MOLECULAR GENETIC ANALYSIS OF 178 I-A^{bm12}-REACTIVE T CELLS

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T cell immunity is highly dependent upon interactions between the TCR and polymorphic glycoproteins encoded by the MHC locus (HLA in man, H-2 in mice) (1, 2). In several cases, it has been shown that the ligand for the TCR consists of a degraded fragment of foreign antigen bound to a self-MHC-encoded glycoprotein (3, 4). This interaction is highly specific in that only ~1:10^5 to 1:10^6 T cells can recognize a given peptide/self-MHC complex (5). In contrast up to 1% of T cells can recognize cells bearing allo-MHC-encoded molecules in the absence of obvious exogenous antigen (6). Considering the degree of MHC polymorphism (~100 H-2 alleles in the mouse) (7), it has been speculated that nearly all TCRs have specificity for some alloantigen (8).

Three theories have been invoked to explain the high frequency of alloreactivity. The first suggests that an allo-MHC protein structurally resembles self-MHC plus antigen. Since the thymus selects for T cells that react with self-MHC plus antigen, they coincidentally recognize homologous allo-MHC alone; this is the "altered self" hypothesis (1). A second theory suggests that MHC gene products and TCRs have coevolved such that there is a germline bias of the TCR genes to recognize MHC molecules; both antigen/MHC complexes as well as alloantigens fall into this category (9, 10). Finally, it has been suggested that the high density of MHC molecules on presenting cells is sufficient to stimulate many T cells through low affinity interactions with their receptors (11). Obviously, some of these theories need not be mutually exclusive. Nevertheless, any theory must explain the high frequency of alloreactivity to mutant H-2 molecules, which in some cases differ from the wild-type protein by only a few amino acids. It has been speculated that MHC-encoded glycoproteins contain many epitopes for T cell recognition and that these are highly dependent

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on three-dimensional structure (12). Thus, even a small change in amino acid sequence could alter the three-dimensional structure and result in many new epitopes. Alternatively, the recognition by T cells of an epitope present on allo-MHC molecules might be degenerate, i.e., many different TCRs may recognize a single alloantigenic epitope. Support for this possibility comes from the recent report that most T cells expressing a *Vβ17a*-encoded TCR react with I-E molecules (13, 14). This allo-I-E reactivity does not appear to depend on other TCR gene segments (Vα, Jα, Dβ, or Jβ) (15).

In this study, we wished to examine the molecular basis of the TCR repertoire to an alloantigen in order to define the importance of α and β chain V region genes as well as the contribution of junctional diversity to allorecognition. We chose to examine the repertoire of B10 mice (I-A^b) to the mutant class II alloantigen, I-A^{bm12} (16). As a result of a spontaneous gene conversion event, the *I-A^{bm12}* gene differs from the *I-A^b* gene by a cluster of five nucleotides in the second hypervariable domain of the β chain of the I-A^b molecule. This results in only three amino acid replacements: Ile to Phe^{67}, Arg to Gln^{70}, and Thr to Lys^{71} (17). Despite the limited number of substitutions, mice bearing I-A^b and I-A^{bm12} mount reciprocal skin graft rejections; lymphocytes from these mice respond to each other vigorously in MLC; and I-A-bearing cells from these strains have been demonstrated to differ in their antigen presentation capacities (16, 18, 19). We have generated a large panel of I-A^{bm12}-reactive hybrids and determined TCR V gene expression by hybridization analysis. In selected hybrids, TCR sequence was obtained using the polymerase chain reaction (20–23) to amplify and directly sequence α and β chain cDNAs. The results show that the TCR repertoire to I-A^{bm12} is extremely heterogeneous at the level of variable gene expression, but that the frequencies of V gene expression are significantly different from those in the random B10 TCR repertoire. Furthermore, there is no evidence of conserved junctional (VJα, VDJβ) amino acid residues among I-A^{bm12}-reactive TCRs.

Materials and Methods

Mice. C57BL/10 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. B6.C-H-2^{bm12} mice were bred in the facilities of National Jewish Center, Denver, CO.

Generation of T Cell Hybrids. I-A^{bm12}-reactive T cell blasts were generated by coincubating lymph node and spleen cells from individual B10 mice, (1–2 × 10^8 cells), with 2–3 × 10^8 irradiated (2,000 rad) B6.C-H-2^{bm12} spleen cells in CTM supplemented with 10% FCS at 37°C for 4–5 d. The responding blasts were purified over Ficol-Hypaque and were either fused directly or were first expanded by culture for 1–2 d in the presence of IL-2. α methyl mannose neutralized culture supernatant from a Con A-stimulated T cell hybridoma was used as a source of IL-2. T cell blasts were fused with the thymoma BW5147, as described (24). In two fusions, a variant of BW5147, BW/α$^-$, selected for loss of the endogenous rearranged TCR Vα1 gene, kindly supplied by Dr. Willi Born (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO), was used as the fusion partner (J. White, M. Blackman, J. Bill, J. Kappler, P. Marrack, and W. Born, manuscript in preparation). The resulting HAT-resistant hybrids were tested for specific reactivity to I-A^{bm12} by their ability to produce IL-2 when cocultured with spleen cells from B6.C-H-2^{bm12} but not B10 mice. IL-2 production was assayed by the ability of these culture supernatants to support growth of the Il-2-dependent cell line HT-2, as described (24). The I-A^{bm12}-reactive hybrids were expanded to ~3 × 10^7 cells and were retested for I-A^{bm12} reactivity at the time of freezing. For the second specificity test, I-A^{bm12}-bearing lymphoma cells, 3LBM13.1, were used as the stimulators and I-A^b-bearing lymphoma cells, LB15.13 (25), as the negative control. Both lym-

phomas also expressed surface I-A^d and I-E^d. Pellets of frozen cells were stored at −70°C before RNA isolation.

Analysis of TCR V Gene Expression. Total cellular RNA was isolated from frozen cell pellets and blotted onto nitrocellulose by a modification of the method of Cheley and Anderson (26). Briefly, pellets from 2 × 10^7 hybridoma cells were dissolved in 0.85 ml of 7.6 M Guanidine HCl, 0.1 M potassium acetate, pH 5.0. The lysates were sonicated for 10–20 s using a fine probe to shear genomic DNA after which 0.6 ml of 100% EtOH was added to each sample. The samples were mixed, stored at −20°C overnight, and then centrifuged at 12,000 *g* for 20 min in an Eppendorf centrifuge (Brinkman Instruments, Inc., Westbury, NY). This process is reported to recover ~50% of the RNA while removing 97% of the DNA and 99% of the protein. The pellets were dissolved in a buffer containing 50% formamide, 40 mM morpholinoprane sulfonic acid, pH 7, 10 mM sodium acetate, 1 mM EDTA, pH 8, and 6% formaldehyde. RNA samples were stored at −70°C before analysis. RNA samples were heated to 55°C for 10 min, dissolved in 15× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), and 5 × 10^5 cell equivalents of each RNA were applied to each of 30 replicate nitrocellulose filters (8 cm × 12 cm) using a 96-well blotting manifold and a 12-channel pipette. BW5147 RNA was applied to each filter and served as a positive control for Cα, Cβ, and Vα1 expression and a negative control for all other Vα and all Vβ probes used in these experiments. RNA from the B cell lymphoma A20/2J was applied to each filter and served as a negative control for all probes. Variable region probes, Vα (1–8, 10, 11, and 13), Vβ (2–17), and constant region probes (Cα and Cβ) were labeled by nick translation (27) or by random priming (28) and were hybridized to the hybridoma RNA in 5× SSC, 1× Denhardt's solution, 100 μg/ml salmon sperm DNA, 10% dextran sulfate, and 50% formamide for 16 h at 42°C. Filters were washed nonstringently in 2× SSC at room temperature and stringently in 0.1× SSC at 50–55°C. These conditions do not distinguish individual members within a V gene family. The characteristics and sources of the probes used in these experiments are listed in Table I. While many of the probes were isolated in this laboratory, others were generously provided by Drs. D. Loh (Vβ9, 11, 12), L. Hood (Vβ14, Vα2), D. Raulet (Vβ6, Vα3), S. Hedrick (Vα10, 11), J. Kappler, and P. Marrack (Vβ17) (13, 29–38, J. Yagüe, W. Born, and E. Palmer, unpublished results).

Statistical Analysis of the Data. The distribution of TCR V gene expression in the panel of B10-derived, I-A^{bm12}-reactive, T cell hybrids was compared with the distribution of V gene expression in a panel of B10-derived, mitogen-stimulated, T cell hybrids by χ^2 analysis (*Vβ14, 15, 16*) or a Fisher 2 × 2 test (*Vβ5.1, 5.2*). From the χ^2 and Fisher values, *p* values were determined. The determination of the likely number of I-A^{bm12}-reactive Vα/Vβ pairs relied on standard sampling statistics as previously used to estimate the number of murine Vβ genes (40).

PCR Amplification and Sequencing. TCR α and β chain RNAs from selected hybrids were amplified and converted to single-stranded template for DNA sequencing by two modifications of the polymerase chain reaction (20–23). The basic technique requires the following steps: (*a*) construct oligonucleotides 5′ and 3′ to the region of interest, in this case, the V-C junction of the α and β chain cDNAs; and (*b*) use these oligonucleotides as primers to repetitively resynthesize the intervening DNA. The first modification of the PCR (21), developed at Cetus Corp. (Emeryville, CA) is required to permit amplification of mRNA. This is necessary because the variable and constant regions of the α and β chains of the TCR become contiguous only as a result of gene rearrangement and RNA splicing. The second modification permits direct sequencing of the amplified TCR gene. Direct sequencing is preferable both because it eliminates the requirement to clone the amplified cDNA and because misincorporated nucleotides are not detectable when sequencing the entire amplified product. The method is shown in Fig. 1. A set of nested oligonucleotides are synthesized (Fig. 1 *A*) such that A and B are identical to, while C and D are complementary to, the mRNA sequence. The oligonucleotides used in these experiments were: Vβ14 external (oligo A) 5′ACTATCCATCAATGGCCAGTT3′; Vβ 14 internal (oligo B) 5′TCGGTGGTGCAACTGAACCTC3′; Cβ internal (oligo C) 5′TGATGGCTCAAACAAGGAGAC3′; Cβ external (oligo D) 5′GCCAAGCACACGAGGGTAGCC3′; Vα3 external (oligo A) 5′GAGTTCAGCAAGAGCAACTCT3′; Vα3 internal (oligo B) 5′TCCTCCACCTGCGGAAAGCC3′; and Cα internal (oligo C) 5′CGAGGATCTTTTAACTGGTA3′; Cα external (oligo D) 5′AGAGGGTGCTGTCCTGAGAC3′.

Total cellular RNA was isolated from each hybrid using standard techniques (guanidine isothiocyanate ultracentrifugation over a CsCl cushion (41) and converted to cDNA as shown in Fig. 1. The reaction mixture (100 μl) consists of: 1 μg total cellular RNA, external V and external C region oligonucleotides (oligos A and D) at 1 μM each, dNTPs (Pharmacia Fine Chemicals, Piscataway, NJ) at 100 μM each, 2 U human placental ribonuclease inhibitor (Amersham Corp., Arlington Heights, IL), 2 mM DTT, and 2 U AMV reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 10 mM Tris, pH 8.3, 50 mM KCl, 2 mM MgCl, and 0.01% gelatin. After incubation at 42°C for 1 h, the reaction mixture is heated to 95°C for 5 min to denature the DNA/RNA duplex, the tube is briefly centrifuged, 0.5 U of TAQ polymerase (New England Biolabs, Beverly, MA) is added, and the reaction mixture is overlaid with a thin layer (~50 μl) of heavy mineral oil (Sigma Chemical Co., St. Louis, MO). Repetitive cycles of denaturation at 95°C for 2 min, annealing at 37°C or 50°C for 4 min, and primer extension for 2 min at 70°C, are carried out. Generally, 30 cycles have been optimal. In the first cycle, the second strand of cDNA is primed by the external variable region oligonucleotide (oligo A). Subsequent cycles are primed by both external oligonucleotides (oligos A and D) resulting in amplification of a 400-bp fragment that is visible on an ethidium bromide-stained agarose gel (Fig. 1 *C*).

The initial double-stranded PCR product is gel purified and then used as template for a second PCR in which it is further amplified and converted to ssDNA. Initially, the fragment was electroeluted onto DE81 paper, eluted with high salt, and passed through an Elutip (Schleicher & Schuell, Inc., Keene, NH) column. Subsequently, we found it much easier to electrophorese the product of the first PCR reaction through a 1% low-melting agarose gel (Sea Plaque; FMC BioProducts, Rockland, ME) made up in Tris-acetate-EDTA buffer and cut out the double-stranded band. The plug of low-melting agarose (~100 μl) is melted by heating to 95°C for 5 min and 1–10 μl (~5 ng) of the product is used to generate single-stranded (plus) DNA by a second 30 cycles of PCR using unequal amounts of the two primers. The concentration of the internal V region oligonucleotide (oligo B) is 1 μM and the concentration of the external constant region (oligo D) oligonucleotide is decreased by 100-fold to 10 nM. In the case of generating the plus strand of a Vβ14 cDNA, this results in further amplification of a 300-bp fragment until the concentration of the constant region oligonucleotide (oligo D) becomes limiting. Subsequent cycles of polymerase chain reaction lead to the accumulation of plus strand DNA, which is seen below the brighter 300-bp fragment (Fig. 1 *D*). The intensity of fluorescence does not accurately reflect the amount of ssDNA, since ssDNA binds much less ethidium bromide than does dsDNA (41). To generate the minus strand for DNA sequencing, the second amplification was carried out using the internal constant region oligonucleotide (oligo C) at 1 μM and the external V region oligonucleotide (oligo A) at 10 nM.

The overlying oil is removed from the second PCR and the remainder of the reaction is extracted once with chloroform and once with phenol/chloroform to remove the TAQ polymerase and any residual oil. The aqueous phase is then diluted to 2 ml with Tris-EDTA (TE) and is centrifuged three times through a Centricon-30 (Amicon Corp., Danvers, MA) membrane to remove excess dNTPs and oligonucleotides. The amplified DNA, in a residual volume of ~50 μl of TE, is then precipitated with EtOH. 40 μl of TE, 10 μl of 3M NaOAc, and 250 μl of EtOH are added, and the tube is centrifuged for 20 min in an Eppendorf centrifuge. The pellet is redissolved in 20 μl of TE and one third is used as single-stranded template for dideoxy sequencing reactions (42) using a Sequenase™ (United States Biochemical Corp., Cleveland, OH) kit according to the manufacturer's instructions. The sequencing primer for minus strand template is the internal variable region oligonucleotide (oligo B) and for the plus strand template, the internal constant region oligonucleotide (oligo C) is used. Fig. 1 *E* shows the sequence of Vβ14 obtained from hybrid 15BBM21 as primed by the Vβ14 internal oligonucleotide (oligo B). Plus and minus strands were sequenced for *V*β14 and *V*α3 genes from four hybridomas. Because of the position of the Vα3 sequencing primer, the 18 5′ nucleotides could not be confirmed from the minus strand template. Occasional band compressions are sometimes observed with both the dGTP and dITP reactions but generally occur at different locations in the sequence.

Preliminary experiments performed at Cetus Corp. (Emeryville, CA) utilized TAQ poly-

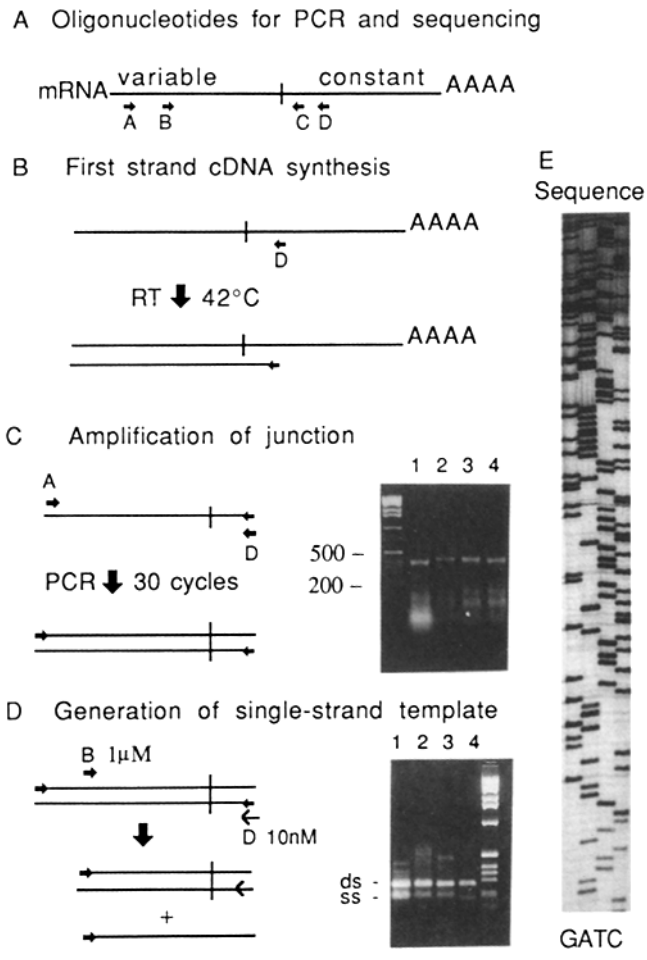


FIGURE 1. Sequencing strategy for TCR genes. (*A*) Oligonucleotides are constructed to bound the junctional region of TCR mRNA. Oligonucleotides A and B are sense and C and D are antisense. (*B*) First-strand cDNA is synthesized using oligonucleotide D and reverse transcriptase (RT). (*C*) The junctional region of the TCR is amplified by 30 cycles of polymerase chain reaction (PCR) using oligonucleotides A and D as primers and DNA polymerase from Thermus aquaticus. 30 cycles of denaturation at 95°C for 2 min, annealing at 50°C for 4 min, and primer extension at 70°C for 2 min are carried out. The photo shows an ethidium bromide–stained agarose gel after electrophoresis of 1/20th of the amplification reactions for Vβ14 RNAs. On the left is the 1-KB ladder (BRL); lane *1*, hybrid 15BBM21; lane *2*, 18BBM7; lane *3*, 18BBM138; lane *4*, 18BBM142. Each reaction shows a prominent band at ~400 bp, the predicted size for amplified Vβ TCR. (*D*) The amplified TCR junction is converted to single-strand template for sequencing by reamplifying ~5 ng of product from the first PCR in the presence of an unequal ratio of primers (B/D, 100:1 for generation of plus strand or C/A, 100:1 for generation of minus strand). Shown is generation of plus strand for Vβ14. Since oligonucleotide B is 3′ of oligonucleotide A, this yields a prominent ds band at 300 bp and a less bright band below this of ssDNA. Lanes *1–4* as in *C*. (*E*) Single-strand template from *D* is sequenced using a Sequenase kit. Shown is the sequence of the minus strand of Vβ 14 TCR from hybrid 15BBM21.

merase supplied by Cetus, the Perkin-Elmer Cetus thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT), and published conditions (20–23). For subsequent experiments, performed at National Jewish Center (Denver, CO), we have found that an Autotechnicon™ (Technicon Instruments Corp., Tarrytown, NY) tissue processor equipped with a 1-h timing clock is easily adapted to perform the polymerase chain reaction. The machine is designed to rotate baskets of samples through 12 baths, some of which are heated. The only necessary modification is to reconfigure the baths such that there are three cycles of: (*a*) one bath adjusted to 95°C for denaturation; (*b*) two unheated baths for primer annealing; and (*c*) one bath adjusted to 70°C for primer extension. All baths are filled with enough mineral oil to cover the bottom of the reaction tubes, and the heat from the adjacent 95°C and 70°C baths maintains the temperature of the unheated baths at ~50°C. If a lower annealing temperature is required, removal of the unheated baths from the machine allows the samples to hang in air at ~37°C. The timer is set to incubate the tubes 2 min at each stage and ~30 s is required to change positions. Thus, a cycle of PCR is completed every 10 min. The timer

TABLE I
TCR Variable and Constant Region Probes

Probe	Size	Fragment	Source	Originating lab	Reference
	bp				
*V*α1.2	371	R1/Alu1	B6 thymus	Palmer	-
Vα2	194	Bal1/Mbo2	B6 thymus	Hood	29
Vα3	305	BstN1/Hinf1	T cell clone 2C	Tonegawa/Raulet	30
Vα4.4	346	R1/Rsa1	Hybridoma 3DT52.5	Palmer	31
Vα5.1	250	R1/Pvu2	B6 thymus	Palmer	-
Vα6.1	298	R1/Tha1	B6 thymus	Palmer	-
Vα7.2	172	Hinf1	B6 thymus	Palmer	31
Vα8.4	297	R1/Rsa1	B6 thymus	Palmer	31
Vα10	300	R1/Hinf1	T cell clone D6	Hedrick	32
Vα11	227	Pst1	Hybridoma 2B4	Hedrick	33
Vα13	286	R1/Rsa1	Hybridoma DO11.10	Palmer	31
Vβ1	319	R1/BamH1	B6 thymus	Palmer	-
Vβ2	200	R1/Pst1	B6 thymus	Palmer	-
Vβ3	296	Rsa1	Hybridoma B03H.25	Hood	34
Vβ4	310	R1/Hinf1	B6 thymus	Palmer	-
Vβ5.1	200	R1/Ava1	B6 thymus	Palmer	-
Vβ6	326	R1/Ava1	BALB/c spleen	Raulet	35
Vβ7	307	R1/Nhe1	B6 thymus	Palmer	-
Vβ8.1	286	R1/Pvu2	B6 thymus	Palmer	-
Vβ9	380	R1/Sac1	BALB/c thymus	Loh	36
Vβ10	372	R1/Msp1	B6 thymus	Palmer	-
Vβ11	300	R1/Sac1	B6 spleen	Loh	36
Vβ12	330	R1/Pvu2	B6 spleen	Loh	36
Vβ13	130	-	Oligonucleotide	Miles/Freed	-
Vβ14	222	R1/Mbo2	Genomic	Hood	37
Vβ15	349	R1/Sau3A	B6 thymus	Palmer	-
Vβ16	253	R1/Hinf1	B6 thymus	Palmer	-
Vβ17	706	Sca1/Hind III	Hybridoma QK24.1	Kappler/Marrack	13
Cβ	800	R1/Hind III	Hybridoma DO11.10	Palmer	38
Cα	896	Pst1/Ra	B6 thymus	Palmer	-

can be easily adjusted to permit other incubation times if necessary. The samples in 1.5- or 0.5-ml microfuge tubes sit in plastic racks and up to 240 samples can be easily accommodated at one time.

Results

Generation and Characterization of I-A^{bm12}-reactive T Cell Hybrids. To study the TCR repertoire to the mutant I-A molecule, I-A^{bm12}, we generated a large panel of I-A^{bm12}-reactive hybrids. Peripheral T cells from C57BL/10 (B10) mice were stimulated in vitro with irradiated spleen cells from B6.C-H-2^{bm12} mice and the resulting blasts were fused to the thymoma, BW5147. 12 fusions, each using I-A^{bm12}-stimulated T cell blasts from an individual B10 mouse, yielded 178 I-A^{bm12}-reactive hybrids (Table II). In fusions 3 through 7, only a fraction of the resultant hybrids were picked. In the remaining fusions, all resultant hybrids were tested for I-A^{bm12} reactivity and the number of hybrids reflects the success of the fusion. I-A^{bm12}-reactive hybrids were initially identified by their ability to produce IL-2 when stimu-

TABLE II
I-A^{bm12}-reactive Hybrids

Fusion No.	No. of hybrids
3	7
4	6
5	6
6	10
7	7
8	4
9	2
14	6
15	11
16	19
17*	38
18*	62

* 22 of the hybrids from fusion 17 and all hybrids from fusion 18 were made with a variant of BW5147 (BW/α^-), which has deleted the rearranged Vα1 gene. All other hybrids were made with BW5147.

lated by B6.C-H-2^{bm12} but not B10 spleen cells. Positive hybrids were expanded and reactivity to I-A^{bm12} was confirmed at the time of RNA isolation. Table III lists the IL-2 titers against I-A^b and I-A^{bm12} of the hybrids included in this study. The titers against I-A^{bm12} vary from 10 to 640 U of IL-2. Most I-A^{bm12}-reactive hybrids showed no detectable crossreactivity against I-A^b. However, 11 hybrids, did crossreact on I-A^b but had at least a 16-fold higher IL-2 titer against I-A^{bm12}. Although minor genetic differences may exist between B6 (the background onto which the I-A^{bm12} mutation has been made congenic) and the responding strain, B10, these differences should not be apparent under the conditions of primary in vitro MLC used in these studies (43). As a further check on specificity, 10 B10 (I-A^b) hybridomas reactive

TABLE III
IL-2 Titers of I-A^{bm12}-reactive Hybrids

IL-2 titer* vs. I-A^b	IL-2 titer vs. I-A^{bm12}	No. of hybrids
<10	10	23
<10	20	17
<10	40	21
<10	80	24
<10	160	25
<10	320	23
<10	640	34
10	160	2
10	320	4
10	≥640	3
20	320	1
40	≥640	1

* IL-2 is measured by the ability to support the growth of the IL-2-dependent cell line HT-2.

with B6.C-H-2^{bm12} (I-A^{bm12}) splenocytes were stimulated with B6 (I-A^{b}) spleen cells and none were found to be reactive (data not shown).

An additional 66 I-A^{bm12}-reactive hybrids were obtained but were not included in our panel for the following reasons. 13 I-A^{bm12}-reactive hybrids were excluded because the analysis with a Cβ probe revealed that insufficient RNA had been obtained. 24 hybrids were excluded because they were determined to be nonclonal, defined by the expression of more than two Vα genes or more than two Vβ genes. 23 hybrids were excluded because they showed significant crossreactivity on I-A^{b}. Finally, six hybrids were excluded because they reacted repeatedly with I-A^{bm12} on spleen cells as used in the initial screen but not with the I-A^{bm12}-bearing lymphoma used in the confirmatory test.

Analysis of TCR Variable Gene Expression in I-A^{bm12}-reactive Hybrids. To determine which TCR variable genes are expressed by these I-A^{bm12}-specific hybridomas, ~5 × 10^5 cell equivalents of total cellular RNA from each hybrid was spotted onto 30 replicate nitrocellulose filters. One filter was hybridized with a Cβ probe to assure the presence of RNA. Other filters were then hybridized with each of the TCR variable gene probes listed in Table I. An equivalent amount of RNA from BW5147 was included on each filter to serve as a positive control for Cα, Cβ, and Vα1 and as a negative control for all other probes. RNA from A20/2J was included on each filter to serve as a negative control for all probes. All V region probes were free of D and J region nucleotides although a few probes include some of the leader sequence. In the mouse, there are ~60 TCR α chain variable genes that fall into at least 15 families (31, 44, 45). Probes to 11 of these families were available to us for the analysis. Hybridization and wash conditions were chosen so as to detect all family members. In contrast to the TCR Vα genes (44), only 21 TCR Vβ genes have been found and these comprise 17 Vβ chain families, most of which are unique genes. The exceptions are Vβ5, which has two, and Vβ8, which has three functional members, respectively. Probes to all 17 Vβ gene families were available for these studies. The fusion partner, BW5147, expresses Vα1 and Vβ1 RNA, and thus in most cases, expression of these variable genes could not be analyzed in our hybrids. A variant of BW5147 that has deleted its rearranged Vα1 gene has recently become available and was used in fusions No. 17 and 18, resulting in 86 hybrids that could be examined for Vα1 expression.

Table IV shows the number of Vα and Vβ genes detected in the panel of I-A^{bm12}-reactive hybrids and demonstrates a limitation of the analysis. While examination of RNA directly spotted onto filters permits a large number of hybrids to be examined, it does not enable one to distinguish functionally rearranged TCR genes from those that are out of frame. Despite the fact that T cells generally express only one surface TCR-α/β (34), they can potentially rearrange both α chain and both β chain alleles and thus, express mRNA for as many as two Vα genes and/or two Vβ genes. The most informative hybrids, therefore, are those that express only a single Vα (or Vβ) gene, since in these hybrids the variable region responsible for I-A^{bm12} reactivity can be determined, if not with certainty, at least with a high degree of probability. For this reason, only the 80 hybrids that express a single TCR Vα gene were used to calculate the frequency of Vα expression in I-A^{bm12}-reactive hybrids and only the 110 hybrids that expressed a single TCR Vβ gene were used to calculate the frequency of Vβ gene expression by I-A^{bm12}-reactive hybrids. 24 hybrids (13%)

TABLE IV

Number of TCR Vα and Vβ Genes Expressed by I-A^{bm12}-reactive Hybrids

No. of Vα genes expressed	No. of Vβ genes coexpressed		
	0	1	2
	hybrids		
0	16 (9%)	25 (14%)	8 (5%)
1	6 (3%)	54 (30%)	20 (11%)
2	2 (1%)	31 (17%)	26 (9%)

RNA from each hybrid was blotted on nitrocellulose filters that were then hybridized with ^{32}P-labeled probes to Vα gene families 1-8,10,11,13, and to Vβ 2-17.

expressed no RNA detectable by any of the 16 Vβ probes used. These may express Vβ1 or a Vβ gene that has yet to be identified. 49 hybrids (27%) expressed no RNA detectable by any of the 11 Vα probes used. Some of these hybrids could not be tested for Vα1 and may transcribe a Vα1-encoded mRNA. Moreover, we lack probes from at least four other Vα families (Vα9, Vα12, Vα13 [Hood], and Vα42H11 [Huber]). Given our collection of V gene probes, it is not surprising that a larger number of hybrids lack detectable Vα gene expression compared with those that lack Vβ gene expression.

Vα Gene Expression in I-A^{bm12}-reactive Hybrids. Fig. 2 *A* displays the frequency of I-A^{bm12}-reactive hybrids expressing particular TCR Vα genes. These data are derived from 80 hybrids that expressed RNA for a single Vα gene. Clearly, most Vα families can generate TCRs that recognize I-A^{bm12}. However, among I-A^{bm12}-reactive hybrids, the frequency of expression of a given Vα family ranges from 25% for the Vα3 family to 0% for Vα7 and Vα10 families. Data from a companion study of TCR variable gene expression in unselected mature B10 T cells (46) is included for comparison. Briefly, 485 random hybridomas were generated by fusing BW5147 cells to Con A-stimulated, peripheral T cells from B10 mice; RNA from these hybrids was analyzed in parallel with RNA from the I-A^{bm12}-reactive hybrids. A χ^2 analysis of the frequencies of Vα expression in I-A^{bm12}-reactive and random hybrids revealed no significant differences. Because individual genes within a Vα family are not distinguished by this method, and because probes to 4 of the 15 known families of Vα genes were not available, it is possible that individual Vα genes or entire Vα gene families are over or underrepresented in the panel of I-A^{bm12}-reactive hybrids.

Vβ Gene Expression in I-A^{bm12}-reactive Hybrids. Fig. 2 *B* displays the frequency of I-A^{bm12}-reactive hybrids expressing particular TCR Vβ genes. Again, hybrids expressing two Vβ genes were excluded, leaving 110 hybrids that expressed a single Vβ gene. As above, the data is compared with Vβ expression in a panel of random hybridomas derived from Con A-stimulated B10 T cells. As with Vα genes, many Vβ genes can participate in the I-A^{bm12} response. However, unlike the expression of Vα, the frequency of expression of Vβ genes in I-A^{bm12}-reactive compared with that in random hybrids is significantly different ($p < 0.0002$ by χ^2 test). The most dramatic difference is seen in the case of the Vβ5 gene family, which is expressed in 12% of random hybridomas but is undetectable among I-A^{bm12}-reactive hybrids ($p < 0.000007$ by Fisher exact test). In contrast, *Vβ14*, *Vβ15*, and *Vβ16* genes are

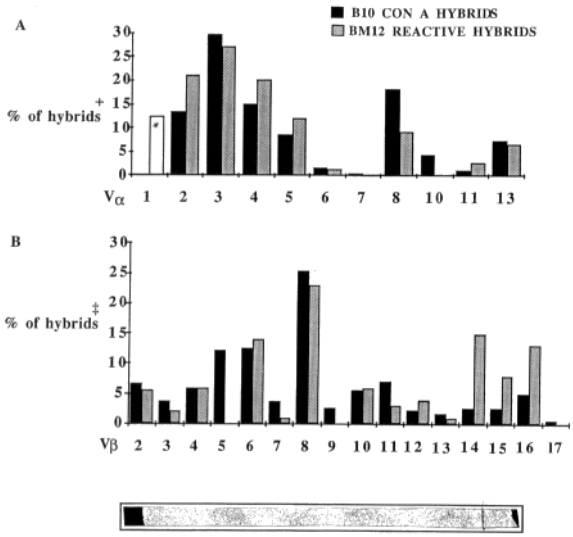


FIGURE 2. (*A*) Histogram showing TCR Vα expression by I-A^{bm12}-reactive and random hybrids. (+) The 80 I-A^{bm12} hybrids that expressed a single TCR Vα RNA have been used to construct the histogram. Reproduced for comparison are companion data from a panel of 186 random B10-derived hybrids (46). (*) The percent of hybrids expressing RNA from Vα1 TCR was extrapolated from the hybrids made with the Vα1$^-$ variant of BW5147 (BW/α$^-$). No comparison data are available from random hybrids. (*B*) Histogram showing TCR Vβ expression by I-A^{bm12}-reactive and random hybrids . (‡) The 110 I-A^{bm12}-reactive hybrids that express a single TCR Vβ RNA have been used to construct the histogram. Reproduced for comparison are companion data from a panel of 272 random B10-derived hybrids (46).

more frequently expressed among I-A^{bm12}-specific compared with random hybridomas ($p < 0.001$, $p < 0.05$, and $p < 0.025$, respectively, by χ^2 test).

Size of the Anti-I-A^{bm12} Repertoire. Fig. 3 shows the frequency of Vα/Vβ pairs among 54 I-A^{bm12}-specific hybridomas that express a single Vα gene and a single Vβ gene (see Table IV). Considering these 54 hybrids, 32 distinct Vα/Vβ combinations are found. Using standard sampling statistics, it is possible to estimate the total number of Vα/Vβ combinations that can recognize I-A^{bm12}. This number is not likely to be <37 nor >67 ($p < 0.05$). The calculation is based on the assumption that all I-A^{bm12}-reactive TCRs are equally likely to be detected. If the recurrence of certain combinations is due to the overrepresentation of a few I-A^{bm12}-reactive TCRs, then the actual size of the anti-I-A^{bm12} repertoire would be larger.

Sequence Analysis of Selected I-A^{bm12}-reactive TCR. From the above results, it appears that most TCR V region genes can participate in the recognition of I-A^{bm12} although

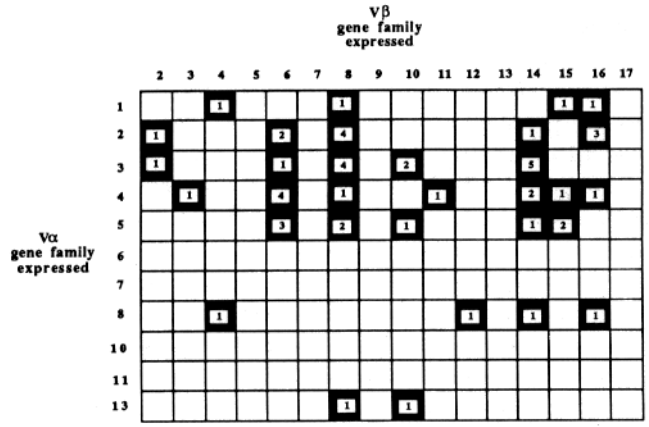


FIGURE 3. Coexpression of TCR Vα and Vβ genes in 54 I-A^{bm12}-reactive hybrids that express a single TCR Vα RNA and a single TCR Vβ RNA. Expressed Vα/Vβ pairs among these 54 hybrids are shown by black boxes and the number of hybrids expressing each combination is inscribed in each box.

not with equal probability. We decided to study a panel of hybrids expressing the same Vβ gene and a member of the same Vα family to determine the contribution of junctional (VJα, VDJβ) diversity to I-A^{bm12} reactivity. Four hybrids, 15BBM21, 18BBM7, 18BM138, and 18BBM142, each expressing only Vα3-encoded and only Vβ14-encoded RNAs were chosen for the analysis. Fig. 1 shows the sequencing strategy for the plus strand of the Vβ14 TCR. A complete description of the technique is given in Materials and Methods.

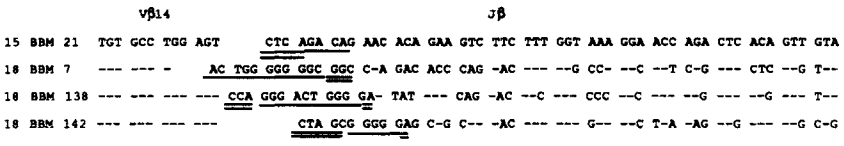
Fig. 4 *A* shows the nucleotide and derived amino acid sequences of the Vβ14 chains from four I-A^{bm12}-reactive hybrids. As expected, since no other Vβ RNA was detected by hybridization, all sequences are in frame and encode a functional protein. Notably, all four sequences are unique as each carries a different Jβ gene segment. Furthermore, N region or D region encoded amino acids are not conserved between these β chains. Fig. 4 *B* shows the nucleotide and predicted amino acid sequences of the Vα chains from the same hybrids. One hybrid, 15BBM21, contains an out-of-frame message and thus does not use Vα3 to recognize I-A^{bm12}. The three in-frame Vα3 sequences all differ from published Vα3 sequences (30, 47) by the presence of a G residue at nucleotide 350 in place of a T residue. Although only 45 nucleotides were sequenced at the 3′ end of Vα3, it appears that at least two different Vα3 family members are used by these hybrids. It is further evident that all hybrids use different Jα genes. Hybridoma 15BBM21 uses a previously identified Jα gene, *Jα28* (29), while the remaining hybrids use previously undescribed Jα gene segments. The conservation of amino acid residues generated by Vα/Jα joining is not apparent among these Vα3 cDNAs.

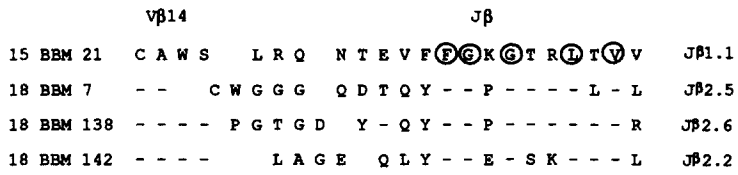
The fact that the predicted sequences were obtained from all eight cDNAs demonstrates the accuracy of the hybridization analysis and supports the assumption that a singly expressed Vα or Vβ mRNA is likely to be functional. The only exception is the out-of-frame Vα3 gene expressed in hybrid 15BBM21. Given the fact that our collection of Vα probes is incomplete, we most likely have not detected the functional Vα gene in this hybrid.

Discussion

To define the genetic complexity of the TCR repertoire to the mutant class II molecule, I-A^{bm12}, we generated T cell hybridomas from B10 T cell blasts that had been stimulated in primary MLC with I-A^{bm12} splenocytes and determined the expression of TCR α and β chain V genes by hybridization analysis of T cell hybridoma RNA. We were able to generate and analyze 178 of I-A^{bm12}-reactive hybridomas. With hybridomas, it is straightforward to obtain sufficient RNA to determine the expression of TCR V genes. The method however, has three shortcomings. (*a*) Out-of-frame (nonfunctional) mRNA cannot not be distinguished from in-frame (functional) mRNA. Consequently, we could only infer the Vα or Vβ gene contributing to the I-A^{bm12} reactivity in those hybrids that expressed only a single Vα or Vβ gene segment. (*b*) The hybridization analysis could only assign a given TCR RNA to a particular Vα or Vβ gene family. This presents no great problem for the analysis of Vβ genes, since only two families, Vβ5 and Vβ8, have more than one member; however, most, if not all, Vα families contain multiple genes. Thus, differential expression of one Vα family member may not be appreciable. (*c*) It is possible that the TCR α or β chain from BW5147 could be responsible for I-A^{bm12} reactivity in

A





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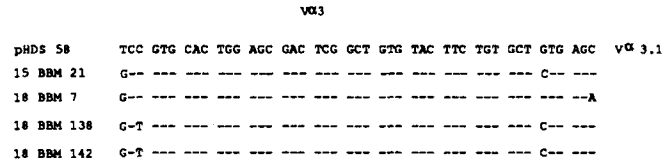

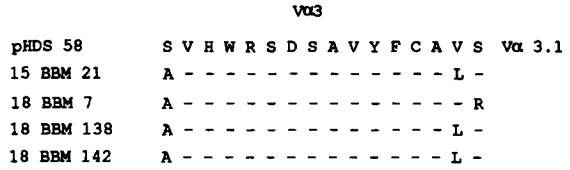

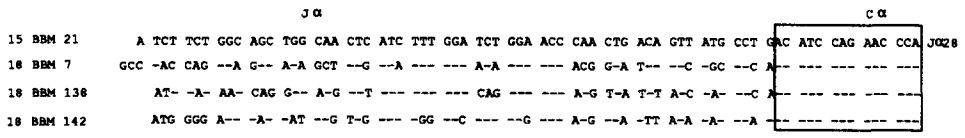

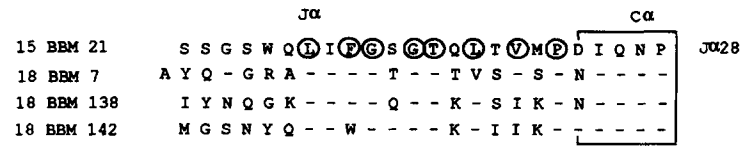

FIGURE 4. DNA and derived protein sequence of TCR junctional regions from 4 I-A^{bm12}-reactive hybrids that express mRNA from Vα3 and Vβ14. (*A*) Vβ14 sequences. N region nucleotides are doubly underlined, D nucleotides are underlined. Circled amino acids are conserved in TCR Vβs. (*B*) Vα3 sequences. The sequence of Vα3.1 is given for reference. Hybrid 15BBM21 uses Jα28 but is out-of-frame. The other three hybrids use previously undescribed Jαs. Circled amino acids are conserved in over half of the published Jα sequences. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00802.

some hybrids. We do not feel that this is a major consideration since nearly one-half of the hybrids resulted from fusion with a new variant of BW5147, BW/α^-, which has deleted its rearranged TCR α chain gene. That these fusions show no difference in the frequency of I-A^{bm12}-reactive hybrids (data not shown) argues against a major contribution of the BW5147 α chain alone or of the BW5147 α/β receptor to I-A^{bm12} reactivity in our hybridomas. A role for the BW5147 β chain towards generating I-A^{bm12}-reactive TCRs cannot be formally excluded but given that over half the hybrids resulting from any fusion show no I-A^{bm12} reactivity (data not shown), this seems unlikely.

Our results indicate that Vα1-6, 8, 10, 11, and 13 genes are expressed in I-A^{bm12}-reactive T cells. Comparing the frequency of expression of Vα genes in I-A^{bm12}-reactive hybrids with the frequency of Vα gene expression in hybrids derived from random B10 T cell blasts reveals no significant differences. Vβ expression is also heterogeneous in this collection of I-A^{bm12}-specific hybrids in that the *Vβ 2-4, 6-8, 10-16* genes can contribute to I-A^{bm12}-specific TCRs. In contrast to Vα genes, the distribution of expression of Vβ genes among I-A^{bm12}-specific hybrids is significantly different from that in random hybrids (χ^2, $p < 0.0002$). This difference is due to the less frequent than expected expression of the *Vβ5.1* and *5.2* genes (Fisher exact, $p < 0.000007$) and the more frequent than expected expression of the *Vβ14*, *Vβ15*, and *Vβ16* genes (χ^2 $p < 0.001$, $p < 0.05$, $p < 0.025$, respectively). The absence of hybrids that express Vβ5 RNA is particularly striking since the *Vβ5.1* and *5.2* genes are expressed by 12% of random B10-derived hybrids. This finding could imply that: (*a*) TCRs encoded by the Vβ5 genes (*Vβ5.1* and *5.2*) are generally incapable of recognizing the I-A^{bm12} gene product; (*b*) Vβ5-bearing T cells that can recognize I-A^{bm12} are not positively selected in the B10 (H-2^b) thymus; or (*c*) Vβ5-containing TCRs that can recognize I-A^{bm12} are eliminated in B10 mice because they crossreact with a B10 molecule (possibly I-A^b) and are deleted from the repertoire.

While no single Vα or Vβ gene can explain the high frequency of I-A^{bm12}-reactive T cells in B10 mice, the frequency of expression of Vβ genes in I-A^{bm12}-reactive T cells does not simply reflect the random usage of Vβ genes. Reimann and Bellan (48) have studied the use of the Vβ8 family of TCR β chains in the response to the mutant class I alloantigens K^{bm1} and K^{bm14}. Using F23.1, which recognizes the Vβ8.1, 8.2, and 8.3 β chain domains, to study CTL clones generated from B6 mice and reactive with K^{bm1} or K^{bm14}, they found that 37% of K^{bm1}-reactive CTL clones and 51% of K^{bm14}-reactive CTL clones express Vβ8-encoded TCRs. In agreement with our own findings, this data implies a bias for V gene usage but not an exclusive expression of any V gene segment. Garman et al. (35) have studied the expression of three Vα genes, (Vα1, Vα3, Vα4) and three Vβ genes, (Vβ6, Vβ7, and Vβ8) in random and alloreactive T cells. Skewed V gene expression was observed in alloreactive cultures; thus, our results agree with those of Garman et al. as well.

To determine the size of anti-I-A^{bm12} TCR repertoire, we counted the number of different Vα/Vβ pairs among 54 hybrids that express only one Vα and one Vβ gene (see Fig. 3) and found 32 distinct Vα/Vβ combinations. If one assumes that these hybrids represent a random sampling of I-A^{bm12}-reactive TCRs, then the number of Vα/Vβ combinations in the pool is not likely to be <37 nor >67 (p <0.05). This is a minimum estimate, since it assumes that I-A^{bm12} TCRs are equally represented in the population of responding T cells. This range (37-67) is likely to be

an underestimate of the actual number of I-A^{bm12}-reactive clonotypes because the analysis does not take into consideration Jα, Dβ, and Jβ regions that may contribute to the I-A^{bm12} specificity of these receptors. Other investigators have studied the genetic diversity of T cells reactive with a single alloantigen. Notably, Sherman (49, 50) has shown the TCR repertoire to K^b to be heterogeneous in that a large number of clones showing many different patterns of crossreactivity with related alloantigens were observed. While Sherman was unable to examine the genetic composition of the anti-K^b repertoire, she estimated it to contain ~50 different clonotypes based on the number of recurring patterns of crossreactivity. Her estimate is in agreement with that derived from the data presented here.

Having established that Vα and Vβ gene expression in I-A^{bm12}-reactive hybrids is extremely heterogeneous, we examined the sequence of α and β chain cDNAs in four hybrids expressing Vα3 and *Vβ14* to determine the importance of D, J, and N region elements to specificity. We obtained these sequences by modifying the polymerase chain reaction to amplify and directly sequence TCR genes. These modifications permitted rapid, accurate sequencing and should be generally applicable. One of the hybrids expresses an out-of-frame Vα3 mRNA and was not considered in this comparison. All three of the remaining hybrids use different Jβ and Jα genes and at least one of these hybrids uses a different Vα3 family member. Furthermore, no amino acid residues encoded by D region or N region nucleotides were found to be conserved in this collection of Vα3-encoded α chains and *Vβ14*-encoded β chains. While we cannot say that these sources of diversity are not important to I-A^{bm12} reactivity, it is unlikely that reactivity to I-A^{bm12} is determined by a single Jα or Jβ segment or by a junctionally derived (VJα or VDJβ) amino acid sequence.

Two fundamentally distinct strategies could render a significant fraction of T cells reactive with a particular alloantigen. Allo-MHC molecules could be recognized by either: (*a*) a large number of structurally related TCRs; or (*b*) by a large number of structurally unrelated TCRs. There are several examples of allorecognition mediated by TCRs of related structure. Kappler et al. (13) have shown that a large proportion of *Vβ17a*-encoded TCRs are reactive with I-E molecules, while our own work (Bill, J., D. Woodland, O. Kanagawa, and E. Palmer, manuscript in preparation) has demonstrated that the majority of *Vβ11*$^+$ TCRs are reactive with I-E as well. Other investigators have shown that *Vβ8.1*- (51) and *Vβ6*- (52 and O. Kanagawa, E. Palmer, and J. Bill, submitted for publication) encoded TCRs are reactive with the Mts^a antigen. In each case, the reactivity of the relevant TCRs is thought to be specified in large part by a single TCR domain. Furthermore, it is conceivable that a group of structurally related receptors may recognize a single ligand. Given these striking observations, it seemed plausible that the TCR repertoire to I-A^{bm12} might be dependent on one or a small number of TCR gene segments. Instead, we have found an extreme degree of gene segment diversity in the composition of the I-A^{bm12} TCR repertoire. This is particularly surprising in light of the fact that the alloantigen I-A^{bm12} differs from the I-A present on the responding strain (B10) by only three amino acid replacements. Thus, the TCR repertoire to a single allelic variant of I-A is mediated by a collection of "structurally diverse" receptors.

The striking result that the TCR repertoire to a single alloantigen is structurally diverse can be interpreted in several ways. Bevan (11) has implied that an alloantigen carries one or a few epitopes and has proposed that these epitopes are present on

the stimulating cell surface at a high concentration relative to the concentration of a self-MHC/conventional antigen complex; thus, alloantigens should be able to drive a small number of high affinity T cells and a larger number of low affinity T cells resulting in the observed high precursor frequency of alloreactive cells. Our data does not rule out and may be consistent with this idea. A second interpretation of our findings, which we favor, is that the TCR repertoire to a single alloantigen is structurally diverse because the allo-MHC molecule is antigenically diverse. While it is conceivable that the three amino acid substitutions in the I-A^{bm12} protein have engendered a large number of conformational changes in its structure relative to I-A^b, we consider this to be unlikely for several reasons. Most antibodies that recognize I-A^b crossreact with I-A^{bm12} (53); thus, at least some of the determinants on the I-A^{bm12} protein have been conserved. Furthermore, Bjorkman et al. (54, 55) have suggested that there may be a generalized conservation of the structure of all class 2 MHC molecules. The idea that the overall structure of the I-A^b and I-A^{bm12} molecules may be quite similar argues against the presence of a large number of conformational differences between these two proteins.

However, the proposed three-dimensional structure of class 2 MHC molecules suggests another possibility. The finding of an electron density in the crystal of the class 1, HLA-A2 molecule led Bjorkman et al. (54, 55) to suggest that peptides may be bound to surface MHC glycoproteins in the groove between the two α helices at all times. Guillet et al. (56) have previously proposed this idea for class 2 molecules. Since the three amino acid residues that distinguish I-A^b from I-A^{bm12} fall within an α helical region creating the presumed peptide binding site (57), the altered amino acids could change the requirements for peptide binding. Thus, I-A^{bm12} may present a different set of self-peptides than does I-A^b. This model implies an antigenic diversity of allo-MHC molecules that is based on the diversity of MHC-bound self-peptides. This idea has also been expressed by Marrack et al. (58). They have carried out experiments describing the differential ability of various cell types to stimulate I-E-reactive hybridomas that is unrelated to the amount of I-E on the presenting cell surface. The implication of these findings is that another cell surface component (possibly a self-peptide) contributes to the ligand recognized by allo-I-E-specific T cells. The data of Marrack et al. (58), Hunig and Bevan (59), and the experiments described here suggest that a population of allospecific T cells actually recognizes the alloantigen plus a variety of self-peptides. This idea is consistent with a high frequency of alloreactive T cells and an allospecific repertoire that is structurally diverse.

Summary

We have studied the genetic diversity of the TCR repertoire to the murine alloantigen I-A^{bm12} by generating a panel of 178 C57BL/10-derived I-A^{bm12}-reactive T cell hybridomas. The expression of Vα and Vβ gene families was examined in this panel and the frequency of expression of Vβ, but not of Vα, gene families differed significantly from that observed in a companion panel of random C57BL/10-derived hybridomas. The Vβ5 gene family was expressed significantly less frequently while the *Vβ14*, *Vβ15*, and *Vβ16* genes were expressed significantly more frequently in the panel of I-A^{bm12}-reactive than in the panel of random hybridomas. The junctional regions (VJα and VDJβ) of TCR Vα and Vβ genes from selected I-A^{bm12}-

specific hybridomas were amplified using the polymerase chain reaction, and directly sequenced. Surprisingly, no conserved Jα, Dβ, Jβ, or N region-encoded sequences among these selected I-A^{bm12}-reactive TCRs were identified. Thus, the T cell response to an I-A alloantigen that differs by only three amino acid residues from the I-A molecule of the responding strain is genetically complex but nonrandom. We have estimated that the repertoire to this alloantigen is comprised of at least 37 different TCRs.

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