Molecular Definition of a Polymorphic Antigen (LA45) of Free HLA-A and -B Heavy Chains Found on the Surfaces of Activated B and T Cells

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Summary

A monomorphic monoclonal antibody (LA45 antibody) reactive with "a new activation-induced surface structure on human T lymphocytes" (LA45 antigen) that resembled free class I heavy chains has recently been described (Schnabl, E., H. Stockinger, O. Majdic, H. Gaugitsch, I.J.D. Lindley, D. Maurer, A. Hajek-Rosenmayr, and W. Knapp. 1990. *J. Exp. Med.* 171:1431). This antibody was used to clone a class I–like heavy chain (LA45 gene) from the HUT 102 tumor cell, which paradoxically did not give rise to the LA45 antigen on transfection into monkey COS cells. We show here that the LA45 gene is HLA-Aw66.2, a previously uncharacterized allele of the HLA-A locus. The previously determined LA45 sequence differs from that of HLA-Aw66.2, from HUT 102, and the CR-B B cell line derived from the same individual as HUT 102 by substitution of tryptophan for serine at position 4 in the α_1 domain. Transfection of HLA-Aw66.2, and of a mutant of this gene with serine 4 substituted for tryptophan, into a human B cell line (C1R) both resulted in expression of the LA45 epitope. Furthermore, we find expression of the LA45 epitope on Epstein Barr virus–transformed B cell lines as well as lectin-activated T cells, but not on long-term T cell lines or unstimulated peripheral blood T cells. The specificity of the LA45 antibody is polymorphic and the presence of the LA45 epitope is precisely correlated with the sequence arginine, asparagine (RN) at residues 62 and 63 of the helix of the α_1 domain. The LA45 epitope is broadly distributed, being associated with half the alleles of both HLA-A and -B loci but none of the HLA-C locus. All the results are consistent with the presence of pools of free HLA-A and -B heavy chains at the surfaces of certain cell types but not others Such molecules are probably responsible for the HLA-associated class I alloantigens of lectin-activated T cells. We hypothesize the free heavy chains result from dissociation of β_2-microglobulin from subpopulations of empty HLA-A,B molecules, or molecules with weakly bound peptides, that vary in size depending on cellular activation and peptide supply.

For >10 yr, it has been known that human T cells express novel HLA-encoded alloantigens, after activation with PHA. These molecules are class I like in their polypeptide composition but are serologically distinct from the classical HLA–A, -B, and -C antigens (1–8). Although it has been speculated that these determinants are human analogues of the mouse nonclassical Qa molecules, evaluation of such hypotheses through molecular cloning of the genes encoding the human class I activation antigens has not been done (9). Recently, Schnabl et al. (10) described a mouse mAb, LA45, with an unusual specificity for class I HLA molecules, which may relate to the PHA-activated alloantigens. This antibody, the result of immunization with the HTLV-1-infected tumor cell line HUT 102, was found to bind to PHA-activated peripheral blood T cells and to T cells activated in vivo by infectious mononucleosis, but not to resting T cells. Immunoprecipitation and cocapping experiments showed that LA45 bound to class I–like heavy chains at the cell surface not associated with β_2-m. Furthermore, binding of the antibody to PHA-activated T cells from a panel of 12 individuals appeared independent of HLA type, suggesting "the LA45 antibody defines a monomorphic determinant on free class I heavy chains" (10).

To identify genes encoding this unusual class I antigen,

Schnabl et al. (10) used the LA45 antibody to screen an expression library made from HUT 102 DNA. Clones derived from a single gene were obtained and these had a novel class I sequence. Transfection of this LA45 gene into monkey (COS) cells resulted in extensive intracellular expression, but only slight expression of the LA45 antigen on the cell surface. These results suggested LA45 was not a conventional classical class I HLA antigen and that its gene may represent a previously uncharacterized nonclassical HLA class I locus. On the other hand, the observation that class I heavy chains bound by LA45 were electrophoretically similar to those of HLA-A,B molecules argued against this thesis. In this paper, we describe experiments that address this paradox, identify the LA45 gene as HLA-Aw66.2, and provide further insight into the molecules and epitope recognized by the LA45 antibody. A relationship between these molecules and the class I-like alloantigens of PHA-activated T cells seems likely.

Materials and Methods

Preparation and Amplification of cDNA. cDNA clones encoding HLA-Aw66 subtypes were from the EBV-transformed B cell lines: MALS (HLA-**Aw66.2**, A3; Bw58), 25-1501 (HLA-**Aw66.1**, A2; B7, B35), CR-B (**A30**, **Aw66.2**; B17, B18), and HUT 102 (**A30**, **Aw66.2**; B17, B18). The boldface alleles are those for which cDNA were cloned. These cell lines were kindly provided by Drs. F. M. Robbins and W. Bias (Johns Hopkins University), and N. M. Lardy and L. de Waal (University of Amsterdam). The construction of cDNA libraries, the isolation of clones, and the sequencing of M13 subclones with synthetic oligonucleotide primers were as described (11, 12). Sequence analyses were made with UWGCG software (13) and a VAX 11/789. Multiple clones were analyzed to obtain a consensus sequence for each allele. In particular, the first 200 bp from a combined total of 41 HLA-Aw66.1 and -Aw66.2 clones were sequenced. All encoded serine at codon 4 of the α_1 domain, none having the tryptophan codon reported for the LA45 clone (10).

PCR Mutagenesis and Transfection. Mutagenesis at nucleotide positions 83 and 84 of the coding region of HLA-Aw66.2 was performed using the PCR mutagenesis method described by Ho et al. (14). A 1.5-kb HindIII/SalI fragment of the mutated clone was subcloned into M13. Mutagenesis was confirmed by sequencing the full-length cDNA. Wild-type mutant clones were inserted into plasmid vector PSRαneo (kindly provided by Dr. R. Tisch, Stanford University) by XbaI and HindIII digestion and ligation of gel-purified fragments. Transfection into HLA-A,B-negative C1R cells and flow cytometric analysis were performed as described (15).

Isoelectric Focusing. This was performed according to the 10th International Workshop protocol (16, 17).

Immunofluorescence. To test for cell surface expression of HLA class I molecules and the LA45 epitope, C1R transfectants, EBV-transformed cell lines, PBL, PHA blasts, or T cell lines were washed, and aliquots of 2.5×10^5 cells were then incubated for 30 min in 100 μl PBS/1% BSA plus 20 μg/ml of one of the following antibodies: W6/32 (monomorphic anti-HLA class I); LA45 (anti-HLA class I heavy chain); CVC.7 (an irrelevant control of the same Ig-G2a isotype [anti-bovine clathrin light chain LC_a]), or saline. The cells were washed twice and labeled with 100 μl of 20 μg/ml affinity-purified goat anti-mouse Ig-FITC (Tago Inc., Burlingame, CA) and incubated for 30 min on ice. Cells were washed in PBS, fixed for 1 min in 100 μl PBS/2% formalin, and diluted to 500 μl with PBS/1% BSA. Cells were then analyzed using a FACScan® cytofluorograph (Becton Dickinson & Co., Mountain View, CA). PHA-activated cells were prepared from PBL by incubation in RPMI/10% FCS in the presence of PHA (1 ml of rehydrated PHA [Gibco BRL]) added to 100 ml of cultured medium], with supplementation on day 3 with 50 U/ml IL-2, and harvesting for analysis on day 5. T cell lines were the gift of Drs. P. K. Wesley, M. M. van Noesel, and A. M. Krensky (Stanford University).

Results

The LA45 cDNA Clone Is Derived from an HLA-Aw66 Gene. From comparisons with a limited number of class I genes, Schnabl et al. (10) found the LA45 gene to be "quite homologous to genes of the HLA class I gene family" and to bear characteristics commonly associated with HLA-A alleles (18). They did not, however, analyze the LA45 sequence with reference to the serological HLA type of the HUT 102 cell from which the LA45 gene was derived (19). The HUT 102 cell line was established from the tumor cells of a Black male patient: an EBV-transformed B cell line (CR-B) was also established from his nonmalignant peripheral blood B cells (20). At that time, 1980, the HLA type of the patient was assessed to be HLA-A30, A31; B17, B18; DR2, DRw6, DQ1 using the available anti-HLA alloantisera.

Comparison of the LA45 sequence with those for the serologically defined HLA-A,B types of HUT 102 showed no particular similarities with HLA–A30, A31, B17, or B18. We then compared the LA45 sequence to a data base of 27 HLA-A, 33 HLA-B, 13 HLA-C alleles, and to eight sequences from nonclassical class I HLA genes. From pairwise comparisons of complete sequences and the pattern of nucleotides at locus-specific positions, LA45 is unequivocally an allele of the HLA-A locus and not of one of the nonclassical class I genes and pseudogenes that are related to HLA-A (Table 1). In addition, the LA45 sequence was revealed to be a hybrid of HLA-Aw68.1 and HLA-A10 alleles, having a leader sequence and α_1 domain similar to HLA-Aw68.1, and the remaining domains similar to the HLA-A10 subtypes HLA-A25 and -A26 (Fig. 1).

This analysis strongly suggested the LA45 gene represented a previously unsequenced HLA-A allele expressed by the HUT 102 cell line, but one that did not correspond to either of the serologically assigned HLA-A antigens. This was not im-

Table 1. *Nucleotide Differences between LA45 and Classical and Nonclassical HLA Alleles*

	HLA-A	HLA-B	HLA-C	Nonclassical HLA-E, F, G, and H
LA45	10–59*	82–120	95–119	84–159
Intralocus comparison: A v A, B v B, C v C				1–62
Interlocus comparison: A v B, B v C, C v A				57–132

* Values given are the range of nucleotide differences in pairwise comparison of complete coding sequences.

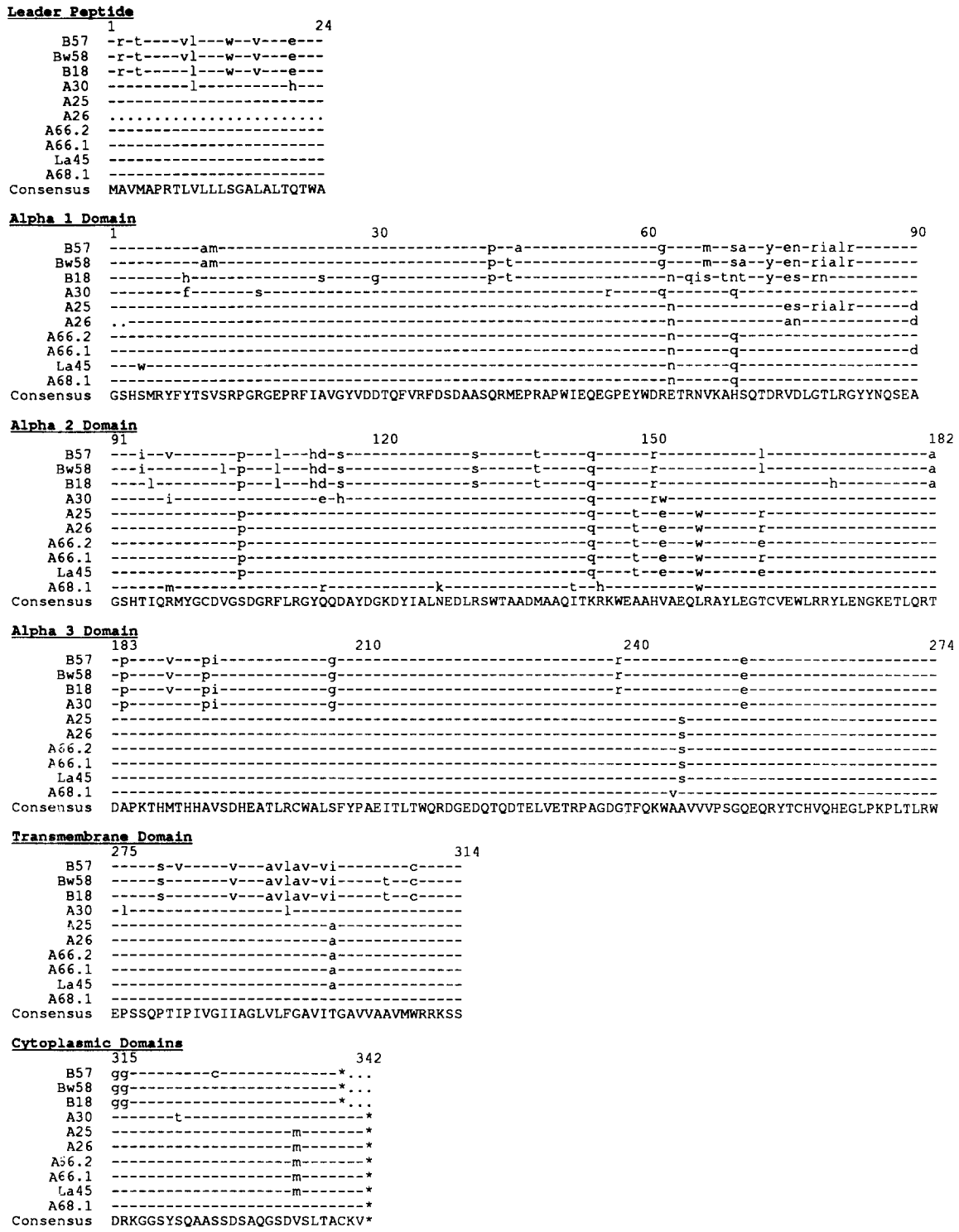

Figure 1. Amino acid sequence comparisons of LA45 with other HLA-A and -B molecules. The consensus is derived from 31 HLA-A sequences. The dots indicate incompleted sequences; dashes indicate identities with a consensus sequence. Asterisks indicate a termination codon. Sequences were obtained from the following sources: HLA-A68.1 (27); LA45 (10); -A26 (43); -A25, -B57 (12); -A30 (44); -B18 (18); -Bw58 (45).

plausible as various factors could have contributed to imprecision in the typing. These include poor definition of HLA alleles of African origin, the well-established crossreactivity of the HLA-A30 and HLA-A31 antigens, and difficulties associated with typing lymphocytes from cancer patients. Given this hypothesis, the question then became whether the LA45 allele corresponded to a defined serological antigen or one that was totally uncharacterized.

A strategy that answered this question came from consideration of the unique structure of LA45 as an Aw68-A10 hybrid and postulation that it would have antigenic properties in common with one or both of these groups of antigens. For two reasons, alloantigenic similarities with HLA-A10 were considered more likely: first, because sequences for all known serological subtypes of HLA-Aw68 were known (21) and shown to be distinct from LA45, whereas various antigens, including HLA-Aw34, Aw43, and Aw66 of the HLA-A10 group, had yet to be characterized in molecular detail; and second, HLA-Aw68 is a relatively weak alloantigen compared to HLA-A10, indicating that the HLA-A10-like aspect of the LA45 structure would be likely to dominate. We therefore predicted that LA45 would correspond to one of the more recently characterized antigens of the HLA-A10 group (22).

Preliminary sequence analysis of HLA-A alleles from serologically well-characterized B cell lines and isoelectric focusing of their expressed HLA-A,B proteins revealed HLA-Aw66, originally described by Mesman et al. (23), to be the most likely candidate for LA45. The isoelectric focusing analysis also revealed heterogeneity in molecules typed as HLA-Aw66 (Fig. 2). The Aw66 molecules of the 25-1501 cell of Caucasian origin was different from that of the MALS cell line, which came from an American Black individual. Complete nucleotide sequence determination of Aw66 alleles from these two cell lines showed the two sequences were highly related, both to each other and to that of the LA45 clone (Fig. 3). The HLA-Aw66.1 subtype from cell line 25-1501 is a perfect hybrid of A68.1 and either A25 or A26, and is likely to have arisen by recombination between these alleles. Flanking markers limit such putative recombination to within codons 77–89 of exon 2. The HLA-Aw66.2 subtype from MALS

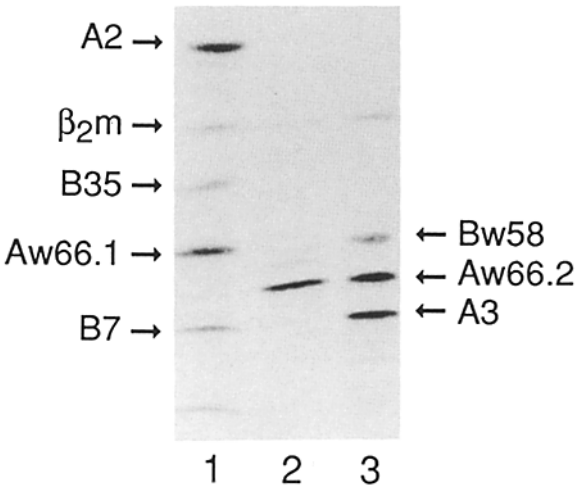

Figure 2. Isoelectric focusing pattern of HLA-Aw66 molecules from the 25-1501 (lane *1*), CR-B (lane *2*), and MALS (lane *3*) cell lines. Immunoprecipitates were with the W6/32 mAb after depletion of HLA-B and -C molecules with the 4E antibody. Depletion of HLA-B was incomplete for lanes *1* and *3*. Two subtypes of HLA-Aw66 (Aw66.1 and Aw66.2) are resolved. The CR-B B cell line is from the same individual as the HUT 102 tumor cell line.

differs from Aw66.1 by four nucleotide substitutions resulting in amino acid substitutions of D→A at position 90 and R→E at position 163. It is HLA-Aw66.2 that is most like LA45, differences being seen at just three nucleotide positions. First is a single nucleotide deletion in exon 1 of LA45. This is almost certainly the result of a sequencing error, as Schnabl et al. (10) showed the LA45 clone directs synthesis of an antigenically recognizable class I heavy chain of normal length, which would not occur with the change in reading frame created by this deletion. The other two are adjacent substitutions in codon 4 of exon 2 and lead to substitution of tryptophan for serine at position 4 of the α_1 domain. This substitution is highly unusual as serine 4 is conserved in all other HLA-A, -B, and -C sequences. Three alternative origins for these latter two differences were considered: first, that LA45 represents a third subtype of HLA-Aw66 found in the human population, second, that LA45 represents a mutant HLA-Aw66.2 allele that arose during either the in vivo or in vitro growth of the tumor cells, and third, that the differences between LA45 and the HLA-Aw66. sequences represent experimental artifacts.

To distinguish these possibilities, we analyzed the HLA-A alleles expressed in HUT 102 (the source of the LA45 cDNA clones) and in the B cell line (CR-B) derived from the same individual. If the LA45 sequence is that of a third HLA-Aw66 subtype, then the LA45 sequence should be obtained from both HUT 102 and CR-B, whereas if the differences were due to somatic mutation in the tumor cells, then they should be seen in HUT 102 but not the B cell line. Clones corresponding to two distinctive HLA-A alleles were isolated from both cell lines. No differences in the sequences of clones derived from the B cell line and the tumor cell line were found. One allele was identical to the HLA-Aw66.2 isolated from the MALS cell line, and the second allele was a subtype of HLA-A30. Finding identical HLA-Aw66.2 genes in the HUT 102, CR-B, and MALS cell lines argues against the the first two possibilities and therefore indicates that the differences between the LA45 and HLA-Aw66.2 sequences are either due to mutations specific to the subline of HUT 102 cells used by Schnabl et al. (10), to mutations introduced during cloning procedures, or to sequencing errors. Of interest is that both HLA-A alleles of HUT 102 have "new" sequences, representing alleles that have not been clearly defined by serology and are possibly subtypes that are preferentially found in African Blacks and their descendants. This probably explains the discrepancy between the "molecular typing" reported here and the original serological typing. For example, the HLA-A30 subtype of HUT 102 is not common and may well have given reactions with alloantisera directed against HLA-A30 and -A31. Reassessment of the serological typing of the HUT 102 and CR-B cell lines showed reactivities with alloantisera defining HLA-Aw66.

The Unique Substitution at Position 4 in the LA45 Heavy Chain Does Not Affect Cell Surface Expression or Detection of the LA45 Epitope. The single difference between the class I heavy chain encoded by the HLA-Aw66.2 and LA45 sequences is the substitution of serine for tryptophan at position 4. To examine the effect of this difference upon cell surface expression, we

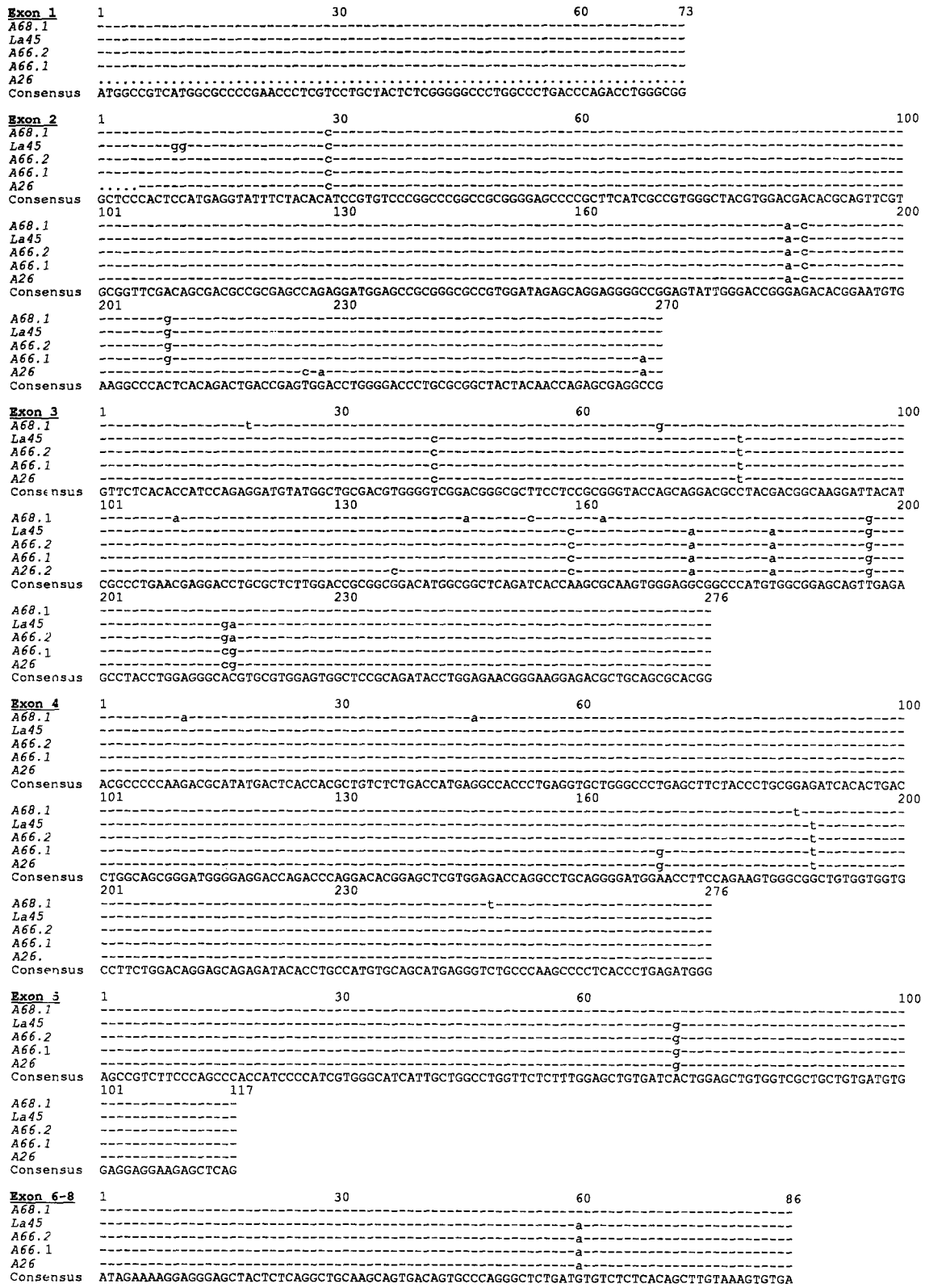

Figure 3. Nucleotide sequence comparison of LA45, A68.1, A26, and the Aw66.1 and Aw66.2 subtypes. Allelic sequences are compared to the consensus derived from 31 HLA-A sequences, and identity is denoted by a dash. The LA45 and HLA-A68.1 sequences were from references 10 and 27, respectively. The Aw66.1 and Aw66.2 sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession numbers X61711 and X61712, respectively.

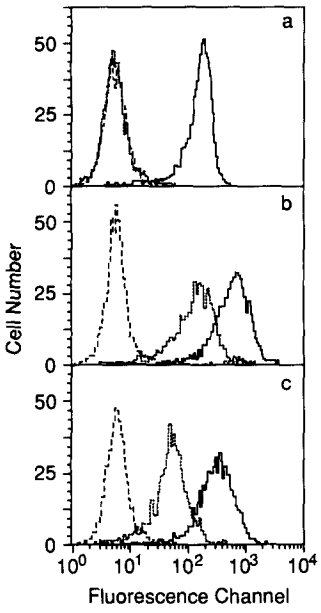

Figure 4. Analysis of the cell surface expression of LA45 and W6/32 epitopes by untransfected C1R cells (*a*) and by C1R cells transfected with LA45 (*b*) or HLA-Aw66.2 (*c*) cDNA clones. The plots are immunofluorescence of FITC goat anti-mouse Ig as analyzed by flow cytometry. Binding of W6/32 is given by the solid line, of LA45 by the dotted line, and of the negative control antibody by the dashed line.

made a mutant of HLA-Aw66.2 with tryptophan at position 4. Mutant and wild-type genes were subcloned into the PSRαNeo expression vector and electroporated into the human HLA-A,B-negative cell line C1R. After 2 wk of drug selection and culture, considerable and comparable increases in the amount of β_2-m-associated class I heavy chains were found at the surface of cells transfected with both the wild-type and mutant genes (Fig. 4). These complexes were detected with the antibody W6/32, which is dependent upon the association of class I heavy chain and β_2-m (24). High expressing cells were selected using the FACScan®, and the mutant HLA-Aw66.2 gene was reisolated and sequenced to confirm the mutation. Thus, the apparent difference between LA45 and HLA-Aw66.2 has no gross effect upon the assembly, transport, and stability of the class I heavy chain.

Furthermore, C1R cells transfected with wild-type or mutant HLA-Aw66.2 genes bound the LA45 mAb, whereas untransfected C1R cells did not. This experiment provides a direct demonstration that the LA45 antigen is the product of a classical HLA-A gene and that no qualitative difference in its expression is caused by the substitution at position 4 that distinguishes LA45 from HLA-Aw66.2. As the C1R mutant is derived from an EBV-transformed cell, a further important conclusion from this analysis is that expression of the LA45 antigen is not restricted to activated T cells. The failure of the LA45 epitope to be expressed when the LA45 gene was transfected into COS cells (10) is therefore likely to have been a function of the recipient cell rather than of the LA45 clone.

Table 2. *Expression of LA45 and W6/32 Epitopes by C1R Cells Transfected with Single HLA-A or -B Genes and by EBV-transformed B Cells Lines*

		mAb			
Cell	HLA type	W6/32	CVC-7	LA45	No mAb
Aw68/C1R	Aw68	911	5.4	**169.3**	3.2
Aw69/C1R	Aw69	394	5.2	**188.6**	2.7
A2/C1R	A2	1,062	3.9	14.7	2.1
B27/C1R	B27	1,743	3.9	7.0	1.9
B7/C1R	B7	1,697	3.3	**153.0**	2.1
Aw66.2/C1R	Aw66.2	312	3.8	**19.3**	2.7
LA45/C1R	LA45	581	4.7	**74.6**	4.1
GRC138	A2; B40,62	2,068	4.2	8.2	5.4
JESTHOM	A2; B27	1,775	4.2	7.5	1.9
JY	A2; B7	2,050	6.8	**160.0**	3.3
DEM	A2; B57	2,125	4.5	10.7	2.4
SPOO10	A2; B44	2,013	2.8	16.0	2.2
BM16	A2; B18	1,295	3.6	**145.3**	2.6
HOM2	A3; B27	1,072	3.5	19.8	2.4
OLGA	A31; B62	1,857	3.4	11.5	2.6
DAUDI	-	4.4	3.5	3.2	2.4
C1R	-	111.9	7.0	6.8	3.0

Cells were labeled using W6/32 (monomorphic anti-HLA class I), CVC.7 (irrelevant control, LA45, or saline as the first step, and FITC goat anti-mouse Ig as the second step. They were then analyzed using a FACScan® cytofluorograph. Numbers represent the mean channel fluorescence for each antibody. The bottom two lines show the staining of the HLA-A,B,C-negative DAUDI cell line and the HLA-A,B-negative C1R transfectant recipient cell line.

The LA45 Epitope Is Polymorphic, Expressed in EBV-transformed B Cells and Is Determined by Residues 62 and 63 of the α_1 Helix of HLA-A and -B Heavy Chains. To determine if the results obtained with class I transfectants of the C1R mutant extend to normal EBV-transformed B cell lines, we compared binding of the LA45 antibody to panels of each type of cell. Among both sets of cells there were some that were positive for LA45 binding and others that were negative, demonstrating polymorphism in the distribution of the antibody epitope. From analysis of the transfectants it was clear that LA45-positive and -negative molecules derived from both the HLA-A and HLA-B loci: HLA-Aw68, -Aw69, and -B7 were positive, whereas HLA-A2 and -B27 were negative (Table 2). Comparison of HLA types and reaction patterns of the EBV lines and transfectants reveals that HLA-A3 is negative and HLA-B18 is positive for the LA45 epitope.

Inspection of the amino acid sequences of the LA45-positive and -negative class I heavy chains shows that substitutions at positions 62 and 63 are the only ones to correlate with the distribution of the LA45 epitope. These residues are at the NH_2-terminal end of the helix of the α_1 domain, and both contribute to the antigen recognition site: LA45-positive molecules have arginine at position 62 and asparagine at position 63, whereas LA45-negative molecules have a variety of other sequence motifs. This analysis suggested that the LA45 epitope is critically dependent upon arginine 62 and asparagine 63. One possibility is that the antibody directly contacts these residues, and another is that they have conformational effects on distant residues that form the actual epitope. If the latter were true, then one might expect denaturation to change the polymorphic specificity of the LA45 antibody. This was not the case, as shown by Western blot analysis of cell lysates made from the panel of C1R transfectants. Heavy chains from HLA-Aw66.2, -Aw68.1, -Aw69, and -B7 transfectants reacted with the LA45 antibody, whereas those from HLA-A2 and -B27 did not. All heavy chains except HLA-A2 reacted with the HC10 mAb (25), which has a distinctive specificity for free, denatured class I HLA heavy chains (data not shown). Our results therefore support a model in which LA45 is a linear epitope centered on arginine 62 and asparagine 63.

This allows HLA-A,B,C molecules to be divided into groups predicted to be positive and negative for the LA45 epitope. All HLA-C alleles are predicted to be negative, whereas HLA-A and -B molecules fairly evenly distribute between the two groups (Table 3). To test these predictions, we analyzed the distribution of the LA45 epitope on resting and PHA-activated T cells from four HLA type donors (Table 4). Confirming the results of Schnabl et al. (10), we found no binding of

Table 4. *Expression of LA45 and W6/32 Epitopes on Resting and Activated Lymphocytes*

		mAb			
Cells	HLA type	W6/32	CVC-7	LA45	No mAb
AML-PBL	A1,2; B44,8	565	1.6	3.7	1.5
AML-PHA		1,537	1.8	**24.4**	1.5
CL-PBL	A2,24; B44,62	472	1.3	2.1	1.3
CL-PHA		1,111	1.4	2.3	1.5
AMF-PBL	A3,w68; B65	555	1.3	3.9	1.2
AMF-PHA		1,367	1.2	**55.4**	1.2
AMK-PBL	A3; B7	464	2.2	8.0	1.7
AMK-PHA		1,482	2.6	**41.5**	2.2
AMK-EBV		2,263	3.2	**81.6**	3.2
AMK-AJY		1,121	2.2	4.4	1.3
HUT78	A1; B62	2,031	5.3	7.8	5.6
HSB	A1,2; B12,44	1,637	3.2	4.2	3.2
BG	A3,23; B50,55	1,227	2.0	13.3	2.1
JURKAT	A3; B35	690	2.0	3.7	2.0
B27/C1R	B27	2,068	1.9	13.3	1.7
B7/C1R	B7	1,537	2.1	**173.9**	2.2
A2/C1R	A2	903	2.0	17.9	1.9
C1R	-	156	4.1	11.0	3.9

PBL and day 5 PHA blasts were from four donors (AML, CL, AMF, and AMK). In addition, an EBV-transformed B cell line (AMK-EBV) and a long-term alloreactive CTL line with specificity for HLA-A2 (AMK-JY) derived from the PBL of donor AMK were also tested. HUT 78, HSB, BG, and Jurkat are T tumor cell lines. Cells were labeled with W6/32 (monomorphic anti-HLA class I), CVC.7 (irrelevant control), LA45 antibody, or saline as the first step, and FITC goat anti-mouse Ig as the second step. They were then analyzed using a FACScan® cytofluorograph. Numbers represent the mean channel fluorescence for each antibody.

Table 3. *Predicted Polymorphic Specificity of the LA45 Antibody*

Predicted to be LA45-positive R62, N63-containing	Predicted to be LA45 negative
A25, A26, Aw33, Aw66, Aw68, Aw69	A1, A2, A3, A11, A23, A24, A29, A30, A31, A32
B7, B8, B14, B18, B35, B38, B39, Bw42, B51, Bw53, Bw65, Bw78	B13, B27, B37, B40*, Bw41, B44, Bw46, B47, B49, B52, Bw57, Bw58, Bw60, Bw62
	All HLA-C

the LA45 antibody to resting PBL. On activation, the T cells from three of the donors became positive, while the fourth remained negative. This last donor, CL, expresses HLA-A2, 24; -B44, 62, which are all molecules predicted to be LA45 negative. In contrast, the other three donors all have at least one HLA-A, or -B molecule predicted to be LA45 positive.

Given the striking difference in binding of LA45 to resting and PHA-activated peripheral T cells, we were interested to assess other types of human T cells for the presence of this epitope. Five T cell–derived cell lines were examined and none expressed the LA45 epitope (Table 4). For HUT78 and HSB, this is simply explained by the fact that neither cell types for HLA-A, -B, and -C molecules predicted to be LA45 positive. Jurkat and two recently stimulated alloreactive cytotoxic T cell lines, however, all express HLA-B molecules predicted to be LA45 positive. In the case of donor AMK, we know the prediction to be true, as PHA-activated T cells and an EBV-transformed B cell line from the same individual were both LA45 positive. The absence of the LA45 epitope on the AMK alloreactive CTL is not simply correlated with it being of CD8 phenotype, as many of the LA45 positive cells in the culture of PHA-activated T cells are of this type. Rather, it seems that the LA45 epitope is differentially expressed in T cells at various activation states. Clearly, the LA45 epitope is not found on all types of activated T cells.

Discussion

Nature of the LA45 Gene. We have shown that the novel class I HLA gene, LA45, isolated from HUT 102 cells by Schnabl et al. (10), corresponds to a subtype of HLA-Aw66, a recently defined antigen of the HLA-A locus. This antigen is difficult to distinguish by HLA serology (serologists in North America and Europe do not agree on its definition [26]), and knowledge of its sequence, its characteristic hybrid structure, and the existence of two subtypes should improve this situation. We isolated and sequenced identical HLA-Aw66.2 alleles from the HUT 102 T cell tumor line, the CR-B B cell line derived from the same individual as HUT 102, and the B cell line MALS derived from an unrelated individual. We also sequenced the HLA-Aw66.1 subtype from the 25-1501 B cell line. In none of the 41 HLA-Aw66.2 clones analyzed have we seen the two unusual nucleotide substitutions in codon 4 of the LA45 sequence, leading to the conclusion that these differences are either unique to the subline of HUT 102 used by Schnabl et al. (10), misincorporations produced during cDNA cloning, or sequencing artifacts. It is unlikely there has been any mix-up of cell lines due to the rarity of the HLA-Aw66 antigen. Either way, these substitutions are unlikely to be found in HLA-Aw66 alleles in the human population.

HLA-Aw66.1 is the third example of a class I HLA allele that is a simple recombinant of two other alleles, the other examples being HLA-Aw69 formed from A2.1 and Aw68.1 (27) and HLA-Bw42 formed from B7 and B8 (28). In each case, the recombination is such that the two halves of the antigen recognition site derive from different alleles, and this may be a particularly effective way for the creation of new antigen-presenting functions from existing alleles.

Specificity of the LA45 Antibody. In contrast to the earlier conclusion that the LA45 mAb has monomorphic specificity (10), we clearly demonstrate that its specificity is polymorphic. Moreover, LA45 reactions are precisely correlated with the presence of both arginine at position 62 and asparagine at position 63 in the α_1 helix, indicating these residues form a critical part of the LA45 epitope. Roughly half of the alleles at the HLA-A and -B loci are predicted to be positive for the LA45 epitope, and the high probability with which any individual will have at least one LA45-positive HLA-A,B molecule probably explains why Schnabl et al. (10) found that PHA-activated T cells from all 12 of the individuals they analyzed were positive for the LA45 epitope. Of the three T cell samples from patients with infectious mononucleosis, one was negative for LA45 binding, which could now be interpreted in terms of the polymorphic specificity of the antibody. Their observation, in an isoelectric focusing experiment, that LA45 precipitates had the same binding patterns as precipitates with the monomorphic anti-class I antibody W6/32 (10) would be consistent with all HLA-A and -B molecules of the cells tested being LA45 positive. In such experiments, the lower levels of HLA-C are rarely visualized. The HUT 102 tumor cell line that was the immunogen for generating the LA45 antibody expresses HLA-Aw66.2 and -B18 molecules that are positive for the LA45 epitope, and HLA-A30 and -B17 molecules that are negative.

The second issue regarding the specificity of the LA45 antibody is its tissue distribution. On the basis of reactions with lectin-activated T cells and in vivo activated T cells from infectious mononucleosis patients, LA45 was described as "a new activation-induced structure on human T lymphocytes" (10). First, we have confirmed the specificity for PHA-activated PBL and the absence of reactivity with unstimulated PBL, second, we show that human T cells activated by other means need not necessarily express LA45 determinants, and third, that the LA45 epitope is ubiquitously found on EBV-transformed B cell lines providing they express an appropriate "LA45-positive" HLA-A or -B allele.

Origin of LA45-reactive Free HLA-A,B Heavy Chains on Cell Surfaces. Our results and those of Schnabl et al. (10) are entirely consistent with the LA45 antibody having specificity for free R62/N63-containing HLA-A,B heavy chains, both in solution and at the cell surface. For human cells, it has consistently been reported (29, 30) that class I HLA-A,B heavy chains are only transported to the cell surface when associated with β_2-m, though one mouse class I molecule, H-2D^b, has been shown to reach the plasma membrane in the absence of β_2-m (31). Thus, the free HLA-A,B heavy chains at the surface of EBV-transformed B cells and PHA-activated T cells are likely to have originated from complexes of heavy chain and β_2-m from which the β_2-m has dissociated. The population of such molecules on resting T cells is undetectable but increases to 25,000–60,000 molecules per cell on PHA activation (10). This difference is not associated with gross changes in the level of class I HLA, which increases two-

to threefold in PHA-activated T cells compared to resting PBL (32). Hansen and colleagues (33) have reported a similar phenomenon for the mouse H-2L^d molecule in which free heavy chains with altered conformation and protease susceptibility were found on all cell types they examined.

One explanation for the occurrence of free HLA-A,B heavy chains on cell surfaces is they represent intermediates in the general pathway of degradation of class I molecules. This scheme, however, cannot explain the difference between resting and activated T cells without postulating different degradative pathways in the two cell types. A second explanation, and one we favor, is that LA45 reactivity is the property of a subpopulation of HLA-A and -B molecules produced in PHA-activated T and EBV-transformed B cells but not in resting cells.

A possible mechanism is for LA45-reactive heavy chains, which from the estimates of McCune et al. (32) and Schnabl et al. (10) may comprise 10–20% of the total heavy chains, to be derived from empty molecules, that is complexes of heavy chain and β_2-m that arrive at the cell surface without a bound peptide. Empty molecules have been detected on the surfaces of mutant and normal cells (33–36), and we recently provided evidence for their presence on the surface of EBV-transformed B cell lines (37). A characteristic of empty class I molecules at the cell surface is they more rapidly dissociate from β_2-m than molecules with bound peptide. Thus, empty HLA-A,B molecules at the cell surface would be expected to give rise to a pool of free and potentially LA45-reactive heavy chains. Alternatively, the free heavy chains may derive from HLA-A,B molecules that arrive at the cell surface with weakly bound peptides. Such molecules are likely to be less stable than those with strongly bound peptides and could, by dissociation of both peptides and β_2-m, be a source of free heavy chains.

From recent investigations, it appears that an abundance of suitable binding peptides is not always available to class I molecules (33–37). Increased synthesis of class I heavy chains and β_2-m in activated T cells may therefore not be accompanied by an equivalent increase in available peptides in the endoplasmic reticulum, thereby leading to greater numbers of empty molecules and molecules with less tightly bound peptides. In this manner, quantitative changes in the amounts of peptides and heavy chains can lead to qualitative differences in the HLA-A,B molecules formed. This model can explain the observation that monkey (COS) cells transfected with the LA45 cDNA failed to express the LA45 epitope, although plenty of LA45-reactive free heavy chains were found within the cell (10). If peptide supply in COS cells is not limiting, then all LA45 heavy chains will be incorporated into molecules with tightly bound peptide. In this case, free heavy chains will not be produced at the cell surface in detectable amounts and the phenotype of the transfected cells will be like that of resting PBL, i.e., LA45 negative. That the result of transfection is indeed a function of the recipient cell is indicated by our finding that transfection of LA45 cDNA into an EBV-transformed B cell line gave cell surface expression of the LA45 epitope.

Relationship to HLA Alloantigens on Activated T Cells. Multiple reports of HLA-linked alloantigens found on activated T but not resting T cells have been described (1–8). The presence of these alloantigens on EBV-transformed B cells is not known and probably difficult to examine due to the many B cell antibodies in human alloantisera. These antigens have usually been correlated with haplotypes encoding particular HLA-A alleles and may, like the LA45 antigen, represent antigenic determinants formed by distinctive conformations of the HLA heavy chain. Recently, Crepaldi et al. (38, 39) have in fact shown, in the case of activation alloantigens correlating with HLA-A1- and HLA-A2-containing haplotypes, that the alloantisera precipitate class I heavy chains that by isoelectric focusing patterns are identical to HLA-A1 and HLA-A2. Conformational distinction in the molecules containing these heavy chains was demonstrated by their lack of reactivity with the monomorphic anti–class I HLA antibody W6/32 (39). In contrast to the LA45 determinant, the T cell alloantigens are the property of β_2-m-associated class I molecules as they are cocapped/blocked and precipitated with anti-β_2-m. Given the similarities in cellular occurrence and HLA-A association of the LA45 and T cell alloantigens, it is conceivable that both sets of antigenic determinants are manifestations of the same phenomenon. The apparent difference in β_2-m association need not rule this out. For example, the target molecules of LA45 and the alloantibodies may come from the same set of empty and relatively labile β_2-m-associated molecules: the alloantibodies binding them while they are still associated with β_2-m, LA45 binding the free heavy chains after dissociation of β_2-m. Also possible is that binding of the LA45 mAb may actually induce dissociation of β_2-m from empty molecules.

That alloantigens of activated T cells are formed by class I molecules such as HLA-A2 and HLA-A24(6), which do not have the LA45 epitope, suggests formation of the conformationally distinctive or empty form is not restricted to molecules with this particular epitope and sequence motif. Supporting this view is the recent finding of a free HLA-C heavy chain on the surface of virus-infected cells (40). Thus, the phenomena described for LA45-reactive HLA-A,B molecules may be a more general property of the products of class I HLA alleles. Furthermore, there is no reason to believe their expression will be limited to cells of the lymphoid lineage, in which case the alloantigens of free class I heavy chains may be a feature of other types of activated cells.

During primary activation, human T cells are likely to undergo considerable changes in their patterns of antigen presentation. Expression of class II HLA molecules is turned on (41) and that of class I is increased by a factor of two to three (32). If the activation pathways of protein degradation and peptide transport are not commensurately increased, then limited peptide supply may create an effective competition for endogenous peptides between class I and class II molecules. The presence of increased numbers of class I molecules on the surfaces of activated cells may facilitate the presentation of peptides of exogenous origin by class I molecules (37). Finally, the degradation of proteolytically susceptible free class

I heavy chains in the endocytic pathway may lead to increased presentation of class I peptides by class II molecules (42). Such changes in antigen presentation could be involved in the initiation of regulatory T cell responses. The lack of the LA45 epitope on lines of human T cells that have undergone multiple stimulations with antigen indicates that the changes leading to the expression of conformationally distinct empty molecules on activated T cells are reversible. Thus, cell surface expression of antigenically distinct free HLA-A,B heavy chains may mark particular stages of human T cell differentiation.

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