→ B10.BR chimera origin.
e) C57BL/10 origin.
f) C57BL/6 origin.
g) B10.BR origin.

Table 4. Analysis of the LCMV fine specificity of the CTL clones



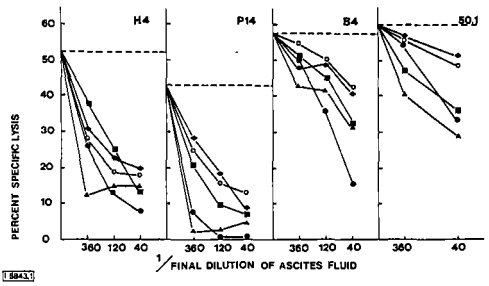


Figure 1. Inhibition of CTL activity with mAb anti-H-2D^b. CTL clones were examined for cytotoxic activity on LCMV-WE-infected MC57G cells. Antibodies from ascites fluid at the indicated concentration were added first to test wells followed by target and effector cells. Assay time 5 h, spontaneous release 22%. mAb: (●) T21.460; (▲) 141-51; (■) 172-93.2; (○) 141-31; (◆) 166-32.1; (---) medium control.

cytolytic activity of the various LCMV-specific CTL clones. From 11 tested anti-D^b mAb, 6 mAb (T21.440, B22-249, 28-8-65, BY-3, T17-433, K15-25) exhibited no or only marginal inhibition (data not shown), whereas 5 mAb (172-93.2, 141-31, 166-32.1, T21-460, 141-51) inhibited the cytolytic activity of the H-2D^b-restricted clones. As shown in Fig. 1 inhibition of chimeric CTL clones (H4 and P14) by anti-D^b mAb was more extensive than that of the clones derived from nonchimeric mice (50.1 and B4).

From 10 tested anti-K^k mAb, 6 mAb (16-3-1M, 3.83, H100-27/R55, 11-4-1, 15-3-1, H116-27/7) had no or low blocking capacities (data not shown) whereas 4 mAb (E.3.25, 12-2-2, 15-1-5, H100-528) inhibited the cell-mediated lysis of the K^k-restricted clones in a dose-dependent fashion (Fig. 2). Interestingly, only mAb 12-2-2 blocked the activity of the clones 527, 531, 532 from H-2^k mice completely, whereas clones derived from H-2^b→H-2^k chimeras were blocked only to about 50%.

3.4 Pattern of LCMV recognition by LCMV-specific CTL clones

Earlier results had demonstrated that CTL may see LCMV antigens differing among various LCMV isolates [35]; also susceptibility to LCM disease is dependent upon the LCMV isolate used for infection. Therefore, it was of interest to determine the pattern of viral antigen recognition by these LCMV-specific CTL clones by using target cells infected with different LCMV isolates.

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a) Infected for 2 days with LCMV-WE (0.1 PFU/cell), LCMV-Arm (0.1 PFU/cell), LCMV-AGG (0.01 PFU/cell) and LCMV-DOC (0.01 PFU/cell).
b) C57BL/10 origin.
c) Effector to target ratios were 3, 1, 0.3, 0.1:1 for cloned CTL and 100, 30, 10, 3:1 for primary immune SC.
d) C57BL/6 origin.
e) B10.BR→C57BL/10 chimera origin.
f) B10.BR origin.
g) C57BL/10→B10.BR chimera origin.

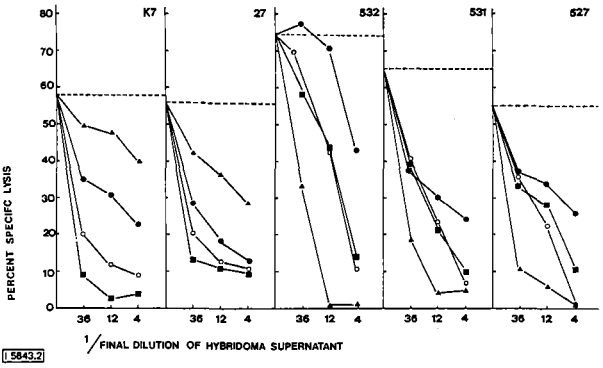


Figure 2. Inhibition of CTL activity with mAb anti-H-2K^k. CTL clones were examined for cytotoxic activity on LCMV-WE-infected FS9 cells. Antibody hybridoma supernatant at the indicated dilutions was added first to test wells followed by target and effector cells. Assay time 3 h, spontaneous release 24%. mAb: (■) 15-1-5; (○) H100-5.28; (●) E3.25; (▲) 12-2-2; (---) medium control.

The clones were tested on fibroblast target cells (MC57G and 4R) infected with LCMV strains WE and Armstrong (Arm) and with two plaque variants of the LCMV-UBC strain called Aggressive (AGG) and Docile (DOC). As shown in Table 4, exp. A, all H-2D^b-restricted clones (50.1, B4, H4, P14) lysed target cells infected with the LCMV strain WE, Arm and the AGG variant. However, in contrast to clone P14, the clones 50.1, B4 and H4 did not lyse targets infected with the LCMV-variant DOC.

We also found that the virus antigen specificity of the various H-2^k-restricted CTL clones fell into 3 groups: Table 4, Exp. B: (a) the clones from chimeric mice, K7 and 27 cross-reacted with all tested LCMV isolates; (b) clones 531 and 532 showed cytotoxic activity on target cells infected with the immunizing strain WE only; (c) beside lysis of WE-infected target cells, clone 527 also exhibited weak cross-reaction with the UBC variant DOC.

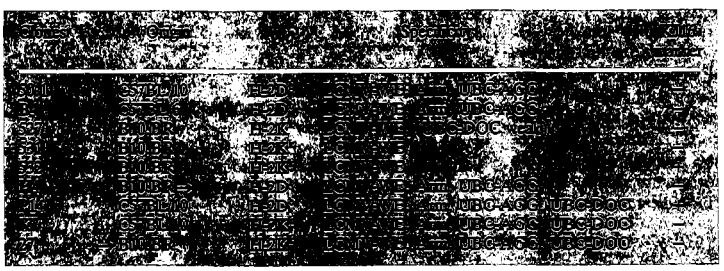
4 Discussion

We have established LCMV-specific H-2D^b- and H-2K^k-restricted CTL clones from normal mice and from allogeneic BM chimeras. The finding that all H-2^b-restricted clones recognize the D^b allele correlates with the finding that in primary responses in C57BL/6 or C57BL/10 mice K^b-restricted responses are usually lower than responses to D^b plus LCMV. The primary anti-LCMV-WE response in CBA (H-2^k) mice favored D^k restriction slightly whereas in B10.BR (H-2^k) mice, LCMV immune SC lysed infected K^k and D^k target cells to about the same extent; this is in contrast to earlier observations [3, 36]; therefore, we are re-examining this question in greater detail. We have no explanation for why all 5 independent H-2^k-restricted clones isolated here all recognized K^k plus the viral antigen.

Antibodies directed against MHC gene products inhibit MHC-restricted T cell recognition [32]. The inhibition of the cytolytic activity of the established LCMV-specific CTL clones with some of the anti-D^b and -K^k mAb helped to define their H-2 restriction specificity. Two points deserve discussion: (a) although the two H-2D^b-restricted clones H4 and P14 have different T cell receptor allotypes as defined by mAb KJ16-133, the blocking pattern with different mAb anti-D^b was virtually identical. Analogue results were obtained with the H-2K^k-restricted CTL clones 531 and 532; (b) one anti-K^k mAb (12-2-2) completely blocked lysis of the CTL clones derived from the B10.BR-mice whereas the clones derived from the B10→B10.BR chimeras inhibited only weakly; this suggests that different epitopes on the K^k molecule are recognized by the K^k-restricted LCMV-specific CTL clones of different origin. We also observed a more extensive inhibition by anti-D^b mAb of CTL clones derived from chimeric mice (B10.BR→B10) than from clones derived from C57BL/6 or C57BL/10 mice.

LCMV-immune T cells induced in C57BL/6 or in B10.BR mice after infection with LCMV-WE or LCMV-Arm lysed target cells infected with any of the tested LCMV isolates (WE, Arm, UBC-AGG, UBC-DOC; data not shown). However, analysis of the virus specificity at the clonal level revealed

Table 5. Summary of results



some heterogeneity. Three (50.1, B4 and H4) of the four H-2D^b-restricted clones were able to discriminate between the two plaque variants (AGG and DOC) of the LCMV strain UBC (Table 4). CTL clones 531 and 532 induced in B10.BR mice primed with LCMV-WE lysed LCMV-WE-infected target cells only, whereas the clones K7 and 27 derived from LCMV-Arm-primed B10→B10.BR chimeras recognized viral determinants expressed on target cells infected with all of the four tested LCMV isolates. Furthermore clone 527 derived from B10.BR mice primed with LCMV-WE showed a weak cross-reactivity with the UBC plaque variant DOC but not with AGG. Ahmed et al. [35] recently reported that 2 out of 13 H-2D^b-restricted CTL clones derived from C57BL/6 mice immunized with LCMV-Arm distinguished target cells infected with LCMV-Traub and LCMV-WE, respectively. Together, these data indicate that isolate-specific LCMV epitopes are recognized by LCMV-specific CTL clones.

Recent studies [14, 37, 38] indicated that the mAb KJ16-133 detected a T cell receptor idiotype encoded by V_β genes. Examination of the KJ16-133-marker among the established LCMV-specific CTL clones revealed that 2 out of the 9 clones were KJ16-133$^+$ (Table 1). Analysis of the specificity of the other clones confirmed findings from others that there is no obvious correlation between expression of the KJ16-133 T cell receptor allotype and their MHC or antigen specificity (Table 5).

Studies dealing with immunocompetence of allogeneic BM chimeras have yielded controversial results [19, 39–46]. We have recently reported [19] that primary anti-LCMV CTL responses in B10.BR→B10 chimeras were comparable to those found in control C57BL/10 mice; in contrast in B10→B10.BR chimeras failed to mount a measurable primary CTL response. The observation that the clones derived from B10.BR (H-2^k)→B10 (H-2^b) mice were H-2^b restricted is consistent with our earlier observation that the primary anti-LCMV response in the allogeneic BM chimeras was restricted entirely to the recipient H-2^b [39]. Although a primary anti-LCMV CTL response was not measurable in B10→B10.BR chimeras [19], CTL clones with high cytotoxic activity could be generated *in vitro* from such chimeras (Table 2).

This might suggest that the relative precursor frequency of LCMV-restricted effector CTL is considerably greater in H-2^k→H-2^b mice compared with H-2^b→H-2^k chimeras. We observed that 4 of 6 long-term bulk cultures from B10→B10.BR chimeras were mostly L3/T4$^+$ and exhibited no or only very low cytolytic activity against LCMV-infected target cells (data not shown). In contrast, all bulk cultures from B10.BR→B10 chimeras showed high lytic activity and Lyt-2$^+$ cells were predominant (data not shown).

The aim of this study was to generate LCMV-specific CTL clones from different origins and to characterize these clones with respect to their self MHC and viral antigen specificity. These cloned CTL can now be evaluated with respect to their capacity to mediate anti-viral protection or cell-mediated immunopathology. Also, the chimeric CTL clones should provide unique starting material for the induction of antibody response to the hypothetical receptor moiety specific for the MHC restriction and for molecular biological analyses.

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CHAPTER 3

FINE SPECIFICITY AND T CELL RECEPTOR β-CHAIN GENE REARRANGEMENTS OF FIVE H-2D^{b} SPECIFIC CYTOTOXIC T CELL CLONES.

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ABSTRACT

A panel of CTL clones that recognize H-2^b target cells has been established. Six different clones were distinguished according to the following criteria. First, the fine specificity of the clones was determined by testing proliferation and cytotoxicity on target cells of recombinant mice. Clone 221 recognized H-$2K^b$, and five other clones recognized H-$2D^b$. Clone 433 distinguished itself from the other five D^b-specific clones because it cross-reacted with an antigen on H-2^k cells. Second, the presence of an idiotypic determinant as defined by the 3F9 clone-specific monoclonal antibodies was investigated in cytotoxicity inhibition experiments. One of the D^b-specific clones, 653, was inhibited with these antibodies and was therefore clearly different from the other D^b-specific clones. The third criterion involved the rearrangement pattern of the DNA coding for the β chain of the T cell receptor. Southern blot analysis showed that each clone had its own characteristic pattern. Interestingly, clone 653, which expresses the same idiotypic determinant as clone 3F9, had deleted the $C_{\beta 1}$ gene cluster, whereas this gene is functionally expressed in clone 3F9.

INTRODUCTION

Cytotoxic T lymphocytes (CTL) recognize antigen in the context of MHC molecules [Zinkernagel & Doherty 1974]. The receptor molecules on the surface of the CTLs that are responsible for the specific interaction with antigen plus MHC have only recently been characterized. During the past few years, several groups have produced monoclonal antibodies that were clone-specific for either tumor T cell lines [Allison et al. 1982; Acuto et al. 1983] or functional T cell clones or hybridomas [Haskins et al. 1983; Meuer et al. 1983; Kaye et al. 1983; Samelson et al. 1983; Staerz et al. 1985; Hua et al. 1985; Acha-Orbea et al. 1985].

By using these monoclonal antibodies it has been possible to isolate a disulfide-linked heterodimeric glycoprotein from the surface of T cells. This T cell antigen receptor consists of two subunits, an α and a β chain. The genes that code for the α chain [Saito et al. 1984; Chien et al. 1984b] as well as those coding for the β chain [Hedrick et al. 1984; Yanagi et al. 1984] have recently been cloned and sequenced. The organization of the genes coding for the two chains of the T cell receptor is very similar to that of the immunoglobulin genes. Variable, diversity and joining gene segments are joined during T cell differentiation and, together with a constant region gene segment, form a functional transcription unit.

Until now, no obvious correlations could be demonstrated between the utilization of certain gene segments and T cell function and/or specificity [Acuto et al. 1985; Barth et al. 1985; Hedrick et al. 1985; Kronenberg et al. 1985; Goverman et al. 1985]. Only in the case of cytochrome C specific helper clones did there seem to be a preferential use of certain variable region genes [Fink et al. 1986]. It has even been shown that helper and cytotoxic T cells can use the same variable region gene segments [Rupp et al. 1985].

In this study we describe the diversification of antigen receptors that recognize one particular alloantigen. The frequency of alloreactive lymphocytes in an unprimed animal is unexpectedly high [Nisbet et al. 1969; Wilson et al. 1970] and the alloreactive T cells have a multitude of distinct fine specificities [Sherman 1980]. By comparing the antigen receptors of a panel of alloreactive T cells, all recognizing the same alloantigen, one may possibly learn more about the recognition of alloantigens by T cells.

We report the generation and characterization of five independent CTL clones of BALB/c origin, directed against the H-2D^b antigen. This alloantigen was chosen because CTL clone 3F9, which recognized this antigen has been isolated, and monoclonal antibodies that reacted with 3F9 in a clone-specific fashion were available [Acha-Orbea et al. 1985]. Also the T cell receptor genes of 3F9 have been isolated and characterized [Rupp et al. 1985, 1986 and manuscript submitted for publication]. The five newly derived clones can be distinguished from each other on the basis of different cross-reactivities with cells of other haplotypes, reactivity with the idiotype-specific monoclonal antibodies directed

against the receptor of 3F9 and the patterns of rearrangement of the DNA coding for the β chain of the T cell antigen receptor as detected by Southern blot analysis.

MATERIALS AND METHODS

Animals

BALB/c, C57BL/6 and B10.BR mice were obtained from the Veterinärmedizinisches Institut für Zuchthygiene der Universität Zürich. B10.A(5R) and B10.HTG mice were purchased from Olac (Bicester, Oxon, UK). Mice of both sexes were used at ages between 2 and 6 months.
Rats (1-3 months of age) were donated by the Institut für Immunologie und Virologie der Universität Zürich.

Media

Single cell suspensions of spleen cells were prepared in Hanks' Balanced Salt Solution. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, KC Biological Inc., Lenexa, KS) supplemented with 3,024 g/l $NaHCO_3$, 100 U/ml penicillin and 100 µg/ml streptomycin (KC), $5x10^{-5}$ M 2-mercaptoethanol and 10 % (v/v) heat inactivated fetal bovine serum (Gibco, Grand Island, NY). This medium is referred to as complete medium. All cultures were kept in a water saturated atmosphere containing 5 % CO_2 at 37° C.
Interleukin-2 containing supernatant from rat spleen cells stimulated with phorbol myristate acetate (PMA) and concanavalin A was prepared as described [Kast et al. 1984].

Tumor cell lines

The following tumor cell lines were kept in culture in complete medium: EL-4 (benzpyrene-induced lymphoma, H-2^b) and YAC-1 (murine leukemia, H-2^a).

Monoclonal antibodies

The following hybridomas producing monoclonal antibodies were obtained from Dr. G. Hämmerling, Heidelberg: anti-H-$2D^b$, H141-51 [Lemke et al. 1979], H172-93 [Hämmerling, unpublished]; other hybridomas used were: anti-L3T4, GK1.5 [Dialynas et al. 1983]; anti-Lyt-2, 53-6.7 [Ledbetter & Herzenberg 1979] and 9-1 D10 [Acha-Orbea et al. 1985]; anti-Lyt-1, 53-7.3 [Ledbetter & Herzenberg 1979]; anti-Thy-1, HO-13-4-9 [Marshak-Rothstein et al. 1985]. Two monoclonal antibodies that are T cell clone 3F9-specific, 44-22-1 (IgG_{2a}) and 46-6B5 (IgM) [Acha-Orbea et al. 1985] and one antibody, 2-93-10 [Acha-Orbea et al. 1985] that binds specifically to the NK like cell HY 3-Ag3 were also used.

Generation of T cell clones

Clone 3F9 was generated as described [Acha-Orbea et al. 1985] and is of BALB/c origin with an alloreactive cytotoxic specificity for H-$2D^b$. Clone 3A2 is a subclone of 3F9 with identical properties.
The other cytotoxic T cell clones: 221, 431, 432, 433, 652 and 653 were all cloned from mixed lymphocyte cultures [Acha-Orbea et al. 1983] under conditions of limiting dilution.
On day 0, unprimed BALB/c spleen cells ($2x10^6$/ml) were cocultured with irradiated (2000 R) B10.HTG stimulator spleen

cells ($5x10^6$/ml) in 5 ml complete medium in 25 cm^2 flasks (Falcon Plastics, Oxnard, CA, No. 3013) in an upright position. On day 7 the medium was removed and the cultures were subsequently kept in 5 ml complete medium plus 10 % Con A supernatant. On day 17 and day 24, the responder cells were restimulated with irradiated C57BL/6 spleen cells ($5x10^6$/ml) at densities between 1 and $5x10^5$/ml.
Clone 221 was obtained from a limiting dilution experiment performed 4 days after the last restimulation. Cloning was performed at various cell numbers per well (3-10-30-100-300-1000) in 0.2 ml complete medium (96 well plates, Falcon, No.3072) in the presence of 10 % Con A supernatant and $1x10^6$ irradiated stimulator cells (C57BL/6).
Clones 652 and 653 were obtained from an MLC that had been restimulated eight times with C57BL/6 spleen cells following the original stimulation with B10.HTG.
Clones 431, 432 and 433 were obtained from a second set of MLCs. For these cultures BALB/c responders were cultured with C57BL/6 stimulator spleen cells under the same conditions as described above. After 11 days, responding cells were restimulated with irradiated C57BL/6 spleen cells in the presence of 10 % Con A supernatant. On day 17, cells were cloned under conditions of limiting dilution in 96 well plates using B10.HTG spleen cells as stimulator cells.
The clones were restimulated in the original 0.2 ml cultures until confluent and were then transferred to 2 ml wells and eventually to 25 cm^2 flasks in upright position. The clones were continuously kept in culture for periods longer than 6 months by restimulation once a week with irradiated C57BL/6 spleen cells ($5x10^6$/ml) in 10 % Con A supernatant.

Chromium release assay

Tumor cells and lymphoblasts were used as target cells in this assay. Lymphoblasts were obtained by culturing spleen cells ($1-2x10^6$/ml) in complete medium for two days in the presence of Con A (2.5 µg/ml) or lipopolysaccharide (10 µg/ml, E. Coli 055:B5, Difco Laboratories, Detroit, MI). Cells were centrifuged on a Ficoll gradient (density 1.077, Seromed, München, FRG) and the blasts were harvested from the interphase.
Target cells were labeled in 0.5 ml medium with 100-300 µCi ^{51}Chromium ($Na^{51}CrO_4$, 13-22 GBq/mg Cr, Amersham, Buckinghamshire, UK). The test was essentially performed as described previously [Acha-Orbea et al. 1983]. Three step dilutions of effector cells were made (100 µl) and $1x10^4$ target cells (100 µl) were added. Tests were carried out in complete medium in U-shaped microtiter plates (Greiner, Nüttingen, FRG). When inhibition of cytotoxicity with monoclonal antibodies was tested, a 1 to 4 dilution of hybridoma culture supernatant was present during the assay. Plates were spun (5 min., 400 g) and incubated for 3-4 hours at 37°C.
Supernatant was removed (100 µl) and counted in a γ-counter. Total release values were estimated in the presence of 0.5 N HCl and spontaneous release values in medium alone. Percent specific release was calculated as follows:

$$\% \text{ spec.rel.} = \frac{\text{exp. rel.} - \text{spont. rel.}}{\text{tot. rel.} - \text{spont. rel.}} \times 100\ \%$$

The data represent mean values of duplicate determinations and all experiments were performed at least twice.

Proliferation assay

The proliferation of cells was measured by [^{3}H]thymidine uptake ([^{3}H]dThd, specific activity 3.11 TBq/mmol, Amersham). Responder T cells (2 x 10^4), that had not been restimulated for 1-2 weeks, were cocultured with 1 x 10^6 stimulator spleen cells in flat-bottom microtiter wells (Falcon, No.3072) in a final volume of 0.2 ml complete medium containing 10 % Con A supernatant.
After three days in culture, 1 μCi ^{3}H-dThd (=37 kBq) was added to each well and 10 hours later the cells were harvested on glass fiber filters. The cell-bound radioactivity was counted in a scintillation counter. The data represent the mean of triplicate determinations.

Southern blot analysis

DNA from 2-3x10^7 cells was isolated as described [Blin & Stafford 1976] and 10-15 μg was digested with the restriction enzyme PvuII (New England Biolabs, Beverly, MA). Southern blotting was performed as described [Southern 1975]. The digested DNA was electrophoresed in a 0.8 % agarose gel. The gel was bathed in 0.25 N HCl for 15 min., denatured in 0.5 M NaOH / 1.5 M NaCl and neutralized in 0.5 M Tris/HCl (pH 7.5)/ 1.5 M NaCl. Subsequently, the DNA was transferred to a filter (Gene Screen, New England Nuclear, Boston, MA) in 0.025 M phosphate buffer pH 7.0. The filter was baked for two hours at 80° C.
Nick translated probes were hybridized to the filters in 10 ml hybridization mix (5x SSPE / 10x Denhardts Solution / 10 % $DexSO_4$ / 25 μg/ml Salmon Sperm DNA / 0.1 % SDS) at 68° C for 16 hours and washed in 0.2x SSPE / 0.1 % SDS at 68° C.

DNA Probes

Two genomic DNA fragments cloned in the vector pUC8 were used as probes (Fig. 1). They were kindly provided by Drs. M. Kronenberg and L. Hood, Pasadena, CA. C1A is a 6.2 kb Bam/HindIII fragment containing the $J_{\beta 1}$ and $C_{\beta 1}$ gene segments and J2B is a 2.3 kb EcoRI fragment of the $J_{\beta 2}$ region [Kronenberg et al. 1985]. Inserts were labeled by Nick translation [Rigby et al. 1977] using [α-^{32}P]dCTP and [α-^{32}P]dTTP (3000 Ci/mmol, NEN, Boston, MA). Specific activity was between 1x10^8 and 4x10^8 cpm/μg.

RESULTS

Cloning of alloreactive CTL

Eighteen cytotoxic T cell clones were isolated from several independent MLCs and they were screened for cytotoxic activity against the leukemic T cell EL-4 ($H\text{-}2^b$). These clones could be classified into five groups according to the characteristics that will be described later. For simplicity only the results obtained with one representative of each group will be shown. Clones 221, 431, 432 and 433 were derived from limiting dilution experiments performed soon after initiation of the MLCs. Cloning efficiency and growth stability of these clones were poor in comparison to that of clones that were derived from bulk cultures that had been kept in culture for a few months by repetitive restimulation (i.e. 652 and 653). However, the range of fine specificities of clones that were isolated from such long term bulk cultures was limited. This was demonstrated in a cloning experiment in which we obtained 9 clones with only two different phenotypes (namely, two with the 652 and seven with the 653 phenotype).

All of the clones described here had been kept in culture for at least six months and could be expanded to yield large amounts of cells. The phenotype of all clones was determined by immunofluorescence and was Thy-1^+, Lyt-1^-2^+, L3T4^-.

Table 1. Analysis of cytotoxic specificity of CTL clones

CTL clone	E:T	% Specific ^{51}chromium release* from target cells			
	H-2:	BALB/c† *d, d, d**	C57BL/6 *b, b, b*	B10.A(5R) *b, b, d*	B10.HTG *d, d, b*
221	3:1	6	34	36	4
	1:1	2	26	28	4
	0.3:1	3	15	26	1
431	3:1	8	36	11	31
	1:1	8	22	6	15
	0.3:1	8	14	5	10
432	3:1	6	60	8	50
	1:1	3	35	6	38
	0.3:1	3	23	9	22
433	3:1	6	23	11	25
	1:1	6	13	5	17
	0.3:1	0	5	4	6
652	3:1	ND	ND	1	62
	1:1			1	46
	0.3:1			1	30
653	3:1	6	37	10	43
	1:1	10	24	6	30
	0.3:1	3	14	9	14
3F9	3:1	2	34	5	42
	1:1	1	26	6	30
	0.3:1	1	22	5	15
Spontaneous release		23	20	20	21

* In a 3-h assay. ND, not determined
† Target cells were Con A-induced blasts from various mice
* *H-2* haplotype: *K, I, D*

Table 2. Antigen-specific proliferation of CTL clones

CTL clone	^{3}H-dThd incorporation* after stimulation				
H-2:	BALB/c† *d, d, d**	C57BL/6 *b, b, b*	B10.A(5R) *b, b, d*	B10.HTG *d, d, b*	B10.MBR *b, k, q*
221	0.6‖	10.0	10.3	0.7	16.0
431	ND	38.1	1.3	40.0	2.9
432	2.2	32.1	2.3	28.7	2.3
433	4.0	25.8	4.0	26.9	24.5
652	3.8	118.5	1.8	31.8	5.5
653	2.9	187.5	2.3	57.4	3.3
3F9	3.1	87.5	6.1	33.3	8.2

* Effector cells (2×10^4) were cocultured with 1×10^5 irradiated stimulator cells in the presence of 10% Con A supernatant. After 3 days, 1 μCi ^{3}H-dThd per well was added and incorporation was measured after 10 h
† Irradiated spleen cells from various mice were used as stimulator cells
* *H-2* haplotype: *K, I, D*
‖ Cpm $\times 10^{-3}$. ND, not determined

Specificity analysis

To assign the specificity of the H-2^b reactive clones to the K or D end of the H-2 complex, cytotoxicity was measured using Con A induced lymphoblast cells from recombinant mice as target cells (Table 1). All clones tested lysed targets from C57BL/6 mice but did not lyse BALB/c targets. B10.A(5R) (K^b,I^b,D^d) derived target cells were only lysed by clone 221. Therefore, this clone recognized either the H-$2K^b$ or the I-A^b antigen. All other clones (431, 432, 433, 652 and 653) lysed target cells from B10.HTG (K^d,I^d,D^b) mice, which indicates that these clones recognize the H-D^b antigen. In this respect they show the same specificity as clone 3F9.

The results of the cytotoxicity assay were confirmed by proliferation experiments (Table 2). All clones were stimulated with the H-$2D^b$ antigen except clone 221. This latter clone proliferated with stimulator cells from B10.MBR (K^b,I^k,D^q) mice and was therefore specific for H-$2K^b$.

Another interesting observation was that clone 433 was stimulated with cells from B10.MBR mice (Table 2). In further experiments it was demonstrated that clone 433 recognized the I-E^k antigen [Schilham et al. 1986].

Inhibition of cytotoxicity with monoclonal antibodies

Acha and coworkers [1985] have described two monoclonal rat antibodies (44-22-1 and 46-6B5) that inhibit the cytotoxic activity of clone 3F9 in a clone-specific manner. These antibodies therefore define an idiotypic determinant on the receptor of 3F9. The same idiotypic determinant is also present on the receptor of clone 3A2, which is a subclone of 3F9. The cytotoxic activity of the D^b-specific clones described here was tested in the absence and presence of culture supernatant from the hybridomas 46-6B5 and 44-22-1 (Table 3). Clone 221 (K^b) and clones 652, 431, 432 and 433 (D^b) were not inhibited by the two anti-idiotypic antibodies, whereas clone 653 was clearly affected. Clone 653 was therefore 3F9-idiotype positive and clearly different from the other four H-$2D^b$ reactive clones. The presence of the idiotypic determinant on clones 3F9 and 653

Table 3. Inhibition of cytotoxicity with monoclonal antibodies

CTL clone	% Specific ^{51}chromium release* from target cells								
Hybridoma:	–†	46-6B5*	44-22-1	53-6.7	9-1 D10	53-7.3	2-93-10	H172-93	H141-51
Specificity:	–	3F9	3F9	Lyt-2	Lyt-2	Lyt-1	HY 3-Ag3	H-$2D^b$	H-$2D^b$
221	64	74	64	43	23	71	66	61	66
431	51	60	54	0	0	60	52	3	ND
432	64	57	55	2	1	57	50	13	ND
433	86	88	79	11	3	82	84	7	0
652	61	66	65	22	4	67	70	31	3
653	39	1	9	1	0	46	46	10	ND
3A2	72	7	21	12	2	76	74	65	20

* EL-4 as target cells in a 4-h assay. Spontaneous release, 12%. Effector: target ratio was 3 : 1 (for 3A2 it was 1 : 1). ND, not determined

† Release in the absence of antibodies

* Release in the presence of a 1 to 4 dilution of hybridoma culture supernatant

was also demonstrated by immunofluorescence (data not shown). The cytotoxicity of all the clones was inhibited by Lyt-2-specific antibodies (53-6.7 and 9-1 D10) to various degrees. Irrelevant antibodies (anti-Lyt-1 and 2-93-10, which binds to a surface determinant of the NK cell line HY 3-Ag3) did not affect cytotoxicity. Another observation was that all D^b-specific clones were inhibited by the D^b-specific monoclonal antibody H172-93 except clone 3A2. The fact that clone 653 was inhibited by this antibody might reflect differences in the receptor affinity for the antigen between clone 653 and 3F9, although both cells are 3F9-idiotype positive. 3A2, however, was inhibited by another D^b-specific antibody, H141-51. This antibody has proved to be very effective in inhibiting anti-D^b reactivities, not only of alloreactive cells but also of virus-specific, MHC restricted T cells (Chapter 2).

Southern blot analysis of the CTL clones.

The rearrangement patterns of the genes coding for the β chain of the T cell receptor were examined by Southern blot analysis. Two genomic DNA probes were used (Fig. 1). One probe, C1A, contains the $J_{\beta 1}$ region, the $C_{\beta 1}$ gene segment plus the intervening intron. With this probe, rearrangements and deletions in the $J_{\beta 1}$ gene cluster can be detected. The second probe, J2B, contains the $J_{\beta 2}$ region and part of the intron between $J_{\beta 2}$ and $C_{\beta 2}$, and is used to demonstrate rearrangements involving $J_{\beta 2}$ gene segments.

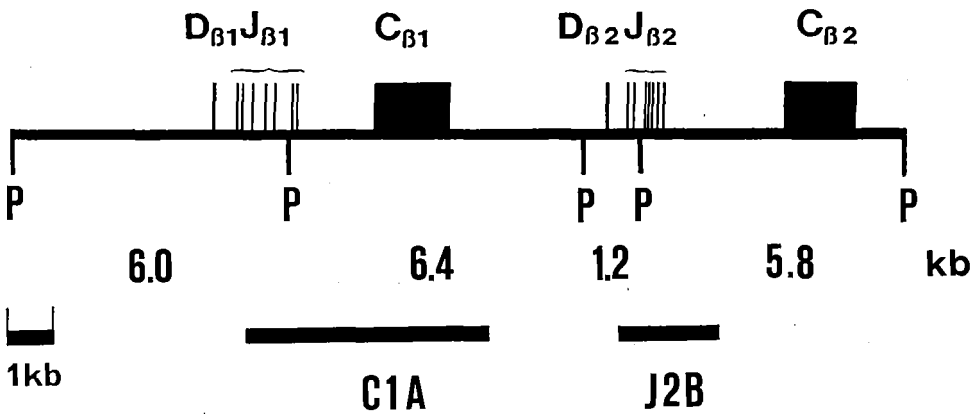

Figure 1. Genomic organization of the germ line DNA coding for the β chain of the T cell receptor [Gascoigne et al. 1984; Chien et al. 1984a]. PvuII restriction endonuclease sites and the sizes of the fragments used as probes are indicated.

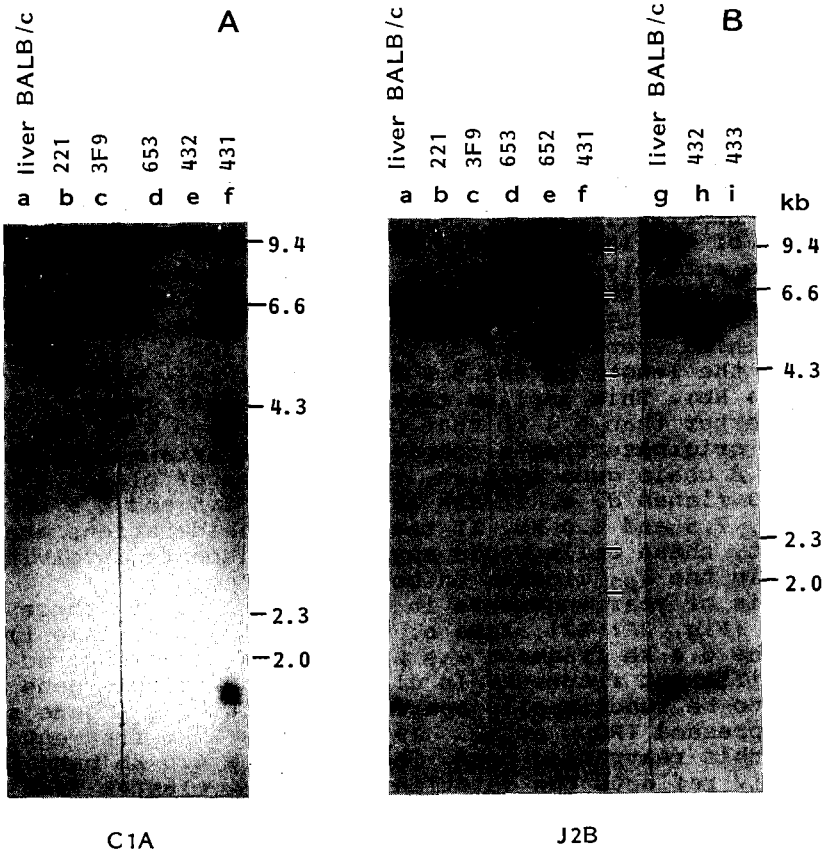

Figure 2. Southern blot analysis showing the rearrangement patterns of β chain DNA in the various CTL clones. The migration distances of some molecular weight markers are indicated. DNA was digested with the restriction enzyme PvuII and hybridized to the probes indicated in Figure 1.
A. Hybridization with the C1A probe. Lane a, BALB/c liver DNA; lane b, 221; lane c, 3F9; lane d, 653; lane e, 432; lane f, 431. **B.** Hybridization with the J2B probe. Lane a and g, BALB/c liver DNA; lane b, 221; lane c, 3F9; lane d, 653; lane e, 652; lane f, 431; lane h, 432; lane i, 433.

The DNA of the clones was digested with the restriction enzyme PvuII, electrophoresed, and transferred to a membrane. The hybridization patterns of the different clones with the C1A probe are shown in Figure 2A and summarized in Table 4. In liver DNA (Fig. 2A, lane a), two germ line fragments, at 6.0 and 6.4 kb, can be detected. In three CTL clones (Fig. 2A, 653, lane d; 652 and 433, not shown), these bands were not detected with this probe (weak bands due to cross-hybridization of the probe to the $C_{\beta 2}$ gene were detected after prolonged periods of exposure). Therefore, the $C_{\beta 1}$ gene regions have been deleted from both chromosomes. These cells were further analyzed with the J2B probe and the hybridization results are shown in Figure 2B and also summarized in Table 4. In liver DNA, representing the germ line configuration, bands were detected at 5.8 and 1.2 kb (Fig. 2A, lane a and g). If only the $D_{\beta 2}$ segment rearranges to $J_{\beta 2.1}$ or $J_{\beta 2.2}$, the 1.2 kb band will become shorter and the 5.8 kb band will remain constant. If the $D_{\beta 2}$ segment rearranges to one of the last five $J_{\beta 2}$ fragments, then the 1.2 kb fragment will be entirely lost and the 5.8 kb fragment will be changed in size. From the published genomic data on the $D_{\beta 2}$-$J_{\beta 2}$ region [Chien et al. 1984a; Siu et al. 1984] it can be deduced that a D-J joining event to one of these last five J fragments will change the length of the 5.8 kb fragment only minimally (+0.1 or -0.4 kb). This implies that all fragments longer than 5.9 kb and shorter than 5.4 kb that can be detected with the J2B probe should originate from a complete V-D-J rearrangement in the second β chain gene complex. The patterns of clones 653, 652 and 433 (lanes d, e, i) are as follows: 8.5 and 6.5 kb, 8.5 and 5.0 kb, 7.5 and 6.0 kb. If the above considerations are correct, these cells would have rearrangements involving V genes in the $J_{\beta 2}$ cluster on both chromosomes.

Analysis of rearrangements in the $J_{\beta 1}$ cluster in three other clones (Fig. 2A; 221, lane b; 3F9, lane c; 431, lane f) showed that the 6.4 kb fragment was present in each clone, as was a new, different fragment. In clone 3F9, a variable gene has been shown to be functionally rearranged to the $J_{\beta 1.1}$ gene segment and expressed [Rupp et al. 1985]. The 3.2 kb fragment should carry this rearranged gene. The lack of a 6.0 kb band in 3F9 probably reflects the deletion of the $J_{\beta 1}$ cluster from the second chromosome. The pattern obtained with the J2B probe (one band of 5.8 kb, Fig. 2B, lane c) indicates that some DNA of the $J_{\beta 2}$ cluster has been deleted (dissappearance of the 1.2 kb fragment). The deletion is probably the result of a $D_{\beta 2}$-$J_{\beta 2}$ joining event, which was not necessarily followed by a V-$D_{\beta 2}$ rearrangement.

A similar pattern was obtained with DNA of clone 431 (Fig. 2A, lane f; Fig. 2B, lane f). One obvious difference with the pattern of 3F9 is the size of the new fragment detected with the C1A probe (1.7 kb instead of 3.2 kb). If this fragment would also contain the productive rearrangement, this would imply the utilization of a different V gene in clone 431 (431 is 3F9-idiotype negative).

The new fragment of 4.6 kb in clone 221 (Fig. 2A, lane b) could be the germ line 6.0 kb band that has been shortened by 1.4 kb as the result of a D-J joining event without the subsequent

Table 4. Summary of the characteristics of the CTL clones

CTL clone	Specificity	Idiotype	Southern blot analysis						Number of clones
			C1A			J2B			
Germline, BALB/c liver			6.4	6.0		5.8	1.2		
653	D^b	id^+	Deleted			8.5	6.5		7
652	D^b	id^-	Deleted			8.5	5.0		6
433	D^b, H-2^k	id^-	Deleted			7.5	6.0		2
3F9	D^b	id^+	6.4	3.2		5.8			1*
431	D^b	id^-	6.4	1.7		5.8			1
221	K^b	id^-	6.4	4.6		5.8	1.2	5.5	1
432	D^b	id^-	10			5.8	1.2	5.2	1

Summary of the characteristics of the CTL clones. Indicated are the various CTL clones, their specificity for H-2D^b or H-2K^b and cross-reactivity with unrelated alloantigens, presence of the idiotypic determinant on their receptor as defined by the 3F9-specific monoclonal antibodies, and the sizes of the fragments of DNA (in kb) as detected by the Southern blot analysis. In the last column, the number of clones with similar characteristics is given
* Described by Acha-Orbea and co-workers (1985)

joining of a V gene. In the $J_{\beta 2}$ region (Fig. 2B, lane b) one chromosome seems to be in germ line configuration, while the 5.5 kb fragment represents a rearrangement.
Finally, hybridization of the DNA of clone 432 with the C1A probe (Fig. 2A, lane e) indicated the presence of a single 10 kb band. The absence of the 6.4 kb band suggests that a rearrangement to a $J_{\beta 1}$ segment 3' of the PvuII site has taken place. This could have been a D-J joining event accompanied by the deletion of ±2 kb. The $C_{\beta 1}$ gene cluster on the other chromosome has been deleted or has rearranged identically. Although one chromosome seems to be in germ line configuration in the $J_{\beta 2}$ gene region (Fig. 2B, lane h), the other could have rearranged productively (5.2 kb).
Although alternative interpretations of the rearrangements as demonstrated with this Southern analysis are possible, the results show that all six D^b-specific CTL clones have different patterns of rearrangements and therefore have been derived from different precursors.

DISCUSSION

Eighteen CTL clones of BALB/c ($H\text{-}2^d$) origin with specificity for $H\text{-}2^b$ antigens have been produced. The clones were classified in six groups on the basis of the following criteria. First, the fine specificity of the clones was determined by testing proliferation and cytotoxicity on target cells of recombinant mice. Second, the presence of the idiotypic determinant as defined by the 3F9 clone-specific monoclonal antibodies was investigated by cytotoxicity inhibition experiments. The third characteristic of these clones that was studied, was the rearrangement pattern of the DNA coding for the β chain of the T cell receptor.

Since clone 3F9 with specificity for $H\text{-}2D^b$ had been characterized extensively [Acha-Orbea et al. 1985], we were interested to obtain more T cell clones with this specificity. To encourage growth of clones that recognize D^b, B10.HTG (K^d,I^d,D^b) stimulator cells have been used once during initial cultivation to stimulate the responder cells. As a result of this procedure 17 of 18 clones were D^b-specific and only one recognized K^b. It has been described that T cell clones with a certain specificity may cross-react with alloantigens [Von Boehmer et al. 1979; Braciale et al. 1981; Sredni & Schwartz, 1980]. Therefore, the possibility existed that some of the D^b-specific clones would exhibit such cross-reactivity and the fine specificity of the T cell clones was further characterized by using third party alloantigens. One of the clones, 433, lysed target cells of the $H\text{-}2^k$ haplotype. This cross-reactivity was a feature of clone 433 exclusively and may reflect a unique structure of the antigen receptor of this clone. Similarly, clone 431 could be stimulated with $H\text{-}2^q$ stimulator cells. However, this stimulation was not found consistently and lysis of $H\text{-}2^q$ targets could not be demonstrated. This cross-reactivity might have been of a very low affinity. The presence of a distinct receptor on clone 431 is compatible with the hybridization results obtained from the Southern blot analysis.

The clones were also analyzed with the monoclonal antibodies that define an idiotypic determinant on the antigen receptor of clone 3F9. Although all D^b-specific clones share the same specificity for this allogeneic determinant, only the cytotoxic activity of one, clone 653, was inhibited with these idiotype specific antibodies. This indicates that these two clones share at least one idiotypic determinant on the antigen receptor. As already mentioned, seven clones with the same phenotype as clone 653 have been isolated. Since they were derived from one MLC in one cloning experiment and they also showed an identical pattern of rearrangements, they can be considered to be multiple isolates of the same precursor. The six clones with the 652 phenotype have been isolated from 2 different MLCs and may be distinguished from each other after more detailed analysis (i.e. nucleotide sequences).

The results of the Southern blot analysis show that all seven CTL clones (six D^b- and one K^b-specific) possess unique rearrangements of the DNA segments coding for the β chain of

the T cell receptor. Therefore, they were all derived from different precursor cells and should express different receptors. Clones 652 and 653 have a common band when analyzed with the J2B probe (8.5 kb). It would be interesting to determine whether this fragment also carries the functional gene in both cells. This might imply that these two cells have a similar β chain. However, the two clones differ with respect to reactivity with the 3F9-specific monoclonal antibody.

An interesting finding is that clone 653, which was inhibited with the 3F9-specific antibodies, has deleted the entire $C_{\beta 1}$ gene cluster. It is known that 3F9 expresses a receptor gene that includes $J_{\beta 1.1}$ and $C_{\beta 1}$ [Rupp et al. 1985]. Clone 653, however, must express a different receptor gene that contains the $C_{\beta 2}$ gene and one of the preceding $J_{\beta 2}$ gene segments. Therefore it seems unlikely that the idiotypic determinant, which is present on both cells, is encoded by a J_{β} segment. In addition, the observation that clone 653 was inhibited with the D^b-specific monoclonal antibody, H172-93, whereas clone 3A2 was not, may reflect the difference between the receptors of these two clones.

In this respect, another interesting observation is that an antigen specific, I-A restricted helper T cell, LB2-1, [Giedlin et al. 1986] uses the same V_{α} and V_{β} for its receptor as 3F9 [Rupp et al. 1985 and manuscript submitted for publication]. The receptor of this helper T cell can be detected with the 3F9-specific monoclonal antibodies by immunofluorescence. This suggests that the idiotypic determinant resides in the variable region genes, either V_{α} or V_{β} or both. Further studies of the exact primary structure of the α and β chains of the receptor of clone 653 may provide more information about the nature of the idiotypic determinant that is recognized by these monoclonal antibodies.

From the present results, it can be concluded that H-$2D^b$-specific T cell clones with different fine specificities possess different patterns of rearrangements of T cell receptor β chain genes; unexpectedly, two clones that seemed to be similar, since they were both recognized by the same idiotype-specific monoclonal antibody, also showed patterns that were remarkably different. These data indicate that the diversity of receptors of alloreactive clones specific for one H-2D allele is great. More extensive molecular analysis of the receptor genes of well-defined cloned T cells will be necessary to determine whether there is a preferential use of certain gene segments in clones with specificity for a single alloantigen.

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CHAPTER 4

CHARACTERIZATION OF AN LYT-2$^+$ ALLOREACTIVE CYTOTOXIC T CELL CLONE SPECIFIC FOR H-2D^b THAT CROSS-REACTS WITH I-E^k

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ABSTRACT.

An alloreactive cytotoxic T cell clone (433) possessing the $L3T4^-$, $Lyt\text{-}2^+$ phenotype is described, that shows a double specificity. It has been derived from unprimed BALB/c ($H\text{-}2^d$) spleen cells by repetitive *in vitro* restimulation with C57BL/6 ($H\text{-}2^b$) cells. The specificity of clone 433 was determined in cytotoxicity and proliferation experiments. One specificity was for the class I antigen $H\text{-}2D^b$ and the second for class II antigen $I\text{-}E^k$. Inhibition of cytotoxicity with monoclonal antibodies confirmed these results.
Cold target competition experiments demonstrated that the two specificities were mediated by the same cell population. Anti-Lyt-2 antibodies inhibited only the $H\text{-}2D^b$ but not the $I\text{-}E^k$ specific lysis, suggesting a higher affinity of the antigen receptor for $I\text{-}E^k$ than for D^b. To our knowledge, this is the first description of a T cell clone that is specific for a class I antigen and cross-reacts heteroclitically with a class II antigen.

INTRODUCTION

T cells recognize foreign antigens on the cell membrane in the context of major histocompatibility complex (MHC) antigens. There are two types of MHC antigens: class I antigens that are present on most cells with varying densities, and class II antigens that are expressed mainly on antigen presenting cells (1).
Class I antigens have been shown to be the restricting elements for antigen specific cytotoxic T cells (2) and class II antigens serve a similar function for helper T cells (3). Phenotypically these two subsets of T lymphocytes can be distinguished using monoclonal antibodies directed against surface antigens (4). For murine T lymphocytes, Lyt-2 is considered to be a surface marker of cytotoxic T cells and L3T4 of helper T cells (5).
In humans, similar cell surface antigens have been found. In general, the T4 surface antigen is present on HLA-D-restricted helper cells and T8 is expressed on the membrane of HLA-A,B,C-restricted cytotoxic T cells.
However, this generally accepted triple correlation between function, restriction and phenotype has been challenged by some investigators. Swain (6) has described an unusual helper function of T cells specific for class I antigens that was inhibited by anti-Lyt-2 antibodies. Furthermore, cytotoxic T lymphocytes (CTL) have been generated that were specific for Ia determinants (7,8). T cells have also been described that were able to exert both helper and cytotoxic function (9-11). Therefore, it has been suggested that the phenotype of a T cell would predict only its specificity for class I or class II antigens but not its function (12). However, murine $Lyt\text{-}2^+$ cytotoxic cells specific for class II antigens have been reported (13-17). Also human $T4^+$ T cells that recognized class I antigens (18) and T8-bearing cells that were specific for class II antigens (19) have been described.
It should be noted that in most examples, these exceptions to the general correlation between phenotype and specificity have been documented using cloned alloreactive T cells. Therefore the overall significance of these exceptions remains unclear.

When we analyzed the specificity of a panel of alloreactive $H\text{-}2D^b$-specific cytotoxic T cell clones, we found one clone that may be relevant to the discussion above. This T cell, clone 433, exhibits a double specificity that has not been previously described. The original specificity is directed against a class I antigen ($H\text{-}2D^b$) and the second specificity, of an apparently higher affinity, is for a class II antigen ($I\text{-}E^k$) to which the clone has never been exposed.

MATERIALS AND METHODS

Animals.

BALB/c, C57BL/6, B10.BR and A.TL mice were obtained from Veterinärmedizinisches Institut für Zuchthygiene der Universität Zürich. B10.A(5R), B10.HTG, B10.AKM, B10.T(6R), B10.AQR, B10.MBR, B10.A(4R) and B10.G mice were purchased from Olac (Bicester, Oxon, UK). Mice of both sexes were used at ages between 2 and 6 months.
Rats (1-3 months of age) were donated by the Institut für Immunologie und Virologie der Universität Zürich.

Media.

Single cell suspensions of spleen cells were prepared in Hanks' Balanced Salt Solution. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, KC Biological Inc., Lenexa, KS) supplemented with 3,024 g/l $NaHCO_3$, 100 U/ml penicillin and 100 µg/ml streptomycin (KC), $5x10^{-5}$ M 2-mercaptoethanol and 10 % (v/v) heat inactivated fetal bovine serum (Gibco, Grand Island, NY). This medium is referred to as complete medium. All cultures were kept in a water saturated atmosphere containing 5 % CO_2 at 37° C.
Interleukin-2 containing supernatant from rat spleen cells stimulated with phorbol myristate acetate (PMA) and Concanavalin A (Con A) was prepared as described (20).

Tumor cell lines.

The following tumor cell lines were kept in culture in complete medium: EL-4 (benzpyrene-induced lymphoma, H-2^b), P-815 (mastocytoma, H-2^d), YAC-1 (murine leukemia virus-induced lymphoma, H-2^a) and TA-3 (B cell hybrid, H-$2^{d/a}$, expressing Ia antigens, ref. 21). TA-3 was a gift from Dr. P. Erb, Basel.

Monoclonal antibodies.

The following hybridomas producing monoclonal antibodies were obtained from Dr. G. Hämmerling, Heidelberg: anti-H-$2D^b$, H141-51 (22) and H166-32, H172-93 (Hämmerling, unpublished); anti-I-A^k, 10-2-16 (23), 17-227 (22); anti-I-E_β, K-22-42.2 (Hämmerling, unpublished); anti-I-E^k, 14-4-4S (24).
Other hybridomas used were: anti-I-E^k, 17-3-3S (24), M5/114 (25); anti-L3T4, GK1.5 (5); anti-Lyt-1, 53-7.3 (26); anti-Thy-1.2, HO-13-4-9 (27); anti-Lyt-2, 53-6.7 (26) and 9-1 D10 (28); anti-LFA-1, I-17 (29).

Generation of T cell clones.

Clone 433 was isolated from a mixed lymphocyte culture prepared according to described procedures (30). On day 0 unprimed BALB/c spleen cells ($2x10^6$/ml) were cocultured with irradiated (2000 R) C57BL/6 stimulator spleen cells ($5x10^6$/ml) in 5 ml complete medium in 25 cm^2 flasks (Falcon Plastics, Oxnard, CA, No. 3013) in an upright position. After 11 days, responding cells were restimulated with irradiated C57BL/6 spleen cells in the presence of 10 % Con A supernatant. On day 17, cells were cloned under conditions of limiting dilution in 96 well plates using B10.HTG spleen cells as stimulator cells. Clone 433

resulted from a cloning experiment in which three out of twenty wells showed growth. Two of these clones were lost soon after cloning. Clone 433 was restimulated once a week with C57BL/6 spleen cells in the presence of 10 % Con A supernatant. It has been kept in culture for more than one year.
Two other clones of BALB/c origin were produced after similar procedures (Chapter 3). Clone 221 is H-2K^b specific and clone 432 recognizes H-2D^b.

Chromium release assay.
Either tumor cells or lymphoblasts were used as target cells in this cytotoxicity assay. Lymphoblasts were obtained by culturing spleen cells (1-2x10^6/ml) in complete medium for two days in the presence of Con A (2.5 μg/ml) or lipopolysaccharide (LPS) (10 μg/ml, E. Coli 055:B5, Difco Laboratories, Detroit, MI). Cells were centrifuged on a Ficoll gradient (density 1.077, Seromed, München, FRG) and the blasts were harvested from the interphase.
Target cells were labeled in 0.5 ml medium with 100-300 μCi ^{51}Chromium ($Na^{51}CrO_4$, 13-22 GBq/mg Cr, Amersham, Buckinghamshire, UK). The test was essentially performed as described (30). Three step dilutions of effector cells were made (100 μl) and 1x10^4 target cells (100 μl) were added. Tests were carried out in complete medium in U-shaped microtiter plates (Greiner, Nüttingen, FRG).
To test the inhibition of cytotoxicity with monoclonal antibodies, a 1:4 dilution of hybridoma culture supernatant was added before the assay. For cold target competition experiments, the test was performed in the presence of unlabeled target cells at the indicated ratios. Plates were spun (5 min., 400 g) and were incubated for 3-4 hours at 37°C. Supernatant was removed (100 μl) and counted in a γ-counter. Total release values were estimated in the presence of 0.5 N HCl and spontaneous release values in medium alone. The percent specific release was calculated as follows:

$$\% \text{ spec.rel.} = \frac{\text{exp. rel.} - \text{spont. rel.}}{\text{tot. rel.} - \text{spont. rel.}} \times 100\ \%$$

The data represent mean values of duplicate determinations and all experiments were performed at least twice.

Proliferation assay.
The proliferation of cells was measured by [^{3}H]thymidine (^{3}H-dThd, specific activity 91.7 Ci/mmol, NEN, Boston, MA) uptake. Responder T cells (2 x 10^4), that had not been restimulated for 1-2 weeks, were cocultured with 1 x 10^6 stimulator cells in flat-bottom microtiter wells (Falcon, No.3072) in a final volume of 0.2 ml complete medium containing 10 % Con A supernatant. After three days in culture, 0.5-1 μCi ^{3}H-dThd (=37 kBq) was added to each well and 10 hours later the cells were harvested on glass fiber filters. The cell-bound radioactivity was counted in a scintillation counter. The data represent the mean of triplicate determinations.

RESULTS

Class I specificity of CTL clone 433

Clone 433 was derived from a MLC of BALB/c ($H\text{-}2^d$) anti-C57BL/6 ($H\text{-}2^b$). The phenotype of clone 433 was Thy-1^+, L3T4$^-$, Lyt-1^-, Lyt-2^+ as detected by immunofluorescence (data not shown). This clone showed cytotoxic activity against EL-4 target cells (Table I). To determine whether clone 433 recognized H-2K or H-2D antigens, its cytotoxicity was tested on various target cells (Table I). Con A induced blast cells from C57BL/6 mice were lysed, in contrast to blasts from BALB/c mice. Because B10.HTG (K^d,I^d,D^b) cells were lysed but blast cells derived from B10.A(5R) mice (K^b,I^b,D^d) were not, the specificity must be directed against $H\text{-}2D^b$. The $H\text{-}2K^b$ specific T cell clone 221 served as a positive control for the B10.A(5R) target cells. The lack of cytotoxicity towards YAC-1 cells indicated that clone 433 did not exhibit measurable nonspecific natural killer cell activity.

The specificity for $H\text{-}2D^b$ was confirmed in a proliferation assay. Stimulator cells from B10.HTG (K^d,I^d,D^b) but not from B10.A(5R) (K^b,I^b,D^d) mice were able to induce proliferation (Table II).

TABLE I

Clone 433 recognizes H-2D^b

CTL Clones	E:T	Percent Specific ^{51}Chromium release[a] from Target Cells					
		BALB/c[c] H-2: d,d,d,d[c]	C57BL/6 b,b,b,b	B10.A(5R) b,b,b,d	B10.HTG d,d,d,b	EL-4 b,-,-,b	YAC-1 k,-,-,d
433	3:1	6	23	11	25	41	1
	1:1	6	12	6	16	17	1
	0.3:1	1	5	4	6	9	0
221	3:1	6	34	36	4	71	1
	1:1	2	26	28	5	58	0
	0.3:1	3	15	26	1	31	0
Spontaneous release		23	20	20	21	11	4

[a] In a 3-hr test.
[b] Two day Con A blasts.
[c] K,I-A,I-E,D.

TABLE II

Clone 433 is stimulated by H-D^b and I^k

CTL Clones	[^{3}H]Thymidine incorporation[a] after Stimulation with Stimulator Cells				
	BALB/c[b] H-2: d,d-d,d-d,d[c]	C57BL/6 b,b-b,b-b,b	B10.A(5R) b,b-b,b-k,d	B10.HTG d,d-d,d-d,b	B10.MBR b,k-k,k-k,q
433	4[d]	26	4	27	25
221	1	10	10	1	16

[a] Effector cells (2×10^4) were co-cultured with 1×10^6 irradiated stimulator cells in the presence of 10% Con A supernatant. After 3 days, 1 μCi [^{3}H]thymidine per well was added, and incorporation was measured after 8 hr.
[b] Spleen cells from various mice were used as stimulator cells.
[c] H-2 haplotype: K, I-A(β-α), I-E(β-α), D.
[d] Cpm $\times 10^{-3}$.

To confirm the K^b specificity of clone 221, B10.MBR (K^b,I^k,D^q) stimulator cells were included in this proliferation assay. As expected, clone 221 proliferated extensively. Surprisingly, clone 433 was also stimulated with B10.MBR stimulator cells. This result prompted us to investigate the specificity of clone 433 more extensively.

Class II specific cytotoxicity of CTL clone 433

Proliferation studies with the use of H-2^k and H-2^q stimulator cells indicated that antigens coded by the I^k region from B10.MBR were stimulating clone 433 (data not shown). Therefore, we tested whether clone 433 could also lyse I^k bearing target cells. In Table III, it can be seen that both C57BL/6 and B10.BR LPS-induced target cells were lysed. Lysis of H-2^b targets was consistently lower than that of H-2^k targets. Lysis of H-2^k targets was a unique characteristic of clone 433, because a different H-D^b specific CTL clone, clone 432, that also lysed EL-4 and C57BL/6 target cells, did not lyse B10.BR blasts. YAC-1 cells, which are of the H-2^a (K^k, D^d) haplotype were not lysed because they do not express class II antigens on their surface. However, they could serve as target cells for H-$2K^k$ specific CTL from a primary in vitro MLC.

The activity of clone 433 was also tested against a panel of target cells from recombinant mice (Table IV). Because targets bearing H-$2I^k$ antigens were lysed, it can be concluded that either I-A^k or I-E^k is recognized by clone 433. This recognition is not obviously restricted by allelic class I antigenic determinants, because I^k-specific lysis is independent of the class I antigen expressed on the target cells.

Clone 432 and two primary in vitro MLC were included in this test as negative and positive controls respectively.

TABLE III

Clone 433 lyses H2-I^k-positive target cells

CTL Clones	E:T	Percent Specific ^{51}Chromium Release[a] from Target Cells				
		EL-4 H-2: b,-,-,b[c]	P-815 d,-,-,d	YAC-1 k,-,-,d	C57BL/6[b] b,b,b,b	B10.BR k,k,k,k
433	9:1	80	0	0	33	68
	3:1	77	0	0	43	56
	1:1	56	0	0	27	39
	0.3:1	26	0	0	4	21
432	9:1	60	0	0	47	0
	3:1	46	3	0	37	0
MLC C57BL/6 (H-2^b)	15:1	12	21	54	0	86
Anti-B10.BR (H-2^k)	5:1	6	8	45	2	62
	1.5:1	3	4	26	0	44
Spontaneous release		10	12	10	51	48

[a] In a 3.5-hr test.
[b] Target cells were LPS-induced blasts.
[c] H-2 haplotype: K, I-A, I-E, D.

TABLE IV

Specificity of clone 433 maps to H-2I^k

CTL Clones	E:T	Percent Specific ^{51}Chromium Release[a] from Different Target Cells						
		B10.BR[b] H-2: k,k,k,k[c]	B10.AKM k,k,k,q	A.TL s,k,k,d	B10.T(6R) q,q,q,d	B10.MBR b,k,k,q	B10.AQR q,k,k,d	B10.G q,q,q,q
433	5:1	61	68	51	6	61	60	1
	1.5:1	49	46	35	3	39	47	0
	0.5:1	32	28	18	0	23	23	0
	0.2:1	19	13	8	0	5	8	0
432	5:1	8	1	5	6	3	0	0
	1.5:1	2	2	0	0	0	0	0
MLC C57BL/6(H-2^b)	25:1	82	53	24	42	20	32	17
Anti-B10.BR(H-2^k)	8:1	74	41	13	16	10	23	5
	3:1	32	30	9	7	3	8	0
MLC C57BL/6(H-2^b)	25:1	49	69	30	73	76	63	79
Anti-DBA/1 (H-2^q)	8:1	32	59	22	74	57	42	59
	3:1	16	43	9	30	40	17	41
Spontaneous release		33	26	25	30	28	26	28

[a] In a 3.5-hr test.
[b] Target cells were LPS-induced blasts.
[c] H-2 haplotype: K, I-A, I-E, D.

Inhibition of cytotoxicity of clone 433 with mAb

Because the cytotoxic activity of CTL can usually be inhibited effectively by mAb directed against the H-2 antigen on the target cell (31,32), this approach was used to determine whether I-A^k or I-E^k was recognized by clone 433 (Table V). The cytotoxic activity against H-2D^b-bearing target cells from B10.A(4R) mice (LPS-induced lymphoblasts) or EL-4 cells could be inhibited by several H-2D^b-specific mAb. These results confirm the H-2D^b specificity of T cell clone 433.

In addition, lysis of B10.A(4R) lymphoblasts, which express I-A^k but not I-E^k antigens, was blocked completely with anti-D^b antibodies. Because the I-A^k antigen did not trigger cytolysis, these data suggested that 433 recognizes the I-E^k antigen. When I^k bearing targets (B10.BR LPS induced blasts or TA-3 cells) were used, lysis was unaffected by anti-H-2D^b antibodies. Anti-I-A^k-specific antibodies did not inhibit lysis of I^k-positive cells, but the anti-I-E^k antibodies 14-4-4S and M5/114 and to a lesser degree also 17-3-3S, did inhibit lysis. These data therefore demonstrate that clone 433 recognizes the I-E^k antigen.

The proliferation data presented in Table II show that B10.A(5R) does not induce proliferation of clone 433. In the B10.A(5R) mouse, the I-E antigen consists of E_α derived from the k-haplotype and E_β from the b-haplotype (33). This molecule is expressed on the cell membrane (34) but is not recognized by clone 433. I-E is recognized only if both the α- and the β-chain are derived from the k-haplotype, as is the case in B10.MBR mice.

TABLE V

Inhibition of cytotoxic activity of clone 433 with mAb

Hybridoma	Specificity	Percent Specific ^{51}Chromium Release[a] from Target Cells			
		B10.A(4R)[b] H-2: k,k,-,b[c]	EL-4 b,-,-,b	B10.BR k,k,k,k	TA-3 d/k,d/k,d/k,d
—[d]	—	24	59	54	55
H141-51	D^b	3	2	83	57
H166-32.1	D^b	0	3	62	62
H172-93.1	D^b	1	4	75	57
10-2-16	$I\text{-}A^k$	38	60	83	57
17-227	$I\text{-}A^k$	38	64	83	60
K22-42-2	$I\text{-}E_\beta$	31	64	65	62
17-3-3S	$I\text{-}E^k$	26	65	38	47
14-4-4S	$I\text{-}E^k$	32	71	16	9
M5/114	$I\text{-}E^k$	33	65	3	3
Spontaneous release		30	10	40	6

[a] In a 3.5-hr test. Clone 433 was present at a 3:1 E:T ratio.
[b] Target cells were LPS-induced blasts.
[c] H-2 haplotype: K, I-A, I-E, D.
[d] Release in the absence of antibodies.

Double specificity of CTL-clone 433 for class I and II antigens

To assure that the same population of cells mediated cytotoxicity directed against class I and class II antigens, cold target competition experiments were performed (Fig.1). Because clone 433 lysed H-2^k targets more efficiently than it lysed H-2^b target cells, lysis of H-2^k targets was examined at an effector to target ratio of 1:1 (Fig.1A,C) and lysis of H-2^b targets at an E:T ratio of 3:1 (Fig.1B,D). Inhibition of lysis of TA-3 or LPS induced B10.BR blasts could only be observed with TA-3 or B10.BR blasts respectively (Fig.1A,C), but not with EL-4 cells or C57BL/6 blast cells.
In contrast, lysis of H-$2D^b$ targets could be inhibited with EL-4 or C57BL/6 blast cells, and in addition, very efficiently with either TA-3 cells (Fig.1B) or B10.BR blasts (Fig.1D). In both cases irrelevant cells such as BALB/c blasts or P-815 cells did not significantly inhibit lysis. These latter experiments provide evidence that the two specificities are mediated by the same cells.

Sensitivity of the cytotoxicity to anti-Lyt-2 mAb

It has been suggested that the Lyt-2 antigen plays a role in enhancing the avidity of the T cell to target cell binding (35). It may exert this function by binding to a monomorphic determinant on the class I molecule (12).
To characterize clone 433 (Lyt-2^+) further, the influence of anti-Lyt-2 antibodies on both specific activities was evaluated. The cytotoxicity of clone 433 on tumor cells and LPS induced blasts carrying either the H-$2D^b$ antigen or $I\text{-}E^k$, was tested in the presence of several mAb's (Table VI). The inhibition with anti-D^b or anti-E^k antibodies is in agreement with the results in Table V.

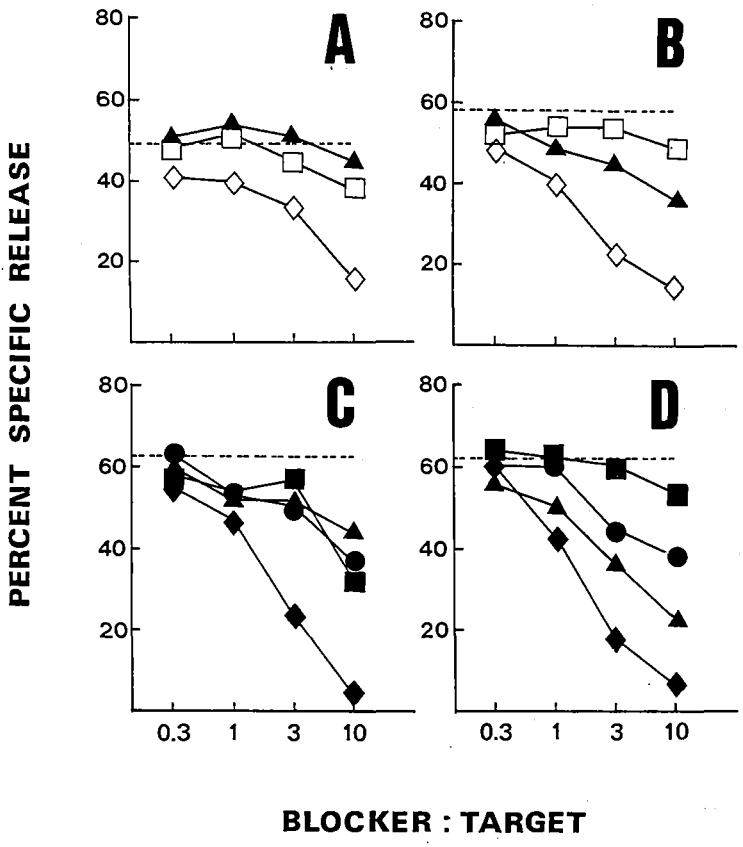

Figure 1. Cold target competition experiments. Cytotoxicity of clone 433 was measured in the presence of various unlabeled target cells at the ratios indicated.
A. Target cells were TA-3 cells and the effector to target ratio was 1:1.
B,D. Target cells were EL-4 cells and effector to target ratio was 3:1.
C. Target cells were B10.BR blast cells and effector to target ratio was 1:1. ▲ = EL-4, □ = P-815, ◇ = TA-3, ◆ = B10.BR, ● = C57BL/6, ■ = BALB/c.

TABLE VI

Inhibition of cytotoxic activity of clone 433 with anti-Lyt-2 mAb

Hybridoma	Specificity	Percent Specific ^{51}Chromium Release[a] from Target Cells			
		B10.HTG[b] H-2: d,d,d,b[c]	EL-4 b,-,-,b	B10.MBR b,k,k,q	TA-3 d/k,d/k,d/k,d
—[d]	—	24	57	49	62
M5/114	I-E^k	24	64	5	3
H141-51	D^b	0	2	62	59
GK 1.5	L3T4	27	59	69	60
53-6.7	Lyt-2	6	8	47	54
9-1 D10	Lyt-2	0	9	57	52
I-17	LFA-1	6	11	5	4
Spontaneous release		25	15	30	6

[a] In a 3.5-hr test. Clone 433 was present at a 3:1 E:T ratio.
[b] Target cells were LPS-induced blasts.
[c] H-2 haplotype: K, I-A, I-E, D.
[d] Release in the absence of antibodies.

Surprisingly, anti-Lyt-2 specific mAb 53-6.7 and 9-1 D10 only inhibited the H-2D^b specific lysis. In contrast, I-E^k specific lysis was not inhibited. Anti-Lyt-2 containing supernatant in a 1 to 64 dilution did inhibit D^b specific lysis but the same supernatant 1:4 diluted did not block I-E^k specific cytotoxicity (data not shown). This illustrates the differential sensitivity of the cytotoxic activities to anti-Lyt-2 antibodies.

As expected, anti-L3T4 had no effect because CTL clone 433 does not express L3T4, as examined by immunofluorescence.

Because anti-LFA-1 antibodies have been found to be very effective in inhibiting cytotoxic activities (36) we tested whether such antibodies could inhibit both specific activities of clone 433. Indeed, the specific cytotoxicity of clone 433 for both class I and class II was strongly inhibited by antibody produced by hybridoma I-17 which is specific for LFA-1.

DISCUSSION

In this report we show that an alloreactive cytotoxic T cell clone derived from a BALB/c (H-2^d) anti-C57BL/6 (H-2^b) MLC recognizes a class I (H-$2D^b$) and cross-reacts to a greater extent (i.e. comparable to heteroclitic antibodies) (37,38) with a class II (I-E^k) antigen. The cytotoxic specificity was confirmed by blocking experiments with mAb. Anti-D^b antibodies could inhibit H-$2D^b$-specific but not I-E^k-specific lysis and conversely, anti-I-E^k specific mAb only blocked the I-E^k specific lysis. The finding that unlabeled I-E^k bearing cells could inhibit lysis of H-$2D^b$ target cells, proved that the two specificities are expressed by the same T cells. The cold target competition experiments do not necessarily imply that the two antigens are recognized by the same receptor. This cytotoxic T cell clone with a double specificity for class I and class II antigens, may illustrate that these two classes of antigens (either alone or together with less polymorphic determinants) are either seen by one receptor with one recognition site or each by one of the two postulated recognition sites of the T cell receptor. Although the presented T cell clone may be a rare case, it illustrates the possibility that alloreactive T cells may use the same lytic mechanism to lyse via class I as well as via class II antigens.

That these two specificities are mediated by the same cloned population of T cells was clearly demonstrated by cold target competition experiments. The H-$2D^b$ specific lysis could be blocked very efficiently with unlabeled target cells bearing I-E^k antigen. The inability of H-$2D^b$ carrying cells to block I-E^k specific lysis probably reflects a lower affinity of the T cell antigen receptor(s) for H-$2D^b$ when compared with that for I-E^k. This view is supported by two additional observations. First, H-$2D^b$ bearing targets were not lysed as well as I-E^k bearing target cells. Secondly, anti-Lyt-2 antibodies inhibited lysis of H-$2D^b$ targets readily, whereas the I-E^k specific lysis could not be blocked.

A relative resistance to anti-Lyt-2 antibodies of class II specific CTL has been suggested previously (17). Uncloned populations of Ia-specific cytotoxic T cells were reported to be less sensitive to inhibition with anti-Lyt-2 antibodies than anti-H-2K CTL. However, anti-class II CTL clones have also been reported that were easily inhibited with anti-Lyt-2 antibodies (14).

MacDonald and co-workers (35) proposed that the interaction of the Lyt-2/3 complex with a structure on the target cell membrane may enhance the avidity of the effector to target cell interaction, e.g. by recognition of a monomorphic determinant on the class I molecule (12). Accordingly, the finding that the clone's activity against H-$2D^b$ targets can be blocked by anti-Lyt-2 antibodies, whereas its anti-I-E^k activity cannot, could be explained in two ways. If Lyt-2 were involved in both cell interactions, then it would be a limiting factor only in the lower affinity recognition of D^b but not in the higher affinity anti-I-E^k activity. Alternatively, if Lyt-2 were directly involved in recognition of class I in the case of

H-2D^b specific lysis, it would not play a crucial role in the recognition of class II in the case of I-E^k specific lysis. Nevertheless, the possibility that Lyt-2 is involved in recognition of class II antigens cannot be excluded, because class II specific cytotoxic T cells that were sensitive to inhibition with anti-Lyt-2 antibodies have been described (14).

Unexpectedly, the cross-reactive specificity of clone 433 for I-E^k is apparently of a higher affinity than its original specificity for D^b. The existence of cytotoxic T cells specific for class I antigens, with a heteroclitic specificity for a third party class I antigen, has been suggested by earlier studies (39,40). The phenomenon of heteroclicity has been described and analyzed in great detail for antibodies (37,38). Various researchers have described antigen-specific, MHC-restricted T cells that cross-reacted with alloantigens (41-43). In at least one example this cross-reactivity was of a lower affinity because it could be inhibited by anti-Lyt-2 antibodies, in contrast to the restricted antigen-specific cytolysis (35). A similar case was recently described for helper T cell activation and anti-L3T4 antibodies (44).

Class II-specific alloreactive cytotoxic T cells with the Lyt-2 phenotype have been described by several groups (13-17). Most of these cells however, had been cultured under conditions in which only class II antigens were stimulatory. Lyt-2$^+$ cells might have had a growth advantage under the culture conditions used, explaining the preferred Lyt-2 phenotype. Differential growth requirements for subsets of lymphocytes have been described (45,46). However, L3T4 positive cytotoxic T cell clones that are either alloreactive against class II antigens (16,18,47) or are antigen specific and class II restricted (10,11,48,49) have also been reported. Such unconventional CTL may in fact not be that rare, because recent studies (50,51) suggest that class II specific alloreactive CTL appear to occur in unprimed spleen cell populations in surprisingly high frequencies, i.e. only two- to tenfold lower than that of class I specific CTL. These primary class II specific CTL were shown to consist of two subpopulations expressing either the L3T4 or the Lyt-2 marker.

Clone 433 is a cytotoxic clone that is Lyt-2$^+$ and recognizes a class I antigen. For this specificity of the clone, the correlation between specificity for the class of MHC antigen, phenotype and function seems to be preserved. The second, cross-reactive and "heteroclitic" specificity for a class II antigen (which it has never encountered during the sensitization <u>in vitro</u>) seems to contradict this general correlation for the same effector T cell.
It is impossible to decide whether the presented cytotoxic T cell clone reflects an exception or a general case for class II specific alloreactive CTL. Because most of the class II specific Lyt-2$^+$ T cells that have been described were alloreactive (one exception, a Lyt-2$^+$ influenza virus specific cytotoxic T cell clone has only recently been described) (52), we like to speculate that such T cells may be comparable to the one described in this report: class I specific Lyt-2$^+$ cytotoxic T cells, that cross-react with an allogeneic class II antigen.

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CHAPTER 5

THE PRIMARY STRUCTURE OF THE ANTIGEN RECEPTOR OF AN ALLOREACTIVE CYTOTOXIC T CELL CLONE.

Data presented in this chapter will be published.

ABSTRACT

Clone 653 is a murine, cytotoxic T lymphocyte clone specific for the H-2D^b alloantigen. The cytotoxic activity of this clone is inhibited by the anti-receptor monoclonal antibody 44-22-1. This antibody was originally generated against the receptor of clone 3F9, another H-2D^b specific T cell clone. The primary structure of the antigen receptor of clone 653 has been determined. The receptor is composed of the following gene segments, $V_{\alpha 4.3}$, $J_{\alpha 653}$, $V_{\beta 6}$, $D_{\beta 1}$, $J_{\beta 2.6}$ and $C_{\beta 2}$. Comparison of the structure of the receptor of clone 653 with that of clone 3F9 reveals that only the $V_{\beta 6}$ gene segment is shared between both receptors. This suggests that the $V_{\beta 6}$ gene segment encodes the determinant that is recognized by the antibody 44-22-1.

INTRODUCTION

T lymphocytes recognize antigen in the context of membrane molecules that are encoded by the Major Histocompatibility Complex (MHC). For cytotoxic T cells the restricting element is usually a class I molecule [Zinkernagel & Doherty, 1974] whereas helper T cells are normally class II restricted [Katz et al, 1975]. The MHC is highly polymorphic [Klein, 1975; Klein et al, 1981] and a high proportion of T cells recognize foreign MHC molecules. The recognition of MHC molecules encoded by different alleles is called alloreactivity.
The structure on T cells, the T cell receptor, which is responsible for the simultaneous recognition of antigen plus MHC, or, in the case of alloreactivity, of foreign MHC, has first been identified with monoclonal antibodies [Allison et al, 1982; Haskins et al, 1983; Meuer et al, 1983]. The T cell antigen receptor has a molecular weight of 90 kD and consists of an α and a β chain. The genes that encode these two polypeptides have been cloned and characterized [Saito et al, 1984; Chien et al, 1984; Hedrick et al, 1984; Yanagi et al, 1984; Marrack & Kappler, 1986; Kronenberg et al, 1986]. The mechanism that is employed by the T cell receptor genes to generate maximum diversity has been shown to be very similar to that of the immunoglobulin genes [Tonegawa, 1983; Yancopoulos & Alt, 1986]. Variable, diversity and joining gene segments are joined during T cell differentiation and form, together with a constant gene segment, a functional gene.

In previous studies, the isolation of an alloreactive, cytotoxic T cell clone, 3F9, with specificity for H-2D^b has been reported [Acha-Orbea et al, 1985]. Two monoclonal antibodies, 44-22-1 (IgG2a) and 46-6B5 (IgM), have been generated that react clone-specifically with clone 3F9 [Acha-Orbea et al, 1985]. No differences in specificity between the two antibodies have been detected so far. Subsequently, a panel of H-2D^b specific CTL has been analyzed for reactivity with these monoclonal antibodies [Schilham et al, 1987]. A correlation between reactivity with 44-22-1 and specificity for H-2D^b was not demonstrated, although the cytotoxicity of one of the independently derived CTL clones, clone 653, was also inhibited with 44-22-1 [Schilham et al, 1987]. In order to characterize the antigenic determinant that is recognized by the monoclonal antibodies 44-22-1 and 46-6B5, the primary structure of the antigen receptor of clone 653 has been determined. Comparison of this receptor structure with that of clone 3F9 [Rupp et al, 1985, 1987] suggests that the $V_{\beta 6}$ gene segment encodes the antigenic determinant for 44-22-1 and 46-6B5.

MATERIALS AND METHODS

Animals

BALB/c and C57BL/6 mice were obtained from the Veterinärmedizinisches Institut für Zuchthygiene der Universität Zürich. Mice of both sexes were used at ages between 2 and 6 months. Rats (1-3 months of age) were donated by the Institut für Immunologie und Virologie der Universität Zürich.

Media

Single cell suspensions of spleen cells were prepared in Hanks' Balanced Salt Solution. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, KC Biological Inc., Lenexa, KS) supplemented with 3,024 g/l $NaHCO_3$, 100 U/ml penicillin and 100 µg/ml streptomycin (KC), $5x10^{-5}$ M 2-mercaptoethanol and 10 % (v/v) heat inactivated fetal bovine serum (Gibco, Grand Island, NY). This medium is referred to as complete medium. All cultures were kept in a water saturated atmosphere containing 5 % CO_2 at 37° C. Interleukin-2 containing supernatant from rat spleen cells stimulated with phorbol myristate acetate (PMA) and concanavalin A was prepared as described [Kast et al, 1984].

Culture conditions

Clone 653 has been generated as described [Schilham et al, 1987] and is of BALB/c origin with an alloreactive cytotoxic specificity for H-$2D^b$. The clone was continuously kept in culture for periods longer than 6 months by restimulation once a week with irradiated C57BL/6 spleen cells ($5x10^6$/ml) in 10 % Con A supernatant.

Isolation of RNA

Seven to ten days after restimulation T cells were removed from the bottom of the culture flasks with PBS containing EDTA (1.45 g/l) [Acha-Orbea et al, 1983]. Cells were washed twice and the cell pellet was rapidly frozen and kept at -70° C. Upon thawing, 2 ml of GTC solution was added (4 M guanidine thiocyanate, 25 mM Na-citrate, 0.5 % sarkosyl, 0.8 % 2-mercaptoethanol) and cells were shaken [Chirgwin et al, 1979]. After addition of 0.5 ml of a 5.7 M CsCl solution (5.7 M CsCl, 0.1 M EDTA, pH 7.0) the lysate was layered on top of 2.5 ml 5.7 M CsCl in an ultracentrifuge tube. The gradient was centrifuged for 20 hrs. at 20° C at 35,000 rpm in a SW50.1 rotor (Beckman Instruments Inc., Palo Alto, CA). After centrifugation, protein and DNA were carefully removed and the RNA containing pellet was dissolved in 0.5 ml TES (10 mM Tris, pH 7.5, 5 mM EDTA, 1 % SDS). After phenol/chloroform extraction (phenol/chloroform/isoamylalcohol, 20/20/1, 0.4 g/l 8-hydroxyquinoline) and ethanol precipitation [Molecular cloning, Maniatis et al, 1982].
Poly(A)$^+$ RNA was isolated on oligo(dT)-cellulose (Boehringer, Mannheim, FRG).

cDNA Synthesis

Six μg of poly(A)$^+$ RNA or 50 μg of total RNA was denatured in 2 mM methylmercuric hydroxide (MeHgOH) for 5' at 20° C. After adjustment to 4 mM 2-mercaptoethanol and addition of 1 μl RNasin (Anglian Biotechnology Ltd., Colchester, UK), cDNA was made in a volume of 200 μl, containing 6000 U/ml Moloney Murine leukemia virus reverse transcriptase (BRL, Gaithersburg, Maryland), 0.5 mM dNTP's (Pharmacia, Uppsala, Sweden), 200 μg/ml BSA (Boehringer), 5 μg/ml oligo(dT)$_{12-18}$ primer (Pharmacia), 50 mM Tris pH 7.5, 75 mM KCl, 3 mM $MgCl_2$. To 10 μl of the reaction, 1 μl [α-^{32}P]dCTP (±10 μCi, Amersham, UK) was added and the reaction was incubated for 1.5 hours at 37°C. cDNA was passed over a Sephadex G50 column to remove free nucleotides, phenol/chloroform extracted and precipitated. Efficiency of first strand synthesis varied between 10 and 30 %.

Second strand synthesis was carried out in 200 μl containing, 150 U/ml DNA polymerase I (Boehringer), 15 U/ml RNase H (BRL), 1μg/ml DNA ligase (NAD$^+$, E.Coli, Pharmacia), 0.1 mM dNTP's, 100 μg/ml BSA, 0.5 mM dithiothreitol (DTT), 0.5 mM β-NAD, 20 mM Tris pH7.5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 100 mM KCl [Gubler & Hoffman, 1983]. Incorporation was monitored by adding 1 μl [α-^{32}P]dCTP to 10 μl of the reaction volume. After 1 hour of incubation at 12°C and 1 hour at 20°C the double stranded cDNA was recovered as described above. Efficiency of second strand synthesis varied between 50 and 100%.

Methylation and blunting

The EcoRI recognition sites of the cDNA were methylated in 20 μl reaction volume with, 500 U/ml EcoRI methylase (New England Biolabs, Beverly, MA), 0.1 mM S-adenosylmethionine, 100 mM Tris pH 8.0, 10 mM EDTA. The reaction was incubated for 45 minutes at 37°C.

After phenol/chloroform extraction and ethanol precipitation, the cDNA was blunted in 30 μl containing 60 U/ml T4 polymerase (Pharmacia), 33 μM dNTP's, 200 μg/ml BSA, 67 mM Tris pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 16.6 mM $(NH_4)_2SO_4$, for 30 minutes at 37°C. The DNA was passed over Elutip (Schleicher & Schuell, Dassel, FRG) before it was used for linker ligation.

Addition of the EcoRI-linkers

To enable insertion of the cDNA-molecules in the EcoRI site of bacteriophage Lambda gt10 [Huynh et al, 1984], EcoRI-linkers were ligated to the blunt cDNA. The reaction volume was 8 μl, containing 20,000 U/ml T4 DNA ligase (Biolabs), 250 U/ml T4 RNA ligase (Biolabs), 5mM rATP, 500 μg/ml BSA, 60 μg/ml 5'-pd(GGAATTCC)-3' (Pharmacia) 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidin. The reaction was incubated overnight at 14°C.

The ligated DNA was heated for 10 minutes at 65°C to inactivate the enzym, and digested with the restriction enzym EcoRI in a volume of 100 μl. The conditions were, 1000 U/ml EcoRI (Anglian Biotechnology Ltd., Colchester, UK), 100 μg/ml BSA, 2 mM spermidin, 50 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. The digestion was incubated at 37°C for 4 hours.

Size selection of the cDNA
To remove free linkers and to obtain cDNA of certain sizes, the cDNA was electrophoresed in a 1% agarose gel. The gel was cut at the appropriate places and the DNA was electroeluted in a Biotrap system (Schleicher & Schuell).

Ligation of cDNA in bacteriophage
The eluted material was ligated in the EcoRI site of Lambda gt10 [Huynh et al, 1984] in 5 µl. 0.4 µg Lambda gt10 (EcoRI digested), 20-40 ng cDNA, 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidin were heated to 70°C and after cooling, 10,000 U/ml T4 DNA ligase and 5 mM rATP were added. The ligation was incubated overnight at 14°C.

Plating and screening of the recombinant phages
The recombinant phage DNA was packaged in commercially available packaging mix (Amersham) and plated on POP 101 (=RH2241,[Lathe & Lecocq, 1977]) which carries a high frequency of lysogeny (hfl) mutation [Huynh et al, 1984]. Under these conditions only phages that have incorporated a cDNA fragment will cause lysis. The libraries were plated on large plates (250 x 250 mM, Nunc, Roskilde, Denmark), transferred to nitrocellulose filters (nitrocellulose HATF 293, Millipore Corp., Bedford, MA) and screened with radiolabeled probes [Feinberg & Vogelstein, 1983].

Probes
cDNA probes containing the constant gene segments of α and β chain isolated from clone 3F9 were used as probes [Rupp et al, 1985, 1987].

Sequencing
cDNA clones of interest were excised from the bacteriophage and subcloned in the polylinker of the plasmid vector pTZ18/19R (Pharmacia). This plasmid, in combination with the M13K07 helper phage produces single strand DNA, which has been sequenced according to the dideoxy chain termination method [Sanger et al, 1977]. Either DNA polymerase I (Klenow fragment, Promega, Madison, Wisconsin) or Sequenase (United States Biochemical Corporation, Cleveland, Ohio) were used.

RESULTS

Initially a cDNA library of clone 653 has been produced that contained 1.2 x 10^6 independent recombinant phages. The cDNA for this library had been selected for inserts greater than 0.3 kb. The frequency of clones that hybridized with a probe of the constant gene segment of the β chain gene, was 1 in 5000. Many of these clones were between 0.4 and 0.8 kb in length and did not contain sequences of the joining or variable regions. The sequence of the beta chain gene that is presented here has been derived from two clones of 0.9 and 1.1 kb length respectively. For the isolation of cDNA clones coding for the α chain, a second library has been produced from cDNA that was selected for inserts greater than 1.1 kb. This library consisted of 2 x 10^5 plaques and the frequency of cDNA clones containing α chain sequences was 1 in 30,000. Half of the library was screened and three α clones were isolated. Two clones represent functional transcipts containing variable and joining gene segments and the third contained intron sequences.

α chain

Three clones of 1.4 kb length containing C_α sequences have been isolated. The sequence of two clones that are transcripts of the same functional gene is shown in figure 1. The variable gene segment is almost the same as the $V_{\alpha 4.3}$ gene segment that is expressed in the T helper clone AP11.2 [Winoto et al, 1986]. Clone AP11.2 is specific for cytochrome c in the context of E^S. The sequence of the variable gene segment of clone 653 extends further in 5' direction than the published sequence of clone AP11.2 and includes two possible translation initiation codons (ATG). The second of these two ATG codons probably represents the major start of translation, because the surrounding nucleotides resemble more the consensus sequence as proposed by Kozak [Kozak, 1987]. In this respect the A at position -3 and the G at position +4 are indicative. The putative site where the leader peptide is cleaved off has been estimated from published data [von Heijne, 1986; Kronenberg et al, 1986]. There are two non-silent nucleotide changes in the sequence of $V_{\alpha 653}$ when compared with $V_{\alpha AP11.2}$, at positions 86 and 106. Two amino acid residue changes (Pro to Gln and Glu to Lys) are the result. From comparisons with other V_α sequences it can be deduced that the 3' border of the $V_{\alpha 653}$ gene segment is at position 339-341. Without the germline sequence of this variable gene segment it is difficult to say exactly which nucleotides belong to the V gene segment.
The rearranged J_α gene segment maintains an open reading frame from V to C. The sequence of this J gene segment has, to our knowledge, not been described before. It contains the typical amino acid sequence G-X-G-T-X-L that is conserved in all J gene segments [Arden et al, 1985; Kronenberg et al, 1986]. Without the genomic sequence of this particular J segment it is impossible to say whether, and to what extend N-region diversity has occurred.

The third cDNA clone that has been isolated from this library contains a normally processed C_α gene as determined by

restriction enzym mapping (data not shown). The 5' end of this clone does not resemble any of the known V_α gene segments. The sequence is shown in figure 2. There are no open reading frames longer than 180 nucleotides. The nucleotides directly 5' to the first C_α exon contain the right splicing junction (acceptor) as reported before [Hayday et al, 1985]. Obviously, this cDNA clone represents a transcript containing part of the intron between the J gene segments and the C gene.

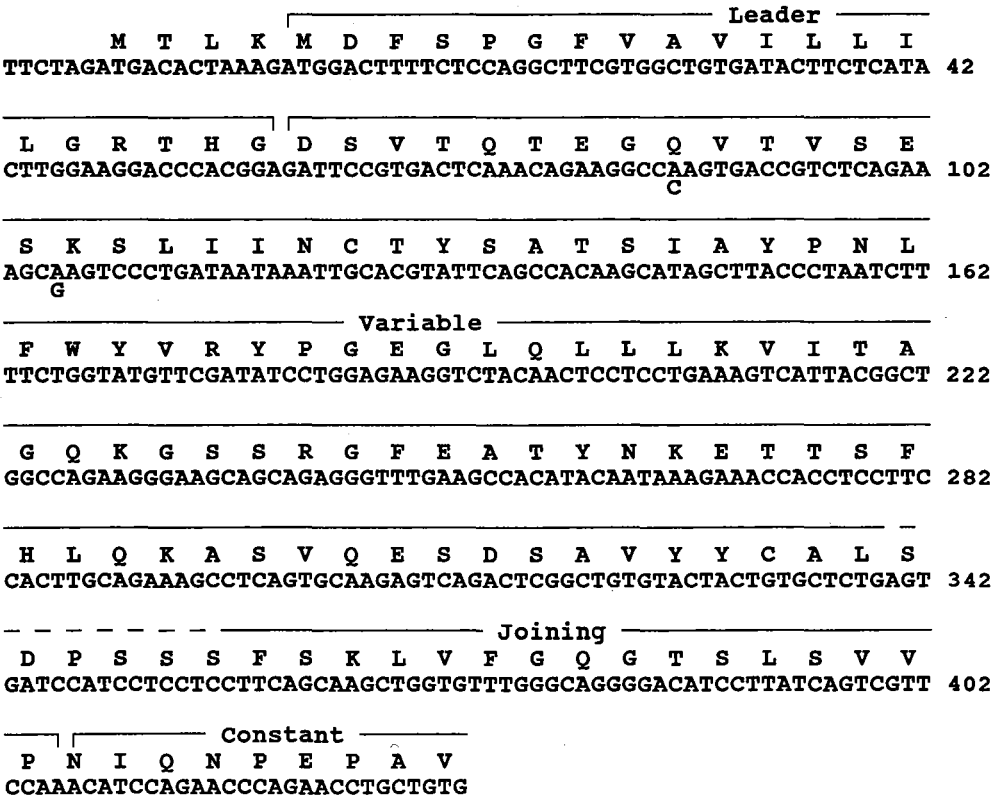

Figure 1. Sequence of the variable portion of the α chain of the antigen receptor of clone 653. The nucleotide sequence as well as the predicted amino acid residue sequence of the leader, variable, joining and first part of the constant gene segment are presented. A previously published sequence of the $V_{\alpha 4.3}$ gene segment has two non-silent substitutions at positions 86 and 106.

β chain

The sequence of the longest cDNA clone coding for the β chain of clone 653 is shown in figure 3. The variable gene segment, $V_{\beta 6}$, is the same as that used by clone 3F9 (BALB/c anti-H-$2D^b$) and clone LB2-1 (anti-Chicken Red Blood Cell plus I-A^b) [Rupp et al, 1985; Giedlin et al, 1986]. The sequences of the three $V_{\beta 6}$ gene segments from these clones are identical except at positions 325 and 349 (figure 3). The differences are between 653 and 3F9 on one hand and LB2-1 on the other. The 3' border of the variable gene segment is probably the C at position 349. Two nucleotides that are present in 3F9 and LB2-1 seem to have disappeared in clone 653 during the process of rearrangement. In clone 653, $V_{\beta 6}$ is functionally rearranged to $J_{\beta 2.6}$, whereas in clone 3F9 rearrangement is to $J_{\beta 1.1}$. Six nucleotides at the 5'end of the $J_{\beta 2.6}$ gene segment have also been lost. Between the V and the J gene segments, five nucleotides derived from $D_{\beta 1.1}$ have been inserted plus two nucleotides (TT) that cannot be assigned to any germline sequence. These nucleotides (N-region diversity) have probably been introduced by the enzym terminal deoxynucleotidyl transferase (TdT) [Desiderio et al, 1984]. When the amino acid residue sequences of the beta chain of 653 and 3F9 in the J region are compared, seven substitutions (but no deletions or additions) are observed (figure 4).

```
CAATGGCCCGGTAGTGTGAGAGATTTTCCAACTTAATGTTAAAATATCACACGCCTTCGT  60

TTCTCACCAACGTCCAGCCTACCTGGGGGAGAGTACTCTAGAGAAGACATCTAAGTCTCT 120

GTTTTCTGAGCTTGTTTTTGTCCTGCCAAAACAAAATTGTTTGACTGGACCCACTGGGTT 180

CTGAAGATCCTGTTAAGTAGAAAAATGACAAGATGGTTTAGTGGTTCCACCTTCCTTTCT 240

GGTTTTCCCAGATACCAGAAAGGACCCCGTGGAGAGGGACTACTGAAAGCTAGAGATCTT 300

GGCTGGCACTCATGACTTGTGCCTGTCCCTAAGCCTGTGTCCATCATGGGCAGCTAGATC 360

CTAGGCTGTGCAATTTCCAATGTGAAGTCTGTGAACAAGACTGGCTAGTCCAGAGAGTTC 420

CGCTCTTGCCTGTCACTGGCATCTGAGTTCTGACCTAAGTTAGGACCCCCAAAAAATCTT 480

                                          ┌──────── Constant ────────
CTAACATCAGTCCTCTTGGCCCACAGACATCCAGAACCCAGAACCTGCTGTGTACCAGTT 540
```

Figure 2. Sequence of the α transcript containing an intron sequence. The right splicing junction (acceptor) sequence directly 5' to the constant gene segment is underlined.

Figure 3. Sequence of the variable portion of the β chain of the antigen receptor of clone 653. The nucleotide sequence as well as the predicted amino acid residue sequence of the leader, variable ($V_{\beta 6}$), diversity ($D_{\beta 1}$) and joining ($J_{\beta 2.6}$) gene segments are shown. Two non-germline nucleotides (TT) have been inserted in between the D and J. At positions 325 and 349 two nucleotide differences with the $V_{\beta 6}$ gene segment of clone LB2-1 (see text) are indicated.

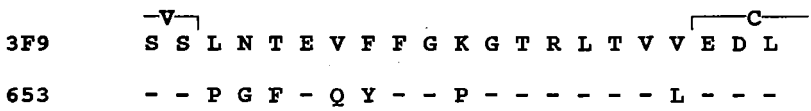

Figure 4. Comparison of the protein structure of VDJ junctions of the β chains of clones 3F9 and 653.

DISCUSSION

The complete primary structure of the T cell antigen receptor of the alloreactive CTL clone 653 has been determined. Clone 653 is particularly interesting because it recognizes the H-2D^b alloantigen, just like the previously described CTL clone 3F9 [Acha-Orbea et al, 1985]. In addition, both clones are inhibited by the 3F9 specific monoclonal antibody 44-22-1, but have distinct patterns of rearrangement of the genes that code for the β chain of the T cell receptor [Schilham et al, 1987]. The receptors of these two clones are therefore different but, on the other hand, must share certain aspects. The specificity and T cell receptor structure of both clones are summarized in table 1. The α chain of clone 653 is composed of a variable gene segment of the $V_{\alpha 4}$ family [Winoto et al, 1986; Kronenberg et al, 1986] and a joining gene segment that has not been described yet. The β chain consists of $V_{\beta 6}$, $D_{\beta 1.1}$ and $J_{\beta 2.6}$ and the second C_β gene. The structure of the receptor of clone 3F9 has been published before [Rupp et al, 1985, 1987].
The sequences of $V_{\alpha AP11.2}$ and of $V_{\alpha 653}$ are identical except at two nucleotide positions. Likewise, the $V_{\beta 6}$ gene segment of 653 (and of 3F9) is slightly different from the sequence published for the $V_{\beta 6}$ gene segment of LB2-1. Three explanations for these minor differences are, cloning or sequencing errors, allelic differences or somatic mutation. If we assume that no cloning errors have been made, allelic differences would be consistent with the results. Clones 653 and 3F9 have been derived from BALB/c mice , clone LB2-1 from C57BL/6 and AP11.2 from B10.S(9R) mice [Schilham et al, 1987; Acha-Orbea et al, 1985; Giedlin et al, 1986; Winoto et al, 1986]. Without the germline sequence of these gene segments in the various mouse strains it is difficult to state that no somatic mutation has occurred but an argument against the occurrence of somatic mutation, at least in the BALB/c derived clones, is the fact that the sequences of the $V_{\beta 6}$ gene segments of 3F9 and 653 are completely identical to each other. In contrast to the immunoglobulin genes [Tonegawa, 1983], the T cell receptor genes have not been found to be subjected to extensive somatic hypermutation [Kronenberg et al, 1986; Marrack & Kappler, 1986].

Table 1. Summary of the characteristics of clone 3F9 and 653.

Clone	Specificity	Reactivity with mAB 44-22-1	Usage of TCR gene segments				
			V_α	J_α	V_β	D_β	J_β
3F9	H-2D^b	+	8	TA19	6	-	1.1
653	H-2D^b	+	4.3	653	6	1	2.6

Clone 653 and clone 3F9 both recognize the H-2D^b alloantigen. For this identical specificity each clone has a unique receptor structure. The only gene segment that is shared between these two clones, is the $V_{\beta 6}$ segment. This example illustrates that two receptors without any significant homology can recognize the same alloantigen. However, both receptors may recognize this antigen differently. The different nature (relative affinity or different epitopes) of the recognition of H-2D^b by both clones, has been suggested by the earlier observation that the two clones show a different sensitivity to inhibition with the monoclonal antibody H172-93 [Schilham et al, 1987].
The isolation of a cDNA clone containing an intron sequence could have two explanations. The first is that the cDNA has been transcribed from an uncompletely processed mRNA molecule. The intron between J and C had not been removed by splicing at the time of cDNA production. The second explanation could be that transcription has initiated at a promoter-like sequence in this intron.
The results presented here suggest that antibody 44-22-1 (and 46-6B5) recognize the $V_{\beta 6}$ gene segment. This suggestion is further supported in Chapter 6.

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CHAPTER 6

TWO MONOCLONAL RAT ANTIBODIES WITH SPECIFICITY FOR THE Vβ6 REGION OF THE MURINE T CELL RECEPTOR

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ABSTRACT

Two rat monoclonal antibodies (mAb) 44-22-1 and 46-6B5 which recognize an alloreactive cytotoxic clone, 3F9, have been further tested on a panel of T hybridomas (T Hy) and cytotoxic T cell (Tc) clones for binding and functional activities. The mAb recognized only those cells sharing the expression of the T cell receptor (TcR) Vβ6 gene with 3F9. All $V\beta6^+$ cells were activated by these mAb under crosslinking conditions and their antigen specific activation was blocked by soluble mAb. Furthermore, depletion of $46\text{-}6B5^+$ normal lymph node T cells eliminated all cells expressing the epitope recognized by 44-22-1 and Vβ6 mRNA.

INTRODUCTION

Several types of monoclonal antibodies (mAb) and antisera have been raised against the α/β T cell antigen receptor (TcR). One group of such antibodies consists of anti-clonotypic antibodies that are able to recognize the unique combination of an individual TcR α/β heterodimer (1-3). The second group includes antibodies which are specific for a single TcR protein chain, such as for example the mAb A2B4 which recognizes the TcR α chain of the T hybridoma (T Hy) 2B4 (4). A third and particularly useful class of TcR specific reagents is comprised of mAb that are specific for individual TcR Vβ regions. This group includes KJ16 (5,6) and F23.1 (7,8) which recognize two or all members, respectively, of the three-membered Vβ8 family. Such antibodies have allowed analyses of V gene expression in different T cell populations that was not possible with anti-clonotypic reagents. Furthermore, the mAb have been crucial tools in studies of thymic T cell development. Another member of this group of reagents is the mAb KJ23 which recognizes Vβ17 (9). This mAb is of special interest, since correlation was found between KJ23 expression and IE^k alloreactivity.

Here we assign new serological reagents to the third group; namely, mAb that are specific for the murine TcR Vβ6 region. Two rat mAb raised against the BALB/c derived anti-$H\text{-}2^b$ cytotoxic T cell, 3F9, were originally described as anti-clonotypes (10). Further analyses with these antibodies which we describe in this report revealed that antibody binding to T cells correlates with expression of Vβ6 gene segment of the TcR. Initial binding studies were confirmed functionally, proving that both mAb are not anti-clonotypes but rather are specific for Vβ6, regardless of Dβ, Jβ or Vα expression.

MATERIALS AND METHODS

Cell lines

All the cytotoxic and helper T cell lines used in this investigation with their origin and specificities are described in Table 1. IL-2 dependent, mycoplasma-free CTLL-2 cells were obtained from American Type Culture Collection. A20, a B cell lymphoma, was obtained from the same source.

Antibodies

Armenian hamster B hybridoma cells, 145-2C11, producing antibodies to mouse CD3 were a kind gift of J. Bluestone (11). The rat mAb hybridoma, 44-22-1 (IgG 2a) and 46-6B5 (IgM), which specifically inhibit T cell clone 3F9, were produced by H. Acha-Orbea et al. (10). The rat mAb 9-1D10 is anti-mouse CD8 (10) specific. The mAb KJ16 which binds to TcR bearing either Vβ8.1 or Vβ8.2, but not Vβ8.3 (5,6), was obtained from J. Kappler and P. Marrack, Denver. Fluorescent antibodies included FITC-conjugated, affinity purified goat anti-rat IgG (heavy and light chain specific) and FITC-conjugated, affinity purified goat anti-hamser IgG (heavy and light chain specific) F(ab')2 fragments from Cappel, Cooper Biomedical, Scientific Division, West Chester, PA, as well as FITC-conjugated goat anti-rat Ig (absorbed on mouse Ig) from TAGO, Burlingham, CA.

FACS - analysis

T Hy: aliquots of 10^6 cells in 100 μl were stained with 44-22-1 or 145-2C11 as neat hybridoma culture supernatants in 0.1% NaN_3 followed by fluorescent goat anti-rat (44-22-1) or anti-mouse (145-2C11) Ig labelling. Flow cytometric analysis was carried out using a Becton-Dickinson FACScan System.
T_C cell clones and normal lymph node cells: aliquots of 10^6 cells in 100 μl were stained at 4°C with 44-22-1, 46-6B5, or KJ16 rat mAb as 25% hybridoma culture supernatants followed by a fluorescent goat anti-rat Ig labeling. Flow cytofluorometric analysis of viable cells were performed on either a Becton-Dickinson FACS II and IV or an Ortho Cytofluorograph 50.H instrument.
Data for both groups are expressed as mean fluorescence intensities in arbitrary units after analyzing 10^4 cells.

Activation with monoclonal antibodies

Sepharose-coupled mAb: rat mAb were purified by ammonium sulfate precipitation of ascites fluid and coupled to CNBr-Sepharose 4B beads following the manufacturer's protocol (Phamacia, Uppsala, Sweden). Two x 10^4 cloned CTL in 0.2 ml complete tissue culture medium in the absence of IL-2 were incubated with 10^3 mAb modified Sepharose beads. Cultures were pulsed with 1 μCi of tritiated thymidine to measure proliferation of the cells.
FcR crosslinking: cultures containing $4x10^4$ T Hy and 10^6 A20 cells bearing FcR were incubated with and without mAb as 25% hybridoma culture supernatants in a total volume of 0.2 ml. The mAb included 44-22-1 and 46-6B5 as test mAb and 145-2C11 as a positive control. Secreted IL-2 levels were measured after 24

hours by incubating IL-2 dependent CTLL-2 cells ($6x10^3$) with 50 μl of cell-free culture supernatant, followed by 1 μCi of tritiated thymidine at 6 hours, and harvesting after 18 hours using a multi-automated harvester. Incorporation of radioactivity is expressed as means measured from triplicate samples.

Blocking of antigen response with 46-6B5

Antigen specific activation of T Hy was measured by a standard IL-2 assay method (12). Cultures containing 3-4 x 10^4 T Hy and 5 x 10^5 irradiated spleen cells, as antigen presenting cells, were incubated with or without various doses (12.5-800 μg/ml) of antigen (beef insulin) for 24 hr in a volume of 0.2 ml. Secreted IL-2 was measured as described above. Blocking of antigen-stimulated IL-2 production was tested by including the mAb 46-6B5 as hybridoma culture supernatant at various concentrations in the 0.2 ml IL-2 production cultures described above. Incorporation of radioactivity was measured and is expressed as means of triplicate samples.

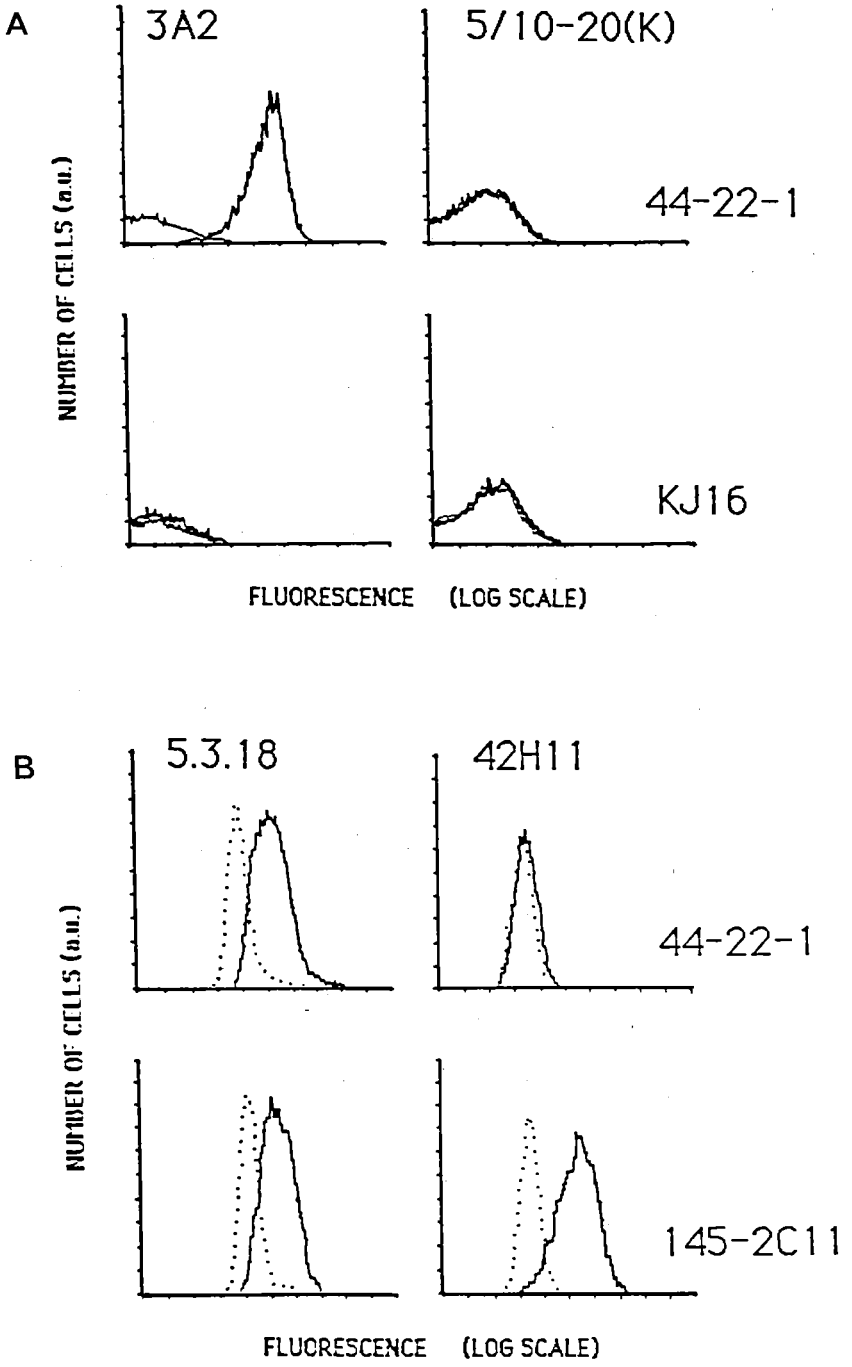
Depletion of lymph node cells using mAb 46-6B5 and complement

Nylon wool purified BALB/c lymph node cells were treated twice with 46-6B5 mAb as 50 % culture supernatant together with 5 % rabbit complement (Cedarlane, Hornby, Ontario) for 30 minutes at 37°C. Untreated and 46-6B5 depleted cells were stimulated with 3 ng/ml PMA, 250 ng/ml ionomycin and 30 U/ml recombinant human IL-2 (rIL-2) at a concentration of 2.5 x 10^5 cells in complete tissue culture medium. Cultures were diluted with fresh medium containing 20 U/ml rIL-2 on day 4 and harvested on day 7. Aliquots of the cells were stained either with mAb 44-22-1 or KJ16 followed by FITC-conjugated goat anti-rat Ig for FACS analysis.

Northern analysis

Total cellular RNA was prepared by lysis of cells in 1% NP-40 and subsequent phenol extraction as described by Scott et al. (13). RNA was fractionated by electrophoresis in a glyoxylate buffered agarose gel (14) and transferred onto Hybond-N nylon membrane (Amersham, U.K.)

Filters were hybridized with ^{32}P-labeled, nick translated DNA probes in 50 % formic acid, 5 x SSC, 50 mM potassium phosphate buffer pH 6.5, 5 x Denhardt's solution, 1% SDS, 0.1 ng/ml denatured salmon sperm DNA at 42°C. Filters were washed twice for 10 min. in 2 x SSC, 0.1% SDS at 42°C and once in 0,3 x SSC, 0.1 % SDS at 42°C for 30 min. The DNA fragments were Vβ6, an EcoRI/AvaI endonuclease fragment of the cDNA clone 653β25 (22). Filters were washed twice in water at 100°C for 5 min. for further hybridization experiments.


A
3A2
5/10-20(K)
44-22-1
KJ16
NUMBER OF CELLS (a.u.)
FLUORESCENCE (LOG SCALE)
B
5.3.18
42H11
44-22-1
145-2C11
NUMBER OF CELLS (a.u.)
FLUORESCENCE (LOG SCALE)

RESULTS AND DISCUSSION

Analysis of the fine specificity of the mAb 44-22-1 and 46-6B5

The mAb 44-22-1 and 46-6B5 were derived from a fusion of the myeloma cell line Y3M and spleen cells from a Lou rat immunized with the alloreactive cytotoxic T cell clone 3F9 (10). They were selected based on their capacity to inhibit the cytotoxic activity of 3F9. They also reacted with the chicken red blood cell specific, I-A^b restricted helper T cell clone LB2-1 that shares variable region genes with 3F9, namely Vα8 and Vβ6, but expresses different D and J region segments (16,17). To further characterize the specificity of the two mAb, they were screened on a panel of helper T Hy and T_C cell clones. Origin, antigen specificity, H-2 restriction, function and usage of Vα and Vβ TcR gene segments of these cells are listed in Table 1. Both mAb specifically bound to all T cells which express Vβ6 irrespective of Vα, Dβ or Jβ segment usage. Specificity for Vβ6 was further confirmed by the negative results obtained with the T cell clone 5/10-20(K) that expresses the identical Vα gene segment as 3F9 and LB2-1, but a different Vβ, and the T Hy 42H11 which expresses a Vα gene 98 % homologous to the Vα utilized by 5.3.18 and Vβ4. Fluorescence histograms for both pairs of cells are shown in Figure 1. All the control T cell lines described in Table 1 were negative in FACS analyses using 44-22-1 and 46-6B5 (data not shown). Reciprocal competitive antibody binding studies with 46-6B5 and 44-22-1 suggest that the two mAb recognize sterically very closely located epitopes on the TcR protein (R. Schneider, unpublished results).

Figure 1. A. Binding of 44-22-1 and KJ16 to 3A2 and 5/10-20(K). The two cytotoxic T cell clones 3A2 (Vα8.3F9, Vβ6) and 5/10-20(K) (Vα8.520K, Vβ7) were cytofluorometrically analyzed after labeling with the rat mAb 44-22-1 or KJ16 (▬▬) followed by FITC-conjugated goat anti-rat IgG antibodies. Staining with the fluorescent second antibody alone is represented by ——.

Figure 1. B. Binding of 44-22-1 and 145-2C11 to 5.3.18 and 42H11. The two T Hy 5.3.18 (Vα3, Vβ6) and 42H11 (Vα3, Vβ4) were cytofluorometrically analyzed after labeling with 44-22-1 or 145-2C11 (——) followed by FITC-conjugated goat anti-rat (44-22-1) or anti-hamster (145-2C11) Ig antibodies. The fluorescent antibody alone is also shown (---).

Table 1. Usage of TcR genes by T cell clones in correlation with mAb binding

T Cell Clone or Hybridoma	Mouse Strain	Specificity	T Cell Type	T Cell Receptor		MAb		Ref.
				α - Chain	β - Chain	44-22-1	46-6B5	
3F9 (3A2)	(BALB/c)	alloreactive D^b	T_c	V_α8.3F9 J_αTA19	V_β6 J_β1.1	+	+	10,15
LB2-1	(B6)	I-A^b-CRC (Mls^a)	T_H	V_α8.3F9 J_αLB2	V_β6 D_β2.1 J_β2.3	+	+	16,17
5.3.18	(bm12)	bm12-Insulin-B	T_H	V_α3	V_β6 D_β2.1 J_β2.3	+	+	18,19
42F7	(bm12)	bm12-Auto	T_H	not V_α3 or V_α AF.3.G7	V_β6	+	+	18,19
C9	(B6)	D^b - AED	T_c	V_α3.C9 J_αC9	V_β6 D_β1.1 J_β1.1	+	+	20,21
653	(BALB/c)	alloreactive D^b	T_c	V_α4.3 J_α653	V_β6 D_β1.1 J_β2.6	+	+	22
24	(BALB/c)	alloreactive D^b	T_c	ND	V_β6 (rearranged)	+	+	np
25	(BALB/c)	alloreactive D^b	T_c	ND	V_β6 (rearranged)	+	+	np
5/10-20(K)	(B6)	K^b - AED	T_c	V_α8.520K J_α520K	V_β7 D_β1.1 J_β1.2	-	-	20,21
5/10-20(D)	(B6)	D^b - AED	T_c	V_α1.520D J_α810	V_β5.2 D_β1.1 J_β2.6	-	-	20,21
52H10	(B6)	B6-Insulin-A	T_H	V_αAF3.G7*	V_β4	-	-	18,19,23
10.10.58	(bm12)	bm12-Insulin-B	T_H	not V_α3 or V_αAF3.G7	not V_β4 or V_β6	-	-	18,19,23
42H11	(bm12)	bm12-Insulin-A	T_H	V_α3	V_β4	-	-	18,19,23

* Vα 52H10 is identical by partial sequencing and restriction enzyme mapping to AF3.G7 (24).
np = not published.

Table 2.

T Hy	Relative IL-2 production induced by mAb crosslinked via FcR (specificity)		
	medium alone	46-6B5 (αVβ6)	145-2C11 (αCD3)
5.3.18	944	9894	26353
42H11	817	2907	30776
52H10	636	771	20238

^{3}H-thymidine uptake after stimulation with crosslinked mAb. 0.2 ml cultures containing T Hy and 1 x 10^6 mitomycin C arrested A20 cells were incubated without and with mAb (46-6B5: 50% culture supernatant; 145-2C11: 25% culture supernatant). Secreted IL-2 was measured as described in Materials and Methods.

Functional activation and inhibition of T hybridomas and T cell clones by mAb 44-22-1 and 46-6B5

As several groups have previously described for other anti-TcR reagents (1,3,17,18), we found that serological crosslinking of the TcR-complex via the FcR on a bystander cell or by mAb coupled to Sepharose beads specifically activated $V\beta6^+$ T Hy and T cell clones, as shown in Table 2. In these experiments the $V\beta6^+$ T Hy 5.3.18 was specifically triggered by 46-6B5 in the presence of FcR bearing A20 cells. The $V\beta4$-TcR α-matched T Hy, 42H11, was not activated by 46-6B5, nor were the 52H10 or 10.10.58 T Hy which have very similar specificities to 5.3.18. Each of the T Hy could be activated through the TcR-complex using the murine CD3 specific mAb 145-2C11. Isotype matched control antibodies to 44-22-1 did not activate any of the T Hy (results not shown). The $V\beta6^+$ T_C cell clone 3F9 was activated by 44-22-1 and 46-6B5 coupled to Sepharose (results not shown).

An identical pattern could be derived for the blocking capacity of 46-6B5. As shown in Table 3, binding of the mAb specifically interfered with antigen recognition by the TcR in the $V\beta6^+$ T Hy 5.3.18. The mAb 46-6B5 blocked the IL-2 response of this T Hy to insulin very efficiently, while insulin responses of $V\beta6^-$ 42H11 and 52H10 were not altered.

Table 3.

	Relative IL-2 production (^{3}H-Thymidin uptake)				
	mAb 46-6B5 (IgM)				no Insulin
T Hy	none	12.5%	25%	50%	
5.3.18	10460	6438	3170	1079	690
42H11	8286	7594	7273	7453	681
52H10	4107	4225	4249	4393	542

Vβ6 specific inhibition of insulin specific activation of T Hy by mAb 46-6B5. T Hy were incubated in the presence of irradiated splenocytes, 500 µg/ml beef insulin (Sigma, Chemical Co.) at the indicated final concentration of mAb 46-6B5 in percentage of hybridoma supernatant. Levels of secreted IL-2 were measured as described in Materials and Methods.

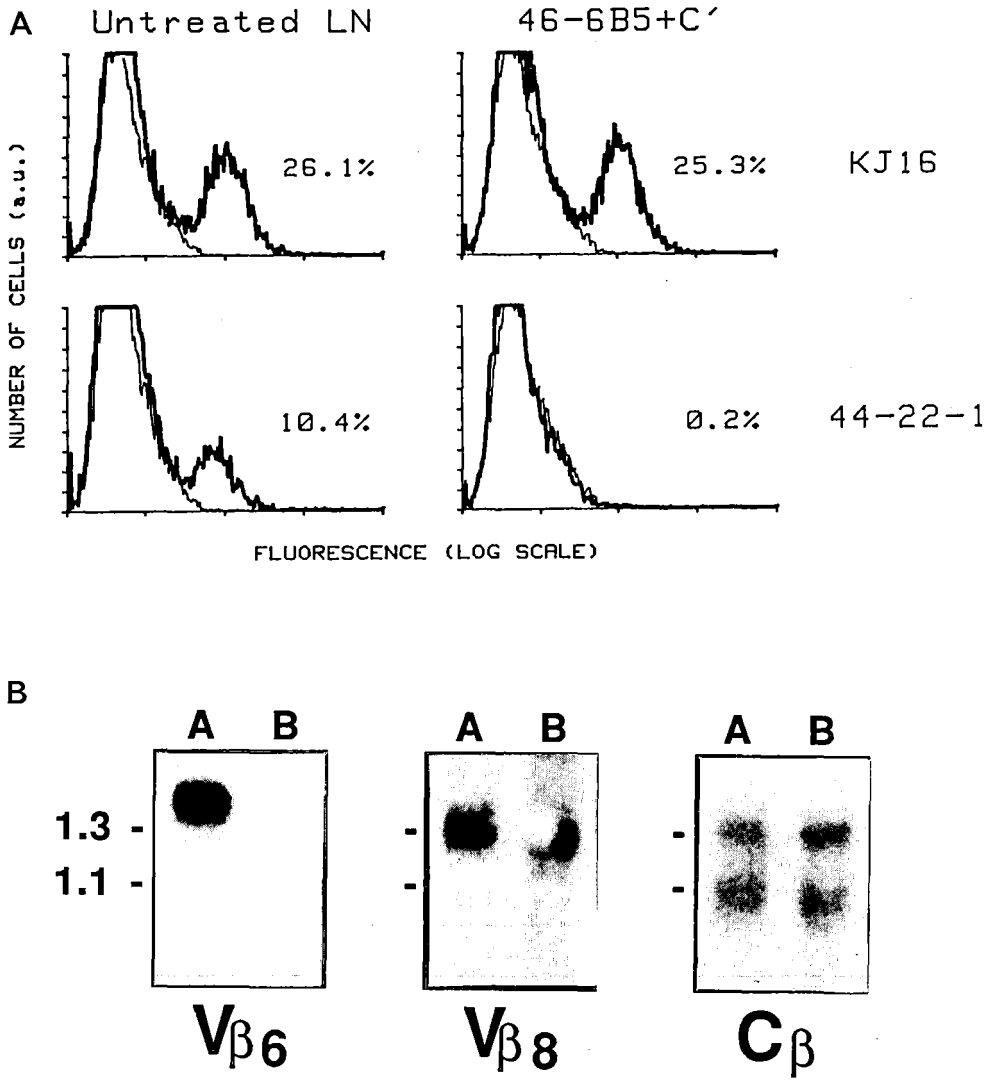


Figure 2. A. Depletion of $V\beta6^+$ T cells by rat mAb 46-6B5 plus complement treatment. Lymph node cells, untreated and 46-6B5 plus complement treated, were labeled with 44-22-1 or, as a control, with KJ16 (anti-Vβ8) and FITC-conjugated goat anti-rat IgG antibodies (——). Staining with the fluorescent second antibody alone is represented by —. Fluorescence analysis was carried out on a FACS II flow cytometer.

Figure 2. B. Northern analysis of the total cellular RNA extract of untreated (A) and 46-6B5 plus complement treated (B) lymph node cells was performed by using radioactive Vβ6, Vβ8 and Cβ TcR probes as described in Materials and Methods.

44-22-1 and 46-6B5 mAb recognize all Vβ6+ TcR expressing normal T lymphocytes

In order to correlate the expression of the epitopes recognized by mAb 44-22-1 and 46-6B5 and the presence of Vβ6 mRNA we performed T cell depletion experiments. Normal lymph node cells were treated with 46-6B5 plus complement and subsequently non-specifically stimulated to proliferate. After 7 days, aliquots of the 46-6B5 depleted and control cell cultures were cytofluorometrically analyzed with the rat mAb KJ16 and 44-22-1 and their cytoplasmic RNA extracted. As shown in Figure 2A, the mAb 44-22-1 stains about 10% of normal lymph node cells. Depletion of the 46-6B5$^+$ cells resulted in the complete loss of 44-22-1$^+$ cells, while the percent of KJ16$^+$ T cells remained unchanged. These results strongly suggest that the 44-22-1$^+$ lymphocytes are identical to those expressing the epitope recognized by 46-6B5. Furthermore, Northern analysis of RNA extracted from these cells using Vβ6, Vβ8.2 and Cβ probes revealed that the mAb 46-6B5$^+$ cells depleted cultures selectively lacked Vβ6 mRNA. In contrast, hybridization with Vβ8.2 and Cβ probes resulted in signals of proportional intensities. These data demonstrate directly that all normal T lymphocytes utilizing the Vβ6 gene segment are recognized by mAb 44-22-1 and 46-6B5. However, the possibility that these mAb recognize some T lymphocytes using other Vβ genes (with the exception of the Vβ8 family) cannot be formally excluded (although no such cells have yet been detected in our analysis of T cell clones).

Conclusions

Our results clearly demonstrate that the mAb 44-22-1 and 46-6B5 recognize not the TcR clonotype of 3F9 but rather an epitope(s) expressed by the Vβ6 region, because both mAb specifically bind to and functionally affect all Vβ6$^+$ T Hy and T cell clones regardless of the combinatorial rearrangements of the remaining TcR gene segments. This finding was confirmed in whole T cell population studies since we found that all Vβ6 expressing normal lymph node T cells were eliminated by mAb 46-6B5 and complement treatment. These mAb will further facilitate analysis of the acquisition and expression of the T cell receptor repertoire.

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CHAPTER 7

GENERAL DISCUSSION

In this study the antigen receptors of murine cytotoxic T lymphocytes have been studied. Since it has been described that T lymphocytes recognize antigen in the context of a membrane molecule encoded by a gene of the major histocompatibility complex (MHC), attempts have been made to characterize the receptor molecule(s) present on T cells that is (are) responsible for this specific interaction. The antigen receptor and the genes coding for it have now been isolated and characterized in great detail (c.f. Chapter 1). The receptor of the great majority of T cells consists of an α and a β chain. Receptors with different specificities are formed by rearrangement of DNA fragments containing variable, diversity and joining gene segments. Together with a constant gene segment a complete functional receptor chain is formed. Although the general structure as well as the mechanism of diversification of the T cell receptor is well understood, the nature of the simultaneous recognition of antigen and MHC is still not clear yet. One way to address this question is to analyze the variable portions of T lymphocyte clones with known specificities. Correlations between structure of the antigen receptor on one hand and specificity of the clone on the other may provide information about the process of recognition. For example, a consistent use of a particular gene segment in receptors that recognize a particular antigen might imply that there is an interaction between that part of the receptor and the antigen.

1. Production and characterization of T cell clones

Because antigen receptors of T lymphocytes are clonally distributed, T lymphocyte clones have to be produced in order to characterize the specificity of a receptor and its structure. In Chapters 2 and 3 the production of functional, stable T cell clones is described. The fine specificity of the clones has been investigated using various methods.
In Chapter 2, the antigen recognized by the cytotoxic T cell clones is the lymphocytic choriomeningitis virus (LCMV). The clones have been derived from normal C57BL/10 (H-2^b) and B10.BR (H-2^k) mice, but also from allogeneic bone marrow chimeras, C57BL/10 → B10.BR and B10.BR → C57BL/10. All H-2^k restricted clones are restricted to K^k whereas all H-2^b restricted clones recognize virus plus D^b. The finding that all H-2^b restricted clones recognize D^b is in agreement with the observation that primary responses against LCMV in C57BL/10 mice are mainly D^b rather than K^b restricted. The primary response of B10.BR mice, however, uses D^k and K^k to the same extent. There is no explanation for the fact that all five H-2^k restricted clones

are K^k restricted.
Analysis of the fine specificity of the various clones, revealed that cytotoxic T lymphocyte clones from normal mice have slightly different receptors than cytotoxic T lymphocyte clones from chimeric mice. The H-$2D^b$ restricted clones of chimeric (B10.BR → C57BL/10) origin were inhibited more easily by anti-D^b monoclonal antibodies than clones of C57BL/10 mice. The H-2^k restricted clones of chimeric (C57BL/10 → B10.BR) origin differed from the clones of B10.BR mice with respect to their fine specificity for LCMV strains. The clones derived from normal mice only recognized the LCMV-WE strain, which had been used for immunization, while the clones from the chimeric mice recognized all four LCMV strains tested. These differences in fine specificity between the clones from normal mice and the clones from chimeric mice might reflect the effects of different selective forces during the process of tolerance induction and will be discussed later.
In Chapter 3, the response against a single murine class I alloantigen (H-$2D^b$) has been chosen. This antigen was selected because several crucial reagents were available. An alloreactive T cell clone, 3F9, had been produced [Acha-Orbea et al, 1985], which specifically lyses H-$2D^b$ bearing targets. This clone had been used to generate anti-receptor antibodies. In order to produce monoclonal antibodies, rats were immunized with 3F9 cells and spleen cells were fused with myeloma cells. The monoclonal antibodies were selected for their ability to inhibit the lysis of target cells by clone 3F9, but not the activity of other cytotoxic T cells. Two monoclonal antibodies, 44-22-1 (IgG2a) and 46-6B5 (IgM), have been characterized which recognize the receptor of 3F9 [Acha-Orbea et al, 1985]. Until now, no differences in specificity between these two antibodies have been found.
In the present study, the question was addressed whether the antibodies directed against the receptor of 3F9, which is H-$2D^b$ specific, recognize receptors of other H-$2D^b$ specific clones as well. It was hypothesized that receptors of other H-$2D^b$ specific T cells can possess a general characteristic that is recognized by the 3F9 specific antibodies.
To this end, a panel of H-$2D^b$ specific cytotoxic T cell clones has been generated (Chapter 3). In order to distinguish between clones of different origin and multiple isolates of one and the same clone, the pattern of rearrangements of the DNA encoding the β-chain was investigated. Rearrangements on both C_β loci were examined. Within a group of 20 clones, 5 distinct patterns of rearrangement were observed, indicating at least 5 different events of rearrangement. Each of these five rearrangements has resulted in a functional β-chain for a receptor that is specific for H-$2D^b$.
The five different H-$2D^b$ specific clones have been analyzed with the 3F9 specific antibodies. One of the clones, clone 653, was inhibited by these antibodies. This suggests that the 44-22-1 antibody does not exclusively recognize the receptor of clone 3F9, but may recognize a determinant encoded by a germline gene segment. On the other hand, the observation that only a minority of H-$2D^b$ specific clones is recognized by

44-22-1 antibody suggests that the determinant recognized by 44-22-1 is not required for specificity of T cell receptors for H-2D^b.

2.Antibodies against T cell receptors

Since two H-2D^b specific T cell receptors, of clones 3F9 and 653, were available, which were both recognized by the same monoclonal antibody (44-22-1), it was of interest to determine the exact structure of these two antigen receptors. The objective was to identify the gene segment (or combination of gene segments) that encodes the determinant recognized by the 44-22-1 antibody. The primary structure of the antigen receptor of clone 3F9 has been determined [Rupp et al, 1985, 1987]. The structure of the receptor of clone 653 is described in Chapter 5. Comparison of the primary structures of the receptors (α and β chains) of both clones revealed that only the $V_{\beta 6}$ gene segment is shared between these two receptors. This suggested that the antibody 44-22-1 recognizes a determinant that is encoded by the $V_{\beta 6}$ gene segment. Comparison of the T cell receptors of a greater number of clones has confirmed this hypothesis. In Chapter 6 a greater number of T cell clones with known receptor structure have been analyzed with the 44-22-1 antibody. The result of this analysis is that whenever a T cell functionally expresses the $V_{\beta 6}$ gene segment, it is recognized by 44-22-1.
During the last few years several antibodies against T cell antigen receptors have been produced. Some of these antibodies are anti-idiotypic in the sense that they only inhibit the function of one particular T cell clone or hybridoma, while others have been found to react with a relatively large fraction of peripheral T lymphocytes. In principle, determinants of different complexities may exist. They can be encoded by 1) one or more germline segment(s), 2) a specific sequence at a particular VDJ-junction formed by rearrangement or 3) a conformational determinant, that involves participation of one or both T cell receptor chains. Each of these possibilities is discussed below.
Ad 1) Antibodies specific for a germline gene segment can be expected to stain a few percent of peripheral T lymphocytes, depending on the frequency of using this gene segment. As shown in Chapter 6, the $V_{\beta 6}$ specific antibody, 44-22-1, stains 10.4 % of BALB/c lymph node cells ($\approx$ 15 % of T cells). In subsequent experiments it has been shown that the frequency of 44-22-1 positive cells varies between 0 % and 11 % in various mouse strains [MacDonald et al, 1988]. Assuming that about 20 V_β gene segments are present in the murine genome, the expected frequency should be around 5 % per V_β gene segment, if each gene segment would be expressed randomly. Several other V_β specific monoclonal antibodies have been described.
The $V_{\beta 8}$ gene family in the mouse has three members, $V_{\beta 8.1}$, $V_{\beta 8.2}$ and $V_{\beta 8.3}$. Monoclonal antibodies recognizing 1, 2 or all three members of this gene family have been produced. F23.1 recognizes all three gene segments [Staerz et al, 1985; Behlke et al, 1987], KJ16 recognizes only $V_{\beta 8.1}$ and $V_{\beta 8.2}$ [Haskins et

al, 1984; Behlke et al, 1987], and a third antibody, F23.2, recognizes only $V_{\beta 8.2}$ [Kappler et al, 1988]. Similarly, a monoclonal antibody KJ23a has been described that recognizes the $V_{\beta 17a}$ gene segment [Kappler et al, 1987a]. In humans, monoclonal antibodies have been produced that react with variable gene segments of the $V_{\beta 5}$ family [Borst et al, 1987] and with a gene family that has first been isolated from the tumor cell line REX [Acuto et al, 1985b].
Ad 2) The second determinant, a specific VDJ-junction sequence, is recognized by a monoclonal antibody directed against the α-chain of a helper T cell [Samelson et al, 1983; Saito et al, 1987; Gascoigne et al, 1987]. Such determinants are expected to occur at much lower frequencies in the T cell population than the first category of antibodies.
Ad 3) The third class of antibodies, recognizing a conformational determinant involving both T cell receptor chains, will only recognize receptors if a particular combination of α and β chains is expressed on the surface. A few examples of such antibodies have been described [Haskins et al, 1983; Hua et al, 1985]. These antibodies are not expected to stain a detectable fraction of peripheral T lymphocytes.

3.Tolerance by clonal deletion

Antibodies specific for the products of variable gene segments have been used to examine the frequencies in which certain variable gene segments are expressed in the peripheral T lymphocyte population. It appeared that for several V_{β} genes, the observed interstrain differences were profound. For the gene segments $V_{\beta 17a}$ [Kappler et al, 1987b], $V_{\beta 6}$ [MacDonald et al, 1988] and $V_{\beta 8.1}$ [Staerz et al, 1985; Roehm et al, 1985; Kappler et al, 1988] it has been shown that the proportion of T cells, expressing a particular V_{β} gene segment, depends on the genetic background of the mouse strains.
The $V_{\beta 17a}$ gene segment has been shown to be preferentially expressed in mouse strains that do not express I-E gene products [Kappler et al, 1987b]. In I-E expressing mouse strains, cells with $V_{\beta 17a}^{+}$ receptors were shown to be present in the immature $CD4^{+}$, $CD8^{+}$ thymocyte population, but not in the more mature, single positive subpopulation. It was also demonstrated that T cell hybridomas expressing $V_{\beta 17a}^{+}$ receptors preferentially react with I-E encoded antigens [Kappler et al, 1987a]. The interpretation of these data is that self-reactive cells have been deleted from the functional repertoire in the thymus [Kappler et al, 1987b].
Similar experiments have recently been performed with the 44-22-1 antibody [MacDonald et al, 1988]. It appeared that $V_{\beta 6}^{+}$ T cells, recognized by the 44-22-1 antibody (Chapters 5 and 6), were not present in mice that carried the Mls^{a} allele. Mls^{a}, which is a strong stimulatory antigen in mixed lymphocyte cultures, was shown to be recognized by T cells that express $V_{\beta 6}$. Tolerance for Mls^{a} apparently involved exclusion of $V_{\beta 6}^{+}$ cells from the peripheral repertoire.
The experiments described above provide important new information concerning the generation of tolerance for self

antigens. In principle there are several mechanisms by which non-responsiveness can be attained. The first is clonal elimination of all self reactive T cells. The second would be suppression of the activity of self reactive T cells in the periphery. The third possibility would be that self antigens are not presented by MHC molecules. Until recently, functional assays were used (i.e. mixed lymphocyte cultures or CTL induction) to demonstrate self tolerance. Lack of response under these conditions does indicate tolerance, but does not reveal whether this tolerance is due to clonal deletion or mechanisms of suppression [Kappler et al, 1987b; Marrack & Kappler, 1987]. The introduction of monoclonal antibodies that recognize antigen receptors of T cells with the same specificity, does provide the most direct means of analyzing a receptor repertoire, without being dependent on functional assays. The fact that in two unrelated examples, described above, deletion of a specific population of T cells occurs, argues in favor of clonal deletion as a mechanism of tolerance induction. Of course, these examples do not prove that self tolerance is always and for all antigens obtained by clonal deletion, but they show that it is definitely occurring. It must be stressed that these observations have been dependent on the availability of a reagent (monoclonal antibody) that reacts with a majority of receptors specific for the same antigen. The correlation between expression of the $V_{\beta 6}$ gene segment, reactivity of the receptor with antibody 44-22-1 and specificity of this receptor for Mls^a encoded antigens has provided such a model system.

4.Correlations between receptor structure and specificity

The discovery of the genes encoding the T cell receptor has inspired many investigators to attempt to find correlations between specificity of T cells for certain antigens and/or MHC molecules and the structure of the antigen receptor. During the last three years it has been tried to assign specificities for antigen or MHC to certain gene segments or receptor chains. Correlations could provide information about the nature of the simultaneous recognition of antigen and MHC by the T cell receptor. The key question is whether there are two distinct recognition sites on the T cell receptor, one recognizing MHC and the other recognizing antigen.
The first indication that a correlation might be difficult to find was that that helper and cytotoxic T cells use gene segments from the same gene pool [Rupp et al, 1985; Borst et al, 1987]. In more detailed analyses, the T cell receptor structures of specific T cells have been studied in several cases of antigen specific immune responses. For this purpose, collections of antigen specific T cell clones have been produced and the primary structure of the receptors has been determined from the nucleic acid sequences of the rearranged T cell receptor genes.
The panel of $H\text{-}D^b$ specific T cell clones described in Chapter 3 provide good starting material for such a study. The question that can be asked is, whether antigen receptors specific for

the same antigen (H-2D^b) possess structural similarities. The multitude of rearrangements of β chain genes illustrates that many different receptors are capable of recognizing H-2D^b. In a more detailed analysis (Chapter 5) the primary structure of the receptor of one of these clones, clone 653, is presented. Comparison of the primary structures of the receptors of clones 653 and 3F9 does not suggest any obvious similarities. The only gene segment that is utilized by both clones is the $V_{\beta 6}$ gene segment. However, four other H-2D^b specific clones, 652, 433, 431 and 432 do not employ this gene segment in their receptors, since they do not react with the 44-22-1 antibody. Therefore, the $V_{\beta 6}$ gene segment is not required in receptors with specificity for H-2D^b. In the future, the structure of the receptors of the other four H-2D^b specific clones should be determined in order to gather more structural information on H-2D^b specific receptors.
Except from being recognized as an alloantigen, H-2D^b does also function as a restriction element for nominal antigen. In both cases H-2D^b is recognized by the T cell receptors. Therefore, certain aspects may be shared by the receptors of alloreactive and D^b restricted T cells. The structures of the α and β chains of three alloreactive CTL clones specific for D^b, one LCMV specific clone and two N-iodoacetyl-N- (5-sulfonic-1-naphtyl) ethylene diamine (AED, a hapten determinant) specific clones restricted to D^b are shown in Table 1. No preferential use of certain gene segments among the T cell clones, which all recognize D^b, can be noticed. These findings are in agreement with the general lack of correlation between specificity and primary receptor structure reported by other investigators. Several of these reports and the possible reasons for the lack of consistent results will be discussed.

Table 1. Antigen receptor structures of H-2D^b recognizing clones.

T cell clones	Specificity	V_α	J_α	V_β	D_β	J_β
3F9	allo-D^b	8*	19	6	-	1.1
653	,,	4.3	653	6	1	2.6
MDA	,,	5	31	8.3	-	2.5
P71	,,	8	P71	ND	ND	ND
P14	LCMV + D^b	2	31	8.1	-	2.4
5/10-20D	AED + D^b	1	810	5.2	1	2.6
C9	AED + D^b	3	C9	6	-	1.1

The structures of the receptors were obtained from the following references: 3F9,[Rupp et al, 1985, 1987]; 653,[chapter 5]; MDA and P71,[Becker et al, 1985]; P14, [Pircher et al, 1987]; 5/10-20 and C9,[Iwamoto et al, 1987].
* - numbers represent families of V genes. For example, 4.3 represents the third member of family 4. For D and J genes, numbers are unique identifiers. ND is not determined.

In order to have a well defined antigen recognized in the context of an MHC molecule, several studies have used haptens as antigens. Haptens are small chemical compounds that can be coupled to proteins. Two haptens that have been used are trinitrophenyl (TNP) and AED. In the TNP specific, H-$2K^b$ restricted CTL response, a preference for the combination of $V_{\alpha 4}$, $J_{\alpha 11 2.2}$ and $V_{\beta 3}$, $J_{\beta 2.6}$ was observed. A relatively great number of clones has been analyzed [Hochgeschwender et al, 1986, 1987]. The authors conclude that this combination of gene segments may be specific for this antigen MHC combination. It would be of interest, however, to analyze the response against TNP in combination with another MHC molecule. Although it is suggested that the antigen (TNP) is the same for all of these T cells, this may not be the case. The hapten TNP can be coupled to many proteins and the repertoire of processed antigenic peptides containing TNP is potentially large. AED, on the other hand, is coupled to free cysteine residues that are relatively infrequent [Pircher et al, 1984]. Therefore, the diversity of antigenic determinants formed by AED will be more limited. The analysis of four AED specific clones has suggested the preferential use of certain J segments [Iwamoto et al, 1987].
In addition to the class I restricted, hapten specific response, the T helper response specific for protein antigens has been studied. No consistent usage of certain gene segments was demonstrated in the response against horse cytochrome C [Baumhüter et al, 1987] and bovine insulin [Spinella et al, 1987]. In contrast, it has been suggested that the I-E^d restricted sperm whale myoglobin specific response preferentially uses V genes of the $V_{\beta 8}$ family [Morel et al, 1987].
Correspondingly, helper T cell clones and hybridomas specific for the COOH-terminal fragment of cytochrome c in combination with I-E molecules of several haplotypes have been analyzed [Fink et al, 1986; Winoto et al, 1986; Sorger et al, 1987]. The preferential use of gene segments of the $V_{\alpha 11}$ family was striking.
The repertoire of receptors specific for alloantigens has also been studied in several reports. A particular V_β gene segment was shown to be used in receptors of alloreactive T cells specific for class I as well as class II antigens [Acuto et al, 1985a]. Within a panel of alloreactive T cell clones specific for HLA-A2 and expressing the same V_β gene segment, considerable diversity exists in J_β or α chains [Borst et al, 1987]. In a study, comparable with the work described in this thesis, the response against the human alloantigen HLA-DPW2 was analyzed [Beall et al, 1987]. In this study the preferential use of a V_β gene segment was suggested, but not proved.
In mixed lymphocyte cultures against alloantigens differential activation of T cells expressing certain V gene segments was demonstrated. However, no consistent correlations between specificity and V gene usage were observed [Garman et al, 1986]. The studies described above suggest that, only in some cases, there may be a correlation between specificity of a receptor for an antigen/MHC combination and the expression of certain gene segments. Most analyses, however, have been

performed on the receptors of cells that had been in culture for a long time. It would be of interest to study the in vivo response directly after immunization or after a short period of in vitro restimulation. In this manner selection of particular clones by long term culture can be excluded.
A clear correlation between V gene usage and specificity under in vivo conditions was demonstrated with the help of V gene specific monoclonal antibodies. The correlations between specificity for I-E encoded antigens and $V_{\beta 17a}$ expression [Kappler et al, 1987b; Kappler et al, 1987a] and also between recognition of Mls^a encoded determinants and the usage of $V_{\beta 6}$ [MacDonald et al, 1988] or $V_{\beta 8.1}$ [Kappler et al, 1988] are striking. For example, in mixed lymphocyte cultures in which Mls^a antigen (and other minor H antigens) induces proliferation, the responder T cell population is enriched for expression of the $V_{\beta 6}$ gene segment, as monitored with the 44-22-1 antibody (Chapters 5 and 6).
Although the experimental data are convincing, some doubt exists whether the reactivity against Mls antigens is a conventional MHC restricted specificity [Molnar-Kimber & Sprent, 1980; Lynch et al, 1985; Janeway & Katz, 1985]. The significance of the correlation between antigen recognition and gene usage for general MHC restricted recognition is therefore uncertain. Nevertheless, the existence of such a clear antigen-receptor correlation, combined with a straight forward detection mechanism, offers the possibility to study the repertoire of T cells against Mls^a encoded antigens under in vivo conditions.
The conclusion that can be drawn from the analysis of T cell receptors until to date, is that there is no obvious correlation between specificity for either antigen or MHC and gene segment usage. It is important to realize that a correlation between receptor structure and antigen/MHC can only be found if the structure of the antigen/MHC complex is well defined. Peptide antigens instead of complex proteins should be used for such analyses and even then it cannot be excluded that different T cell receptors look at this complex from different angles. For alloantigen recognition this could imply that the receptors of alloreactive T cells can recognize many different epitopes on a single allogeneic MHC molecule.
For the panel of alloreactive clones described in Chapter 3, it would be of importance to establish whether different epitopes of the $H\text{-}2D^b$ molecule are recognized by the various clones. Using target cells from $H\text{-}2D^b$ mutant mice it has not been possible to distinguish between the clones. Inhibition of cytotoxicity with a panel of anti-$H\text{-}2D^b$ monoclonal antibodies also did not reveal distinct patterns of inhibition for different clones. Using these two methods it has not been possible to define different epitopes on $H\text{-}2D^b$ that are recognized by the clones.

5.Cross-reactivity between a class I and a class II antigen

The panel of D^b specific CTL clones that has been produced in this study has been analyzed for cross-reactive activities on

third-party alloantigens. This analysis was originally performed in order to try to detect differences in fine specificity between the clones. Only one of the clones, clone 433, has been found to cross-react with cells of the H-2^k haplotype. In Chapter 4, this cross-reactive activity, which is of an unexpected nature, is described. The additional antigen that is recognized by clone 433, appeared to be a class II (I-E^k) antigen. Target cells expressing I-E^k were shown to be lysed by clone 433 and this lysis was blocked by anti-I-E^k antibodies. In cold target competition experiments it was shown that the original class I (D^b) specific interaction as well as the class II (I-E^k) specific cytotoxicity are mediated by the same cell population. Although it has not yet been formally demonstrated that the two specificities are mediated by one receptor molecule, this is likely to be the case.
This finding illustrates that, at least in the case of alloantigen recognition, the difference between class I and class II antigens may be small. For antigen specific, MHC restricted T lymphocytes the distinction between class I and class II antigen is strict. In contrast, alloreactive cells have been reported to be more "degenerate" in their recognition of MHC antigen [Vidovic et al, 1981; Miller & Stutman, 1982; Flomenberg et al, 1983; Spits et al, 1983; Haas & von Boehmer, 1984; Pierres et al, 1984; Shinohara & Kojima, 1984]. Lyt-2^+ T cells specific for class II antigens and L3T4$^+$ cells specific for class I have been described. Structurally, it has been suggested that class I and class II molecules may be similar (c.f. Chapter 1 and Bjorkman et al, 1987a and 1987b), but also functionally, presentation of antigenic peptides seems to be analogous (c.f. Chapter 1). It is therefore not surprising that T cell clones expressing the same variable gene segments in their receptor have been reported to recognize class I as well as class II MHC antigens [Rupp et al, 1985; Acuto et al, 1985b; Borst et al, 1987; Rupp et al, 1987]. It has also been described that two alloreactive T cell hybridomas expressing the same α but different β chains, recognize a class I and a class II antigen, respectively [Blackman et al, 1986]. Given the fact that class I and class II MHC molecules are so similar, the question how they cause a dichotomy in the T lymphocyte population has not been answered yet.
Some responses are primarily class I restricted while others are class II restricted [Bevan, 1987]. Experiments with class I and class II restricted influenza specific CTL suggest that class I molecules present antigens that have been produced endogenously, while class II antigens present antigens that are derived from endocytosed material [Morrison et al, 1986]. Assuming that the physiological function of cytotoxic T lymphocytes is to lyse a target cell, virus synthesizing cells will be killed and virus production will be halted, while cells that phagocytose viral particles and present the antigen on class II molecules will stimulate T helper cells in order to amplify the immune response. This would imply that class I and class II molecules bind the antigenic fragments in different intracellular compartments. Indeed, it has been shown that class I and class II molecules are found in different

intracellular vesicles [Pernis, 1985]. It has been proposed that endocytosed antigens are fragmented in acidic endosomes where they also bind to Ia [Grey & Chesnut, 1985; Cresswell, 1985]. Endogenously produced proteins may be combined with class I molecules at some stage along the pathway of synthesis and processing (Golgi) [Germain, 1986; Chain et al, 1986]. The general validity of the model explaining the difference between class I and class II presentation still has to be proved.

6.Alloreactivity

The phenomenon of alloreactivity has been a puzzle in immunology for decades. In comparison to "naturally" occurring antigens (viruses etc.) the precursor frequency of alloreactive T cells seems to be 100-1000 times higher [Simonsen, 1967; Nisbet et al, 1969; Wilson et al, 1970]. Many different T cell clones are activated in response to a single alloantigen (Chapter 3 and Sherman, 1980). An explanation for the existence of such strong responses against alloantigen does not seem to be provided by an evolutionary pressure. The survival of a species does not depend on a strong response against transplanted organs or tissues. Alloreactivity most likely represents a coincidental byproduct of MHC restricted recognition of antigen. The reason why alloreactivity does occur has been the subject of many speculations. In models of recognition of MHC and antigen by T cells, the existence of alloreactivity has to be explained.
An attractive hypothesis concerning alloreactivity has been presented about ten years ago [Matzinger & Bevan, 1977]. In view of the recently acquired data about antigen presentation, this theory can be rephrased in modern molecular terms. In short, the theory proposes that alloantigens are in fact allogeneic MHC molecules in combination with "self" determinants. In the thymus the T cell population will be tolerized for these self antigens in the context of self MHC. Because the antigen presenting properties are different, allogeneic MHC molecules present a different set of "self" determinants. The multitude of "self" peptides presented in the context of an allogeneic MHC, for which the T cell population has not been tolerized, will provide a large number of "foreign" (=allogeneic) determinants.
Taking into consideration what is known about the presentation of antigen (c.f. Chapter 1), the model described above can be supported as follows. Usually the T cell response against an antigen is used to examine the binding of peptides to MHC because this is, to date, the only way of detecting peptide/MHC complexes. This does not necessarily mean that only antigenic peptides have the property of binding to MHC. At the site where peptides are added to MHC molecules [Germain, 1986] a large variety of debris of normal cellular and extracellular proteins can be expected to be present [Schwartz, 1985; Germain, 1986; Robertson, 1988]. In this mixture of "self" proteins, peptides will occur with adequate structural characteristics (motive) to bind to a particular MHC molecule. Therefore, it can be speculated that every MHC molecule will always contain a

peptide fragment [Townsend & McMichael, 1987; Robertson, 1988]. Indeed, the structure of HLA molecules as determined by X-ray cristallography has revealed that, at least in that particular preparation, the majority of HLA-molecules contain peptide structures not belonging to the HLA molecule itself. This peptide is located in the groove between the two α helices [Bjorkman et al, 1987b].
In contrast, it has been suggested by some authors [Guillet et al, 1987; Schwartz, 1987] that the MHC molecule has an internal receptor-ligand structure which can be opened to accommodate an antigenic peptide which resembles the internal ligand. The authors even suggest that the MHC may distinguish between self and nonself [Guillet et al, 1987]. It seems dangerous to assign the function of self versus non-self discrimination to the MHC molecules because they seem to bind peptides with a low degree of specificity [Sette et al, 1987].
The nature of the "self" peptide fragments bound to the MHC molecules will be typical for a given set of MHC molecules. This is analogous to the situation when a virus infects cells expressing different MHC molecules, these MHC molecules will select which antigenic peptides of the virus will be presented at the cell surface [Taylor et al, 1987]. Likewise, the MHC will select some of the many self peptides that are present. Only peptides with an appropriate motive will be bound to self MHC. Which peptides will actually be presented by the antigen presenting cells will further depend on the concentration of degradation products in the intracellular compartments where association between peptide and MHC occurs.
In an individual with a particular set of MHC molecules, the composition of intracellularly produced (this will bind to class I antigens) and endocytosed (binds to class II molecules) material will give rise to a defined array of peptide/MHC complexes in the membrane [Townsend & McMichael, 1987]. When we assume that a population of T cells has been tolerized (for example by clonal deletion) for this set of peptides, this will represent the immunological "self". Correspondingly, in cells with different (allogeneic) MHC molecules, the peptides selected from the pool of proteins and degraded products will be completely different. The combination of this different set of peptides with the allogeneic MHC molecules will present a multitude of antigenic determinants. The peripheral T cell repertoire will consequently respond to these antigenic determinants.
In principle both sets of peptides are "self" determinants. The difference between both sets is only whether or not they have been presented on the MHC of tolerance inducing cells. Assuming that both sets of peptides do not overlap significantly, a multitude of T cell clones will be stimulated by allogeneic cells.
If indeed a great number of different peptides would be bound to MHC, the actual concentration of one sort of peptide/MHC complex on the cell surface is low. To support the theory, it will be necessary that a small number of MHC molecules carrying the same peptide is able to stimulate an alloreactive T cell. For antigen specific T helper cells, it has been estimated

that, although under artificial conditions, 1000 antigen-Ia complexes are enough to stimulate IL-2 production [Watts & McConnell, 1986]. The presence of 1 x 10^5 to 5 x 10^5 class I MHC molecules on lymphoid cells [Haemmerling & Eggers, 1970; Kindt & Robinson, 1984; Dower & Segal, 1985], can provide up to five hundred different stimulatory molecular complexes on the cell surface. If each different peptide/MHC complex is able to stimulate some T cells, five hundred of such complexes will stimulate a substantial fraction of T lymphocytes. This might explain the high frequency of alloreactive T cells in an unprimed individual [Simonsen, 1967; Nisbet et al, 1969; Wilson et al, 1970].
When antigen enters the antigen presenting cell, it will be degraded into peptides. These peptides will be immunogenic if they are capable of competing with endogenous peptides for binding to MHC. If one thousand MHC/antigen complexes were indeed enough to stimulate T cells, it will not be necessary to compete with all endogenous peptides but competition with one or a few will suffice.
The view presented above predicts that the many determinants seen by alloreactive T cells are not epitopes present on one and the same MHC molecule, but rather are formed by the various peptides bound by allogeneic MHC molecules. Interesting is that the majority of polymorphic residues on HLA-A2 molecules that are recognized by alloreactive CTL, have been shown to be located in the groove where antigen is bound [Bjorkman et al, 1987a]. If indeed the peptides bound by the MHC molecules constitute the determinants recognized by alloreactive T cells, it will be important to identify the peptides that are normally present in the antigen binding site of the MHC molecules and characterize the diversity of these peptides. The H-$2D^b$ specific clones described in this thesis could be specific for peptides combined with the class I MHC molecule. It would be interesting to test this hypothesis by testing the activity of the clones on a set of target cells that express the H-$2D^b$ molecule but possess a lot of different proteins. For example, target cells of nonlymphoid tissues or xenogeneic target cells.
The recognition of MHC antigens across species barriers is a special case of antigen recognition. Xenogeneic antigen recognition may provide information relevant to the discussion about alloreactivity. Experiments have revealed that two distinct principles of recognition of xenogeneic MHC antigens seem to exist. For example, murine cytotoxic T cells can recognize human MHC antigen as nominal antigen in an H-2 restricted way [Maryanski et al, 1986a; Viguier et al, 1987; Achour et al, 1986] or as complete molecules [Engelhard et al, 1980; Achour et al, 1986]. The first situation is illustrated by the fact that murine cytotoxic T lymphocytes specific for HLA, obtained after immunization of mice with murine cells transfected with DNA coding for HLA, recognize murine target cells that have been treated with a peptide derived from HLA [Maryanski et al, 1986b]. The second mechanism actually is unrestricted recognition of the xenoantigen. This mechanism is supported by the fact that murine CTL specific for HLA antigens lyse human target cells expressing the appropriate HLA

[Engelhard et al, 1980; Greenstein et al, 1986; Achour et al, 1986].
The first example shows that HLA encoded antigens can be presented as peptides in the context of H-2 molecules. This indicates that under normal conditions HLA derived peptides may be presented by intact HLA molecules on the cell surface. Some authors have indeed observed that specificity of human alloreactive CTL can be regulated by peptides derived from HLA [Parham et al, 1987; Clayberger et al, 1987]. When MHC derived as well as virus derived peptides can be presented in the context of class I MHC, it is reasonable to expect that all intracellularly produced proteins can be presented. Minor histocompatibility antigens could be products of polymorphic genes and provide antigenic peptides that are presented in an MHC restricted manner [Gordon et al, 1975; Bevan, 1975].
Another interesting observation, that may support the hypothesis that alloantigens are, actually, non-MHC peptides recognized in the context of allogeneic MHC, is that human alloreactive CTL do not always recognize HLA antigens that have been transfected in murine cells. Two populations of CTL seem to exist, one that does recognize HLA in murine cells [Parham et al, 1987; Clayberger et al, 1987] and one that does not [Barbosa et al, 1984; van de Rijn et al, 1984; Koller et al, 1987]. Non-MHC encoded self determinants may not all be shared between mouse and man. Only the human CTL clones that are specific for HLA plus a peptide that is present in human as well as in murine cells, will lyse murine transfected cells, while CTL specific for a human peptide plus HLA will only be able to lyse human cells and not mouse cells. An alternative explanation of this phenomenon may be that interspecies differences exist between accessory molecules [Koller et al, 1987] and that only the high affinity interactions will take place when HLA is expressed on murine cells.
In this discussion arguments have been listed supporting the idea, that the peripheral T lymphocyte population is tolerized for self antigens by clonal deletion. If it would be true that MHC molecules always contain self peptides, the consequence would be that the T cells specific for these particular peptide/MHC combinations are deleted. When different MHC molecules present different sets of non-MHC encoded self peptides, it would be expected that tolerance for non-MHC antigens is MHC restricted. This has indeed been reported [Matzinger et al, 1984].
Tolerance induction by clonal deletion may result in "holes" in the repertoire. An illustration of this phenomenon might be the different fine specificities of the LCMV specific clones (Chapter 2) derived from normal animals versus the clones derived from chimeric animals. In the chimeric animals tolerance for an extra set of H-2 molecules has to be induced. During this process, the LCMV specific clones present in normal mice may have been deleted, giving other clones the opportunity to expand upon immunization with virus. The influence of MHC and non-MHC encoded antigens on the peripheral repertoire of T lymphocytes will be an interesting topic for further studies.
The induction of tolerance has been suggested to occur in the

thymus [Kappler et al, 1987b]. Only self antigens that are expressed in the thymus or transported into the medulla of the thymus (by bone marrow derived macrophages or dendritic cells) will induce tolerance. It can be speculated that organ-specific antigens [Steinmuller, 1984; Londei et al, 1985] which are only expressed in organs that are relatively secluded from the circulation, such as skin or joint, may be less involved in the process of tolerance induction. Under normal conditions this may not present a problem because these antigens may be less accessible to circulating T lymphocytes. If, however, the equilibrium is disturbed, for example by an infection of the skin by a microorganism, the skin antigens may be presented efficiently to T cells and an "autoimmune" process may follow. This may be the consequence of a higher expression of MHC antigens [Bottazzo et al, 1986; Blanden et al, 1987] or the presence of more reactive lymphocytes at the site of infection. It will be interesting to identify and isolate these postulated organ specific antigens [Steinmuller, 1984].

The model presented above explains T cell recognition of major and minor alloantigens as well as the existence of class I and class II restricted responses against antigens. The selection of presented peptides by MHC molecules is the basis for this model, which has been presented before [Claverie & Kourilsky, 1986; Werdelin, 1987]. The major implication is that most of the data can be accounted for by assuming that T cell receptors only recognize the peptide that is presented but not the MHC molecule that presents it. However, several observations suggest that at least some aspects of the MHC molecule are recognized.

The first argument is a rather indirect one. From the data that have been published on the three dimensional structure of the HLA-A2 molecule [Bjorkman et al, 1987b], it seems that the antigenic fragment is embedded between the two α helices. Molecularly, a receptor recognizing the peptide, most likely will also interact with the components of the MHC that are in the immediate surroundings of the peptide.

The second argument is a functional one. It has been observed that the haplotype of the MHC expressed on the thymic epithelium determines the preferential restriction element during an <u>in vivo</u> immune response [Zinkernagel et al, 1978; Fink & Bevan, 1978]. The presence of an A-type thymic epithelium in an animal that is A x B will result in a response restricted to A when the animal is infected with a virus. The repertoire of the peripheral T lymphocyte population seems to be educated to recognize antigen in the context of the same MHC that has been encountered in the thymus during T lymphocyte maturation [Marrack & Kappler, 1987]. Thymic education has been an unexplained phenomenon for the last 10 years. It has led to the hypothesis that T cell receptors with a low affinity for MHC are positively selected in the thymus [Marrack & Kappler, 1987]. Alternatively, cells lacking any affinity for self MHC could be eliminated, leaving only the self MHC restricted cells. It will be important to establish that indeed such selection mechanisms function <u>in vivo</u>. It has also been argued that the education of T cells to recognize antigen primarily in

the context of the MHC of the thymic epithelium is only partial [Klein & Nagy, 1982; Goverman et al, 1986], since non-thymic MHC restricted T cells can be found, although in a lower frequency.
Until now it has been impossible to demonstrate if and where education of T cells does occur. With the availability of the genes coding for complete antigen receptors in combination with the technique of making transgenic mice [Brinster & Palmiter, 1986] or transfection of hemopoietic stem cells [Dick et al, 1985], it might be possible to investigate this problem. The full-length cDNA clones coding for a complete receptor with known specificity, which have been described in Chapter 5, could be used to generate transgenic mice. The receptor has been isolated from a peripheral BALB/c ($H\text{-}2^d$) T cell and is specific for $H\text{-}2D^b$. The intrathymic development of a large proportion of lymphocytes expressing this particular transgenic receptor, can be studied under several conditions. For example in a syngeneic ($H\text{-}2^d$) situation, an allogeneic situation ($H\text{-}2^b$) or an irrelevant situation ($H\text{-}2^k$). These experiments might provide clues as to whether positive or negative selection occurs. The presence of an anti-receptor antibody (44-22-1) greatly facilitates these analyses.

7.Epilogue

Many questions concerning the recognition of antigen plus MHC by the T cell receptor remain unsolved. X-ray cristallographic analysis of T cell receptor and of T cell receptor/MHC complexes will be required to determine the precise nature of the recognition. Some attempts in this direction have been made, but were unsuccessful so far [Gascoigne et al, 1987]. Chimeric genes containing T cell receptor variable regions and immunoglobulin constant domains have been produced in order to produce a secreted form of T cell receptor protein. Secretion of an assembled molecule, however, has not been observed yet. Another aspect of antigen recognition that deserves further attention in the near future is the presentation of antigen by MHC molecules. The structure of the peptides in the binding site of the MHC molecule has to be established. It will also be interesting to identify the intracellular compartment where MHC and peptides are combined. Especially the differences between intracellular processing of antigens presented by class I and class II MHC molecules, respectively, will have to be clarified.
At present, the availability of cloned genes of T cell receptors with defined specificities as well as antibodies directed at certain T cell receptor epitopes constitute valuable probes to be used in biological experiments, that may shed new light on the functioning of the immune system.

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SUMMARY

Organs or tissues transplanted from one individual to another within the same species are usually rejected. This phenomenon is called alloreactivity and is the consequence of genetic differences between the two individuals. Genetic analysis has revealed that one particular complex of loci is responsible for most rejections namely, the major histocompatibility complex (MHC). The genes in this complex encode certain membrane proteins. The MHC of the mouse is called H-2. It encodes class I and class II antigens. Class I antigens are expressed on most cells in varying densities. Class II antigens are expressed mainly on antigen presenting cells. Surface molecules encoded by alleles of the MHC evoke a strong immune response upon immunization and this is the basis of allograft rejection. The cells that account for the response are the T lymphocytes.

In 1974 it became clear that the MHC molecules were also involved in the recognition of antigen by T cells. Whereas B cells recognize free antigen, T cells only recognize antigen in conjunction with MHC molecules. Fragments of complex antigens are presented by MHC molecules to T cells. Class I antigens present antigen (e.g. viral antigen) to cytotoxic T cells while class II molecules serve the same function for helper T cells. The antigen receptors on T cells, which are capable of recognizing antigen as well as MHC molecules, have been a mystery in immunology for a decade. Around 1984, the protein and the genes coding for it have been isolated and characterized. The protein consists of two polypeptide chains, the α and the β chain. Each of these chains has a constant and a variable domain. To create a maximum number of receptors with different specificities without occupying a major part of the available genome, the variable parts of the receptors are constructed by recombination of various germline gene segments. This process is called rearrangement.

In each individual T cell precursor, DNA fragments containing variable, diversity and joining gene segments are rearranged in a unique manner, forming a transcriptional unit for a specific receptor. Already a relatively small number of gene segments are sufficient to generate a high number of different receptors.

The identification of the T cell antigen receptor and especially of the genes that code for it, has offered the possibility to investigate how this molecule simultaneously recognizes antigen and MHC.

Because each T lymphocyte has a distinct specificity it possesses a unique antigen receptor. To study the specificity as well as the structure of a specific antigen receptor, a large number of T cells with identical receptors is required. Therefore, T lymphocytes expressing a receptor of particular interest have to be cloned. Continuous growth of T cells is possible if they are confronted with antigen at regular

intervals in the presence of interleukin-2 (T cell growth factor). By use of a certain antigen for restimulation, T cells specific for that antigen will selectively be promoted in their growth.

In this thesis the response against two antigens, a viral antigen and an alloantigen, has been investigated.
In Chapter 2 the production of a panel of cytotoxic T cell clones specific for lymphocytic choriomeningitis virus (LCMV) is decribed. The clones have been derived from normal C57BL/10 (H-2^b) and B10.BR (H-2^k) mice, but also from allogeneic bone marrow chimeras, C57BL/10 → B10.BR and B10.BR → C57BL/10. All H-2^k restricted clones were found to be restricted to K^k and not D^k, whereas all H-2^b restricted clones recognized D^b and not K^b. By analyzing the fine specificity of the various clones, cytotoxic T lymphocyte clones from normal mice were shown to have slightly different receptors than CTL clones from chimeric mice. The H-$2D^b$ restricted clones of chimeric (B10.BR → C57BL/10) origin were inhibited more easily by anti-D^b monoclonal antibodies than clones of C57BL/10 mice. The H-2^k restricted clones of chimeric (C57BL/10 → B10.BR) origin differed from the clones of B10.BR mice with respect to their fine specificity for LCMV strains. The clones derived from normal mice only recognized the LCMV-WE strain, which had been used for immunization, while the clones from the chimeric mice recognized all four LCMV strains tested. The differences in fine specificities between the clones from normal mice on one hand and the clones from chimeric mice on the other may reflect differences in the T cell receptor repertoire induced by the chimerism.

In the second study (Chapter 3) cytotoxic T cell clones specific for a murine alloantigen have been generated. The cytotoxic T cell clones that have been produced, specifically lysed target cells expressing the class I antigen, H-$2D^b$. Analysis of the pattern of rearrangement of the DNA that codes for the β chain of the T cell receptor, demonstrated that each of the clones was derived from a unique precursor cell and therefore has a unique receptor structure. All receptors, however, were specific for H-$2D^b$. The clones have been further analyzed with a monoclonal antibody, 44-22-1, which has originally been generated against the receptor of another H-$2D^b$ specific T cell clone, 3F9. Only one of the newly derived clones, 653, was inhibited by this monoclonal antibody. Apparently the determinant on the T cell receptor that is recognized by the 44-22-1 antibody is not required for specificity of a T cell receptor for H-$2D^b$.

The primary structure of the antigen receptor of clone 3F9 has previously been determined. The α chain consists of a variable gene segment of the $V_{\alpha 8}$ family and a joining gene segment called $J_{\alpha 19}$. The β chain is composed of $V_{\beta 6}$ and $J_{\beta 1.1}$. Since the antibody 44-22-1 does not react with all T cell receptors, it was suspected that one of the variable (or joining) gene segments or a combination of two of these gene segments forms the determinant that is recognized by 44-22-1. Apparently this determinant is also present on the receptor of clone 653. Therefore, the structure of the antigen receptor of

clone 653 was investigated (Chapter 5). A cDNA library was produced of mRNA of clone 653. The library was screened for the presence of T cell receptor gene transcripts using probes of the constant parts of α and β chain genes. The variable portions of the positive clones were sequenced. The α chain of the receptor of clone 653 appeared to be composed of a variable gene of the $V_{\alpha 4}$ family and a joining segment that has not been published yet. The β chain consists of $V_{\beta 6}$, $D_{\beta 1}$ and $J_{\beta 2.6}$. From comparison of the receptors of clone 3F9 and clone 653 it appears that the only gene segment that is shared between the receptors of 653 and 3F9 is the $V_{\beta 6}$ gene segment. This suggests that the $V_{\beta 6}$ gene segment encodes the determinant that is recognized by the antibody 44-22-1.

The analysis of a greater number of T cell clones with known receptor structures has confirmed this hypothesis. This is described in Chapter 6. Cytotoxic T cell clones expressing the $V_{\beta 6}$ gene segment are inhibited with the 44-22-1 antibody. Helper clones expressing this variable gene segment are inhibited by soluble antibody and stimulated by immobilized antibody. RNA isolated from a mixed T cell population depleted of 44-22-1 positive cells did not hybridize to a $V_{\beta 6}$ DNA probe. Together, these data demonstrate that the 44-22-1 antibody recognizes T cell receptors, that functionally express the $V_{\beta 6}$ gene segment. The frequency of T lymphocytes that are stained with 44-22-1 in an unprimed animal is 15 %. The availability of an antibody that recognizes a variable gene segment offers a useful tool to study T lymphocyte receptor function under in vivo and in vitro conditions.

To define differences in the receptors, the panel of H-2D^b specific clones was tested for cross-reactivities with third party stimulator cells. One of the clones, 433, appeared to to lyse target cells of the H-2^k haplotype. The antigen recognized is the class II antigen, I-E^k (Chapter 4). This dual specificity has been shown to be mediated by the same cell population. Assuming that only one functional receptor is expressed in this clone, it implies that the receptor of clone 433 is able to recognize a class I as well as a class II alloantigen. This would mean that, at least as far as recognition by alloreactive T cells is concerned, class I and class II antigens may be quite similar.

Several aspects of alloantigen recognition are discussed in Chapter 7. Since it has become clear over the last few years that antigen is presented to T cells as degraded fragments bound to MHC molecules, a model is favored that explains alloreactivity in terms of peptide recognition as well. Alloreactivity would be the recognition of self-derived peptides in the context of allogeneic MHC. Allogeneic MHC molecules will present other self-derived peptides than self MHC, for which the T cell repertoire has not been tolerized in the thymus. If the number of different self-derived peptides on an allogeneic cell surface is high, this can explain why alloreactive T cells are more frequent than T cells specific for nominal antigen.

SAMENVATTING

Wanneer organen of weefsels binnen een soort van het ene individu naar het andere getransplanteerd worden, zullen ze meestal worden afgestoten. Dit fenomeen wordt alloreactiviteit genoemd en is het gevolg van genetische verschillen tussen de twee individuen. Een complex van loci, het major histocompatibility complex (MHC), is verantwoordelijk voor de meeste afstotingsreacties. De genen in dit complex coderen membraan eiwitten, de histocompatibiliteitsantigenen. Het MHC van de muis wordt H-2 genoemd. Het codeert voor klasse I en klasse II antigenen. Klasse I antigenen komen op de meeste cellen in verschillende mate tot expressie. Klasse II antigenen komen op antigeen presenterende cellen tot expressie. Oppervlakte antigenen die gecodeerd worden door allelen van het MHC, roepen een sterke immuunreactie op en deze reactie is de basis voor transplantaat afstoting. De cellen die verantwoordelijk zijn voor de reactie zijn de T lymfocyten.

In 1974 werd het duidelijk dat MHC moleculen ook betrokken zijn bij de herkenning van antigeen door T lymfocyten. Terwijl B cellen vrij antigeen in oplossing herkennen, kunnen T cellen alleen antigeen herkennen in combinatie met een MHC molecuul. Fragmenten van complexe antigenen worden door MHC moleculen gepresenteerd aan T cellen. Klasse I moleculen presenteren antigeen (b.v. viraal antigeen) aan cytotoxische T lymfocyten en klasse II moleculen presenteren antigeen aan helper T lymfocyten.

Het heeft ongeveer tien jaar geduurd voor de antigeen receptor van T cellen, die in staat moet zijn om antigeen plus een MHC molecuul tegelijk te herkennen, geisoleerd werd. Tegelijkertijd met de isolatie van het eiwit, werden ook de genen, die voor dit eiwit coderen, gecloneerd en gekarakteriseerd. Het eiwit bestaat uit twee ketens, de α en de β keten, die ieder weer uit een constant en een variabel gedeelte bestaan. Om een maximum aantal receptoren met verschillende specificiteiten te creëren, zonder het grootste gedeelte van het genoom in beslag te nemen, worden de variabele gedeelten geconstrueerd door verschillende gen segmenten aan elkaar te koppelen. Dit proces word genherschikking genoemd.

In iedere T cel voorloper worden een variabel, een diversiteits en een verbindend (joining) gen segment op een unieke wijze aan elkaar gekoppeld, zodat een specifieke receptor ontstaat. De identificatie van de T cel receptor en speciaal van de genen die ervoor coderen, heeft de mogelijkheid geboden om te onderzoeken hoe dit molecuul in staat is om gelijktijdig antigeen en MHC te herkennen.
Iedere T cel heeft een eigen specifieke receptor structuur waarmee een bepaald antigeen herkend wordt. Wanneer de specificiteit en de structuur van zo'n receptor bestudeerd wordt, dient men te beschikken over een groot aantal cellen met dezelfde receptor. T lymfocyten met een bepaalde specificiteit moeten daarom eerst gecloneerd worden. De continue groei van T

cellen is mogelijk wanneer ze regelmatig aan antigeen blootgesteld worden in aanwezigheid van het hormoon, interleukine-2 (IL-2, of T cel groei factor). Door een bepaald antigeen te gebruiken voor de restimulatie, worden T cellen met een specificiteit voor dat antigeen selectief gestimuleerd in hun groei.

In dit proefschrift wordt de productie van T lymfocyten specifiek voor twee antigenen, een virus antigeen en een alloantigeen, beschreven.

In Hoofdstuk 2 staat de productie van een aantal cytotoxische T cel clonen beschreven die specifiek zijn voor het lymfocytair choriomeningitis virus (LCMV). De T cellen zijn oorspronkelijk geisoleerd uit normale C57BL/10 ($H\text{-}2^b$) en B10.BR ($H\text{-}2^k$) muizen, maar ook uit allogene beenmerg chimeren, C57BL/10 → B10.BR en B10.BR → C57BL/10. Alle $H\text{-}2^k$ gerestricteerde clonen waren gerestricteerd door K^k, terwijl alle $H\text{-}2^b$ gerestricteerde clonen D^b herkenden. Door de fijne specificiteit van de T cellen nader te onderzoeken kwam naar voren dat de receptoren van de clonen afkomstig van normale muizen verschilden van de receptoren van de clonen die van de chimeren afkomstig waren. De $H\text{-}2D^b$ gerestricteerde clonen van de chimere muizen (B10.BR → C57BL/10) werden gemakkelijker geremd door D^b specifieke antilichamen dan de clonen van C57BL/10 muizen. De $H\text{-}2^k$ gerestricteerde clonen van de chimeren (C57BL/10 → B10.BR) verschilden van de clonen afkomstig van B10.BR muizen voor wat betreft hun specificiteit voor verschillende LCMV stammen. De clonen van de normale muizen herkenden alleen de LCMV-WE stam, die ook gebruikt was voor immunisatie, terwijl de clonen van de chimere muizen determinanten herkenden van vier verschillende virus stammen. Deze verschillen in fijne specificiteit tussen de clonen afkomstig van normale muizen en clonen afkomstig van allogene beenmerg chimeren zou een illustratie kunnen zijn van een verandering in het receptor repertoire geinduceerd door het chimerisme.

In de tweede studie (Hoofdstuk 3) zijn cytotoxische T cel clonen geproduceerd die specifiek zijn voor een alloantigeen van de muis. De cytotoxische T cel clonen lyseren specifiek doelwitcellen die het klasse I antigeen, $H\text{-}2D^b$, tot expressie brengen. Analyse van de patronen van genherschikking van het DNA dat codeert voor de T cel receptor β keten, toonde aan dat elk van de clonen afkomstig is van een unieke voorloper cel en daarom ook een unieke receptor structuur bezit. Alle receptoren herkennen echter wel het $H\text{-}2D^b$ antigeen. De clonen zijn verder geanalyseerd met een monoclonaal antilichaam, 44-22-1, dat oorspronkelijk gegenereerd is tegen de receptor van een andere $H\text{-}2D^b$ specifieke cloon, 3F9. Van de nieuw geproduceerde clonen werd er maar één, 653, geremd door dit antilichaam. Kennelijk is de determinant, die herkend wordt door het 44-22-1 antilichaam, niet noodzakelijk voor een $H\text{-}2D^b$ specifieke receptor.

De primaire structuur van de antigeen receptor van cloon 3F9 was reeds bepaald. De α keten is opgebouwd uit een variabel gen segment uit de $V_{\alpha 8}$ familie en een verbindend gen segment, $J_{\alpha 19}$. De β keten bestaat uit $V_{\beta 6}$ en $J_{\beta 1.1}$. Aangezien het 44-22-1

antilichaam niet met alle T cel receptoren reageert, was de verwachting dat de antigene determinant voor dit antilichaam gecodeerd wordt door een (of meer) variabele gen segment(en). Deze determinant blijkt ook aanwezig te zijn op de receptor van cloon 653. Daarom werd de structuur van de antigeen receptor van cloon 653 nader onderzocht (Hoofdstuk 5). Van mRNA van cloon 653 werd een cDNA bank gemaakt. Met behulp van DNA probes van de constante gedeelten van de α en β keten genen, werd onderzocht of de bank transcripten van de T cel receptor gene bevatte. Van de variabele gedeelten van zulke transcripten werd de nucleotide volgorde bepaald. De α keten van de receptor van cloon 653 blijkt opgebouwd te zijn uit een variabel gen van de $V_{\alpha 4}$ familie en een nog niet gepubliceerd verbindings gen segment. De β keten bestaat uit $V_{\beta 6}$, $D_{\beta 1}$ en $J_{\beta 2.6}$. Wanneer de structuren van de receptoren van cloon 653 en 3F9 vergeleken worden, volgt hieruit dat het $V_{\beta 6}$ gen segment het enige gemeenschappelijke gedeelte is. Dit suggereert dat het $V_{\beta 6}$ gen sement de antigene determinant codeert die door het 44-22-1 antilichaam herkend wordt.

Deze hypothese werd bevestigd nadat een groter aantal T cel clonen met bekende receptor structuur geanalyseerd werd. Deze analyse staat beschreven in Hoofdstuk 6. Cytotoxische T cel clonen die het $V_{\beta 6}$ gen segment tot expressie brengen worden geremd in hun activiteit door het 44-22-1 antilichaam. Helper T cel clonen die dit variabel gen segment tot expressie brengen worden ook geremd door het antilichaam als dat in oplossing is terwijl ze gestimuleerd worden door antilichaam dat geimmobiliseerd is. RNA, geisoleerd van een T cel populatie waaruit de 44-22-1 positieve cellen verwijderd zijn, hybridiseert niet meer met een DNA probe voor het $V_{\beta 6}$ gen segment. Deze gegevens tonen aan dat het 44-22-1 antilichaam T cel receptoren herkend waarin het $V_{\beta 6}$ gen segment functioneel tot expressie komt. De frequentie van T cellen die met het 44-22-1 antilichaam reageren is ongeveer 15% in een normale muis. De beschikbaarheid van een antilichaam dat met een fractie van de T lymfocyten reageert, kan waardevol zijn bij de bestudering van de functie van de T cel receptor onder zowel *in vivo* als *in vitro* condities.

Teneinde verschillen tussen de receptoren van de $H2D^b$ specifieke clonen te documenteren, zijn de clonen getest op kruisreactiviteiten met allogene cellen van uiteenlopende haplotypen. Een van de clonen, 433, bleek doelwitcellen van het $H\text{-}2^k$ haplotype te lyseren. Het antigeen dat herkend wordt op deze cellen, is het klasse II $I\text{-}E^k$ antigeen (Hoofdstuk 4). Het is aangetoond dat dezelfde populatie T cellen inderdaad verantwoordelijk is voor beide specificiteiten. Aangenomen dat cloon 433 maar één functionele receptor tot expressie brengt, betekent dit dat deze receptor zowel een klasse I als een klasse II antigeen herkent. Dit zou kunnen inhouden dat, althans in het geval van alloantigeen herkenning, klasse I en klasse II antigenen veel op elkaar lijken.

In Hoofdstuk 7 worden verschillende aspecten van alloantigeen herkenning besproken. Sinds een paar jaar is het duidelijk geworden dat antigeen aan T cellen gepresenteerd wordt als gedegradeerde fragmenten (peptiden) die aan MHC

moleculen gebonden zijn. Bij het formuleren van een model voor antigeen herkenning door T cellen, en in het bijzonder bij het verklaren van het verschijnsel alloreactiviteit, moet rekening gehouden worden met deze gegevens. Aldus zou alloreactiviteit de herkenning van, in principe lichaamseigen, peptiden kunnen zijn die gepresenteerd worden door allogene MHC moleculen. Voor deze combinaties van peptiden en MHC moleculen is het T cel repertoire in de thymus niet getoleriseerd en daarom wordt een sterke immuun-respons geinduceerd. Als het aantal lichaamseigen peptiden die op het oppervlak van allogene cellen gepresenteerd wordt hoog is, dan zou dit een mogelijke verklaring kunnen zijn voor het feit dat de frequentie van alloreactieve T cellen zo hoger is dan de frequentie van T cellen die specifiek zijn voor andere antigenen.

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ABBREVIATIONS

AED	- N-iodoacetyl-N-(5-sulfonic-1-naphtyl) ethylene diamine
APC	- antigen presenting cell
BM	- bone marrow
BSA	- bovine serum albumine
BSS	- balanced salt solution
C	- constant gene segment
CD	- cluster determinant
cDNA	- complementary DNA
Con A	- concanavalin A
cM	- centimorgan
CTL	- cytotoxic T lymphocyte
FCS	- fetal calf serum
FITC	- fluorescein isothiocyanate
^{3}H-dThd	- tritiated thymidine
Ig	- immunoglobulin
IL-2	- interleukin-2
IMDM	- Iscove's modification of Dulbecco's medium
Ir	- immune response
J	- joining gene segment
GTC	- guanidine thiocyanate
H-2	- histocompatibility-2
HLA	- human leukocyte antigen
hfl	- high frequency of lysogeny
kb	- kilo base
kD	- kilo dalton
LCMV	- lymphocytic choriomeningitis virus
LFA-1	- lymphocyte function-associated antigen
LPS	- lipopolysaccharide
mAb	- monoclonal antibody
MHC	- major histocompatibility complex
MLC	- mixed lymphocyte culture
Mls	- minor lymphocyte-stimulating
MΦ	- macrophage(s)
PBS	- phosphate-buffered saline
[α-^{32}P]dCTP	- ^{32}P labeled deoxycytidine triphosphate
pI	- isoelectric point
SC	- spleen cells
SDS	- sodium dodecyl sulfate
SPF	- specific pathogen free
RIA	- radioimmunoassay
T_C	- cytotoxic T cell
TcR	- T cell receptor
TdT	- terminal deoxynucleotidyl transferase
T_H	- helper T cell
T Hy	- T cell hybridoma
Ti	- T cell receptor
TNP	- trinitrophenyl
V	- variable gene segment