Cytotoxic T Cell Recognition of an Endogenous Class I HLA Peptide Presented by a Class II HLA Molecule

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Summary

Human leukocytes were stimulated in vitro with peptides corresponding in sequence to the highly variable helix of the α1 domain of various HLA-B and -C molecules. A $CD4^+CD8^-$ cytotoxic T cell line, CTL-AV, that is specific for the HLA-B7 peptide presented by HLA-DR11.1 was obtained. The HLA-DR11.2 molecule, which only differs at three residues from HLA-DR11.1, did not present the HLA-B7 peptide to CTL-AV. Peptides from the α1 domain helix of other HLA-A and HLA-B molecules, but not HLA-C molecules, competed with the HLA-B7 peptide for binding to HLA-DR11.1. A cell line (WT50) that coexpresses HLA-B7 and HLA-DR11.1 was killed by CTL-AV in the absence of any added HLA-B7 peptide. The processing and presentation of HLA-B7 in these cells appears to be through the endogenous, and not the exogenous, pathway of antigen presentation. Thus, Brefeldin A inhibits presentation and chloroquine does not. Furthermore, introduction of purified HLA-B7 molecules into $HLA\text{-}DR11.1^+$, $HLA\text{-}B7^-$ cells by cytoplasmic loading via osmotic lysis of pinosomes, but not by simple incubation, rendered them susceptible to CTL-AV killing. These results provide an example of class II major histocompatibility complex (MHC) presentation of a constitutively synthesized self protein that uses the endogenous pathway of antigen presentation. They also emphasize the capacity for presentation of MHC peptides by MHC molecules.

T lymphocytes with α/β antigen receptors interact with ligands consisting of peptides bound to MHC glycoproteins (1–4). Study of T cell responses to a variety of foreign proteins has shown that peptides arising from endogenously synthesized proteins are frequently presented by class I MHC molecules and that peptides derived from cellular degradation of exogenously synthesized proteins are similarly presented by class II MHC molecules (5–11). There are, however, exceptions to these general rules for antigen presentation (12–15).

It is becoming increasingly clear that, in the absence of foreign antigens, MHC molecules bind peptides derived from self proteins and that these complexes play important roles in thymic selection of the TCR repertoire, in the establishment of tolerance and its subsequent maintenance (16–20). Moreover, the interaction with peptide can, in the case of class I, be critical for the correct assembly and cell surface expression of the MHC molecule (21). It is therefore likely that most, if not all, cell surface MHC molecules have a bound peptide and that peptides contribute directly or conformationally to the structures contacted by all TCRs, including those of alloreactive T cells.

Analysis of the xenogeneic response of murine T cells to the human class I molecule, HLA-Cw3, revealed that the antigenic target was not native HLA-Cw3 but a peptide derived from the processing of the HLA-Cw3 H chain and presented by a murine class I MHC molecule (22). Examination of allogeneic responses has furnished additional examples of the peptides derived from one MHC molecule being presented to T cells by a second MHC molecule (23–25); and raises the question as to the frequency of such composite MHC targets in the immune response to allogeneically transplanted tissues.

In this paper, we describe an in vitro immunization of human peripheral blood leukocytes with synthetic peptides derived from class I HLA H chains. This immunization led to the stimulation of CTL specific for a composite MHC target, consisting of a peptide derived from an allogeneic class I molecule (HLA-B7) presented by a syngeneic class II MHC molecule (HLA-DR11.1). In cells that express both HLA-B7 and HLA-DR11.1, the composite target can be formed "naturally" and is detected by CTL. Processing of HLA-B7 and its presentation by HLA-DR11.1 have properties that are characteristic of the endogenous pathway of antigen presentation.

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These experiments thus demonstrate a further exception to the rules of antigen presentation, in which an endogenous and constitutively synthesized self protein is presented by class II MHC molecules.

Materials and Methods

Peptides. Synthetic peptides corresponding to residues 60–84 of HLA-A,B,C H chains (26) were synthesized by a solid phase method using Fmoc chemistry (27). The peptides were desalted by gel filtration on a P-10 column in PBS (pH 7.3). Homogeneity of the peptides was indicated by reverse-phase HPLC. The HLA-B7.60-84 peptide was sequenced by automatic Edman degradation using a gas phase sequencer and was shown to be >95% pure.

Establishment of Peptide B7.60-84–specific Cytotoxic T Cell Line AV (CTLAV). Responder cells were peripheral blood leukocytes from three healthy donors: BC (HLA-A11, 24; B27, 22; Cw1,w3; DR8,12; DQ1,3), AV (HLA-A2, 29; B27, w58; Cw2, w7; DR2, 11; DQ1, 3), and JL (HLA-A2, 31; B7, w57; Cw3; DR1, 3; DQ1, 2). Peripheral blood leukocytes at 10^6 cells/ml were stimulated with 10 μg/ml of a mixture of nine HLA-derived peptides in triplicate macrowell (Costar, Cambridge, MA) cultures with supplemented RPMI 1640. The immunizing peptides had sequences corresponding to residues 60–84 of HLA-B7, B13, B14, B27.1, B27.2, B41, Cw1, Cw2.2, and Cw3. Medium was supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA). 3 d after initiation of culture, rIL-2 was added at 10 U/ml. The cultures were subsequently fed every 3 d with medium containing rIL-2 at 50 U/ml and restimulated every 14 d with the mixture of peptides using irradiated (4,000 rad), autologous peripheral blood leukocytes (10^6 cells/ml) as APC. After four stimulations, irradiated (10,000 rad) autologous EBV-transformed B cells (5×10^5 cells/ml) were used as APC instead of peripheral blood leukocytes. Cultures from donor AV gave substantial growth after five stimulations. The expanded culture, designated CTL-AV, was aliquoted (10^7 cells per vial) and cryopreserved in medium containing 10% DMSO at −135°C. The CTL-AV line was shown to be $CD4^+CD8^-$ by indirect immunoassay using monoclonal anti-CD4, -CD8, -HLA, and -DR antibodies and radioiodinated rabbit anti-mouse Ig as the second-step reagent.

Cell Lines. EBV-transformed B cell lines (EBV-LCL) were used both as antigen-presenting and target cells. Cell lines JBUSH, SPOO10, SWEIG007, BM21, BRIP, TUBO, JO52839, JVM, BM15, TISI, WT24, KT12, PF97387, BM16, BH, PITOUT, and KASO11 were obtained from the 10th International Histocompatibility Workshop. Cell lines AVOROS and LBA were established by coculturing peripheral blood leukocytes with supernatant containing EBV virus and cyclosporin A (100 ng/ml). Cell lines ARB, WT50, and GMR were obtained from Dr. J. Bodmer of the Imperial Cancer Research Fund, London. All cell lines were maintained by regular passage in supplemented medium.

Cell-mediated Cytotoxicity. CTL-AV was thawed and restimulated with the mixture of nine immunizing peptides (10 μg/ml/peptide) presented by irradiated autologous EBV-LCL in medium containing rIL-2 at 50 U/ml. In experiments after the identification of B7.60-84 as the specific antigen, CTL-AV were simulated solely with the B7.60-84 peptide at 10 μg/ml. Cytotoxicity by CTL-AV was measured 7 d after restimulation. Effector cells were diluted in supplemented medium to yield the E/T ratios of 20:1, 10:1, and 3:1. Target cells were labeled with 250 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, IL) in 0.3 ml of medium for 45 min. The target cells were washed two times in medium, diluted to 5×10^4 cells/ml, and 100 μl of cell suspension was added to microwells containing medium or peptide (2 μg/ml) for 30 min before the addition of effector cells in 100 μl. The cultures were incubated for an additional 4 h in a 5% CO_2 incubator, and supernatants were collected with harvesting frames (Skatron, Sterling, VA) and counted for ^{51}Cr release. Percent cytotoxicity is calculated using the formula: 100× (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release and maximum release values were determined by incubating target cells in medium or 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), respectively. Results are presented as means ± SD of triplicate determinations.

Treatment of Target Cells with Brefeldin A, Chloroquine, and HLA-A1 Peptide. The effect of treating target cells with Brefeldin A and chloroquine on their susceptibility to CTL-AV killing was examined (28–30). Brefeldin A and chloroquine were added to ^{51}Cr-labeled target cells (5×10^3 cells per microwell) at 15 μg/ml and 10 μM, respectively. The inhibitors were preincubated with the target cells for 30 min at 37°C in 5% CO_2 followed by an additional 30-min incubation with B7 peptide, B27 peptide, or medium before adding the effector cells. The inhibitors were present for the duration of the 4-h ^{51}Cr release assay. The concentrations of the inhibitors used in our experiments had previously been shown to inhibit the presentation of either endogenous or exogenous antigens (28–30), and did not show toxic effect when tested on ^{51}Cr-labeled CTL-AV effector cells in a 4-h ^{51}Cr release assay. To test the two inhibitors on WT50 target cells that constitutively synthesize and present B7 antigens, the target cells were preincubated with a HLA-A1 peptide to replace B7 peptides in cell surface B7/DR11.1 complexes before chromium labeling and introduction of the inhibitors. WT50 cells (2×10^6 cells) were incubated with peptide A1.60-84 (20 μg/ml) for 24 h at 37°C in 5% CO_2. The cells were then pelleted, washed with RPMI 1640, and labeled with 250 μCi of ^{51}Cr for 45 min. After being washed twice with RPMI 1640, the target cells were resuspended in supplemented medium in supplemented medium and added to microwells at 5×10^3 cells per well. Inhibitors and peptides were then added to the target cells as described above.

Cytoplasmic Loading of HLA Molecules. HLA molecules were introduced into the cytoplasm of cells by osmotic lysis of pinosomes containing exogenous HLA molecules using a modified method of Moore et al. (31). Soluble HLA molecules were dissolved in hypertonic medium (0.5 mM sucrose, 10% [wt/vol] polyethylene glycol 1000, and 10 mM Hepes in RPMI, pH 7.2) at 0.3 mg/ml. ^{51}Cr-labeled target cells (2×10^6 cells) were washed twice in PBS, resuspended in 0.5 ml of prewarmed hypertonic medium containing soluble HLA-A2 or B7 molecules, and incubated at 37°C for 10 min. Hypotonic medium (10 ml of 60% RPMI in water) was then added to the cells for 3 min and washed twice in PBS. The cell pellets were resuspended in supplemented medium at 5×10^4 cells/ml and used as target cells in a cytotoxicity assay.

mAbs. Hybridomas secreting the anti-DR mAb (L243), anti-CD4 (OKT4), and anti-DR11 (SFR3.DR5) were obtained from the American Type Culture Collection (Rockville, MD). The anti-CD8 mAb (CamPath8c) was a gift from Dr. H. Waldmann (Cambridge University). The anti-DQ (anti-Leu-10) and anti-DP (B7/21) mAbs were gifts from Dr. F. Brodsky.

Results

Cytotoxic T Cell Line CTLAV Recognizes a Peptide Derived from the α1 Helix of HLA-B7 Molecules. Synthetic peptides

corresponding to residues 60–84 from the α1 helices of six HLA-B (B7, B13, B14, B27.1, B27.2, and B41) and three HLA-C (Cw1, Cw2.2, and Cw3) molecules were cultured in vitro with peripheral blood leukocytes from three healthy donors. After repeated stimulation, the cells from one donor (AV: HLA-A2, 29; B27, w58; Cw2, w7; DR2, 11; DQ1, 3) developed cytotoxicity that was specific for the autologous B cell line when sensitized with the peptide mixture. Expansion of these cells in rIL-2 resulted in the $CD4^+$, $CD8^-$ cytotoxic T cell line CTL-AV.

CTL-AV only lysed the AV B cell line in the presence of peptides, showing that the CTL were specific for epitopes provided by one or more of the peptides. Sensitization of AV target cells with individual α1 peptides showed that significant lysis was only seen in the presence of the HLA-B7 peptide. Moreover, the level of lysis obtained with this peptide was equal or better than that obtained with the mixed peptides (Fig. 1 *A*). This demonstrated that the HLA-B7 peptide was the target antigen for CTL-AV. Peptide sensitization of target cells with this peptide was dose dependent, saturation being reached with 2 μg/ml of peptide (data not shown).

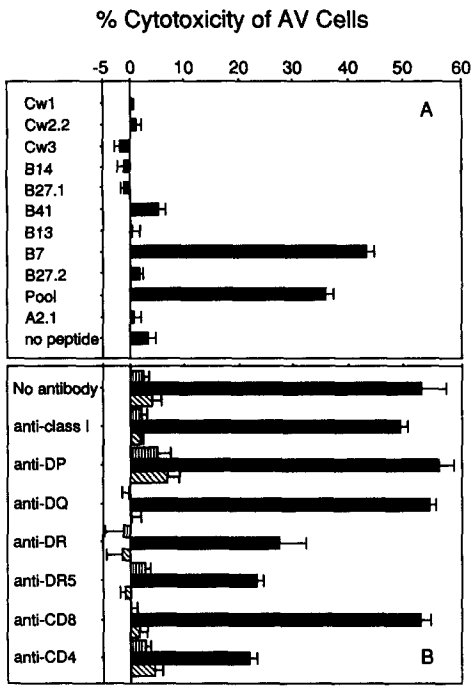


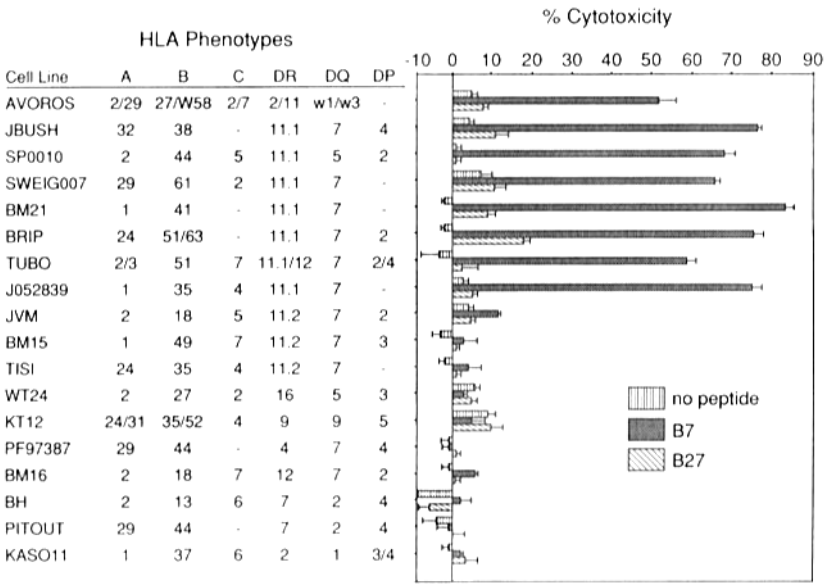
Figure 1. Presentation of an HLA-B7 peptide by HLA-DR11.1 molecules. (*A*) Leukocytes repeatedly stimulated with a mixture of HLA-B,C peptides (at 10 μg/ml/peptide) were tested for cytotoxicity of autologous EBV-LCL with or without the peptides present. Peptides (2 μg/ml) were incubated with target cells for 30 min before the addition of effector cells, and were present throughout the cytotoxicity assay. (*B*) Cytotoxicity of autologous EBV-LCL without peptide (▥), with B7.60-84 peptide (■), or with B27.60-84 peptide (▧) was measured in the presence of mAbs. Antibodies used were monomorphic anti-class I HLA (W6/32), anti-DP (B7/21), anti-DQ (anti-Leu-10), monomorphic anti-DR (L243), anti-DR11 (SFR3.DR5), anti-CD8 (CamPath 8c), and anti-CD4 (OKT4). The target cells were preincubated with mAbs (50 μg/ml) for 30 min before the addition of effector cells. Specific cytotoxicity was measured in a 4-h ^{51}Cr release assay. Mean cytotoxicity of triplicate cultures with SD was obtained at an E/T ratio of 10:1.

HLA-B7.60-84 Peptide Is Presented to CTL-AV by HLA-DR11.1. The CD4 phenotype of the CTL suggested that the antigen receptors of CTL-AV recognize the HLA-B7 peptide when presented by a class II HLA molecule. To address this question directly, we assessed the cytotoxic activity of CTL-AV in the presence of mAbs specific for HLA-DP, -DQ, and -DR, and HLA class I molecules (Fig. 1 *B*). Inhibition of cytotoxicity was observed with a monomorphic anti-DR antibody (L243) and a specific anti-DR11 antibody (SFR3.DR5), but not with anti-DP (B7/21), anti-DQ (anti-Leu-10), and anti–class I (W6/32) antibodies. In addition, inhibition with anti-CD4 and not with anti-CD8 demonstrated that the specific CTL were in fact $CD4^+$ and $CD8^-$. These results show that the antigenic target of CTL-AV is the HLA-B7 peptide presented by the HLA-DR11 molecule of donor AV. The inhibition shown by anti-DR and anti-CD4 mAbs shown in Fig. 1 *B* is ~50%. Total inhibition was observed with higher antibody concentrations, but such concentrations resulted in some inhibition with nonspecific antibodies.

A panel of 18 B cell lines of different HLA types was incubated with the HLA-B7.60-84 peptide and then screened for lysis by CTL-AV. This analysis clearly confirmed that only cells expressing HLA-DR11 were capable of presenting the B7 peptide. However, not all cells typed as DR11 presented the peptide (Fig. 2). Recently, HLA-DR11 has been split into two subtypes, DR11.1 and DR11.2, that differ in the sequence of their β chains by three amino acids (32, 33). Presentation of the B7 peptide correlated precisely with expression of the DR11.1 subtype.

In Vivo Processing of HLA-B7 Results in Presentation by HLA-DR11.1. CTL-AV recognizes a synthetic peptide, with sequence derived from the HLA-B7 H chain, presented by HLA-DR11.1. This finding raised the possibility that cells from individuals who express both B7 and DR11.1 would display this, or a similar, complex upon their surfaces. Four cell lines expressing both HLA-B7 and HLA-DR11 were obtained. However, they had not been previously assigned to one or other subtype of HLA-DR11. On sensitization with peptide HLA-B7.60-84, two of the four cell lines (WT50 and ARB) were lysed by CTL-AV and, on this basis, were typed as HLA-DR11.1; the other cell lines, LBA and GMR, were not lysed by CTL-AV and were assigned as HLA-DR11.2 (Table 1).

In the absence of peptide sensitization, the WT50 cell line, which is homozygous for HLA-B7 and HLA-DR11.1, was lysed by CTL-AV; and this killing was specifically blocked by monoclonal anti-HLA-DR (Table 1). This result, combined with the finding that cell lines expressing either B7, DR11.1, or neither of these molecules were refractory to lysis by CTL-AV (Figs. 1 and 2), shows that functional complexes of B7 peptides bound by HLA-DR11.1 molecules are present on the WT50 cell surface. Although specific lysis of WT50 cells was observed in all of 10 independent experiments, the lysis was never as complete as when a saturating amount of



Cell Line	A	B	C	DR	DQ	DP
AVOROS	2/29	27/W58	2/7	2/11	w1/w3	-
JBUSH	32	38	-	11.1	7	4
SP0010	2	44	5	11.1	5	2
SWEIG007	29	61	2	11.1	7	-
BM21	1	41	-	11.1	7	-
BRIP	24	51/63	-	11.1	7	2
TUBO	2/3	51	7	11.1/12	7	2/4
J052839	1	35	4	11.1	7	-
JVM	2	18	5	11.2	7	2
BM15	1	49	7	11.2	7	3
TISI	24	35	4	11.2	7	-
WT24	2	27	2	16	5	3
KT12	24/31	35/52	4	9	9	5
PF97387	29	44	-	4	7	4
BM16	2	18	7	12	7	2
BH	2	13	6	7	2	4
PITOUT	29	44	-	7	2	4
KASO11	1	37	6	2	1	3/4

Figure 2. Restriction determinant for CTL-AV. Day 6 after being restimulated with B7.60-84 peptide, CTL-AV was assayed for cytotoxicity against a panel of B cell lines sharing either class I or class II allotypes with donor AV. Cytotoxicity was measured in a 4-h ^{51}Cr release assay against target cells in the absence of peptide (▥), preincubated with B7.60-84 peptide (■), or a control peptide B27.60-84 (▧). E/T ratios of 20:1, 10:1, and 3:1 were tested. Results presented are mean cytotoxicity of triplicate cultures obtained at an E/T of 20:1.

Table 1. *CTL-AV Can Lyse HLA-B7$^+$, DR11.1$^+$ Target Cells in the Absence of B7.60-84 Peptide*

A. Target cell	HLA phenotype						Percent cytotoxicity*								
							No peptide			B7.60-84‡			B27.60-84		
	A	B	Cw	DR	DP	DQ	20§	10	3	20	10	3	20	10	3
SWEIG007	29	61	2	11.1	-	7	6 ± 2	4 ± 1	3 ± 3	56 ± 1	46 ± 3	31 ± 1	5 ± 1	1 ± 1	1 ± 1
JVM	2	18	5	11.2	2	7	2 ± 1	1 ± 1	2 ± 1	6 ± 1	2 ± 1	1 ± 1	5 ± 2	5 ± 2	4 ± 1
WT50	3	7	7	11.1	-	7	21 ± 2	14 ± 1	10 ± 1	40 ± 1	35 ± 1	27 ± 1	20 ± 1	15 ± 2	11 ± 1
ARB	26/32	7/51	2/7	11.1/2	-	-	7 ± 1	7 ± 2	6 ± 2	51 ± 4	41 ± 2	34 ± 1	4 ± 4	5 ± 3	4 ± 2
LBA	68/30	7/57	7	11.2/6	-	1/3	−1 ± 2	−2 ± 2	−4 ± 2	1 ± 2	−3 ± 2	−2 ± 2	5 ± 5	0 ± 2	3 ± 2
GMR	3/68	7/8	3/4	11.2/13	-	1/3	1 ± 1	1 ± 1	1 ± 1	7 ± 1	6 ± 2	7 ± 3	7 ± 1	5 ± 2	4 ± 1

B. Target cell	Peptide‡	mAB‡	Percent cytotoxicity in the presence of mAb (μg/ml)				
			0	6	12	25	50
WT50	None	L243	33 ± 3‡	29 ± 4	24 ± 3	16 ± 6	13 ± 4
		W6/32		34 ± 9	30 ± 5	33 ± 4	32 ± 7
	B7.60-84	L243	84 ± 5	81 ± 9	77 ± 6	69 ± 4	55 ± 5
		W6/32		85 ± 5	88 ± 9	82 ± 9	68 ± 5
SWEIG007	None	L243	5 ± 4	5 ± 6	7 ± 4	7 ± 4	2 ± 3
		W6/32		5 ± 5	2 ± 1	−4 ± 5	4 ± 2
	B7.60-84	L243	62 ± 2	61 ± 3	59 ± 4	52 ± 6	41 ± 3
		W6/32		58 ± 6	54 ± 4	55 ± 9	58 ± 6

* Cytotoxicity was measured in a 4-h ^{51}Cr release assay.
‡ Peptides (2 μg/ml) and mAbs were added to target cells for 30 min before the addition of effector cells, and were present throughout the assay.
§ E/T ratios.

exogenous B7.60-84 peptide was added (Table 1). Formation of the appropriate complexes appeared to be limiting, such that certain cells in the target population were killed and others were not. Supporting this quantitative interpretation is the finding that that the ARB cell line, which is heterozygous for B7 and DR11.1, was not killed by CTL-AV unless exogenous peptide was provided.

Presentation of B7.60-84 Is Competed for by Homologous Peptides from HLA-A and B Molecules but not from HLA-C. Of nine homologous α1 peptides used for in vitro stimulation of T cells, only the HLA-B7 peptide was immunogenic. To see if HLA-DR11.1 specifically bound the B7 peptide, we tested whether the homologous peptides from other HLA-A,B,C molecules could compete with the B7 peptide for binding to HLA-DR11.1. SWEIG007 cells, which express HLA-DR11.1 but not HLA-B7, were incubated with peptides from the α1 helix of six HLA-A, seven HLA-B, and three HLA-C molecules before sensitization with the B7 peptide and testing for lysis by CTL-AV. A spectrum of inhibitory capacity was observed for this panel of peptides (Fig. 3), with an apparent hierarchy among peptides derived from HLA-A, -B, and -C products. Peptides from HLA-A molecules were the strongest inhibitors, HLA-C peptides had no significant effect, and intermediate levels of inhibition were seen with HLA-B peptides (Fig. 3 *A*). In no case was the inhibition complete. The observed inhibition was unlikely to result from direct cytotoxic effects upon the CTL, as a 4-h incubation of ^{51}Cr-labeled CTL-AV with each of the peptides produced no peptide-specific lysis. Further evidence for inhibition being due to competition at the level of antigen presentation was the lack of inhibition observed when effector T cells were preincubated with competing peptides, washed, and then assayed for cytotoxic activity (data not shown).

We also examined whether preincubation of WT50 cells with the panel of peptides had any effect upon the presentation of endogenous HLA-B7 peptides by the HLA-DR11.1 molecules of those cells. A similar hierarchy of inhibition to that seen with SWEIG007 cells was observed (Fig. 3, *B* and *C*). However, the degree of inhibition was greater than that seen for SWEIG007 cells, and with peptides from HLA-A1 and HLA-A2, the blocking of cytolysis was almost complete. These results show not only that many of the α1 helical peptides can bind to HLA-DR11.1, but that they can effectively displace the endogenously presented B7 peptide.

Presentation of HLA-B7 Peptides by HLA-DR11.1 in WT50 Cells Is Via the Endogenous Pathway. Processing of HLA-B7 H chain by WT50 cells to give peptides that associate with HLA-DR11.1 molecules could occur by either an endogenous or an exogenous pathway of antigen presentation (34). The endogenous route could involve the breakdown of newly synthesized H chains that failed to associate with β2m, fold correctly, or be routed to the plasma membrane; the exogenous route could involve the internalization of mature HLA-B7 molecules from the cell surface or the endocytic uptake of cellular debris, membrane fragments, or shed B7 molecules from the culture medium.

To examine the latter possibility, we precultured target cells in medium containing irradiated B7 expressing JY cells or a lysate obtained by sonication of these cells. Such treatment might be expected to feed B7 molecules into an exogenous pathway of processing. However, no effects of this treatment were observed. Target cells expressing DR11.1, but not B7, were not sensitized to lysis by preincubation with either a JY cell extract or irradiated JY cells (Table 2), and neither did culture with purified HLA-B7 molecules have any effect (data not shown). These results were not due to a resistance to lysis induced by the preculture, as DR11.1 expressing targets were still specifically sensitized by peptide B7.60-84.

To determine whether HLA-B7 molecules could be processed and presented through the endogenous pathway, soluble B7 molecules were introduced into the cytoplasm of DR11.1$^+$ SWEIG007 target cells by osmotic rupture of pinocytic vesicles (31), and the cells were then tested for their susceptibility to CTL-AV killing. Cytotoxicity was observed against DR11.1$^+$ target cells treated with B7 molecules, but not against DR11.1$^+$ target cells treated with A2 molecules nor against the control DR11.2$^+$ target cells treated with B7 (Table 2 *B*). The consequence of treating these target cells with Brefeldin A and chloroquine was also examined. Brefeldin A has been shown to inhibit endogenous antigen presentation by blocking transport of newly synthesized MHC molecules from the endoplasmic reticulum to the Golgi complex (29, 30), and chloroquine blocks MHC presentation of a va-

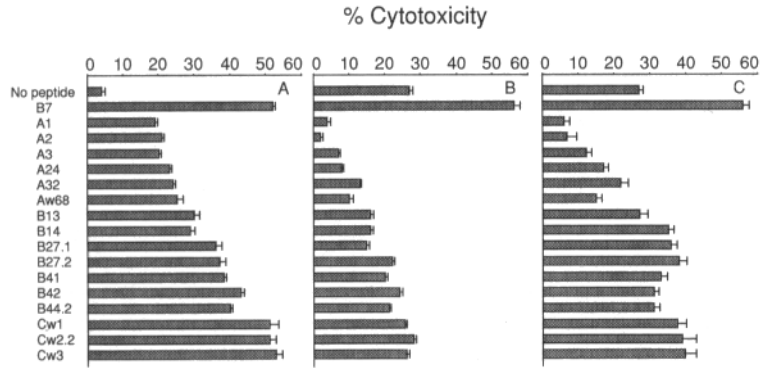


Figure 3. Competition of cytotoxicity by peptides of various HLA allotypes. Target cells were SWEIG007 + B7 peptide (*A*), WT50 (*B*), and WT50 + B7 peptide (*C*). Competing peptides at 100, 30, 10, or 3 μg/ml were incubated with all three target cell populations followed by incubation with B7.60-84 peptide (*A* and *C*) or medium (*B*) for an additional 30 min before adding effector cells. The peptides were present for the duration of the 4-h ^{51}Cr release assay. Only results obtained at an E/T ratio of 10:1 in the presence of threefold excess of competing peptides are shown.

Table 2. *Intracytoplasmic but not Endocytosed B7 Molecules Sensitize Target Cells to Killing by CTL-AV*

A. Target cell	Peptide	Percent cytotoxicity of target cells treated with:* Medium	JY.X‡	JY.Lys§	WT24.X‡	WT24.Lys§
SWEIG007	None	3 ± 1	4 ± 1	2 ± 2	4 ± 1	6 ± 1
	B7.60-84	42 ± 1	49 ± 2	47 ± 3	47 ± 1	44 ± 1
	B27.60-84	3 ± 1	7 ± 1	8 ± 1	3 ± 1	7 ± 1
JVM	None	7 ± 1	2 ± 2	5 ± 1	2 ± 2	4 ± 1
	B7.60-84	8 ± 1	4 ± 1	6 ± 1	7 ± 1	3 ± 1
	B27.60-84	8 ± 1	7 ± 1	8 ± 1	6 ± 1	3 ± 6

		Percent cytotoxicity of target cells treated with:*								
		Medium			Brefeldin			Chloroquine		
B. Target cell‖	Peptide	20¶	10	3	20	10	3	20	10	3
SWEIG007 (B7)	None	13 ± 5	10 ± 1	5 ± 2	5 ± 1	4 ± 2	3 ± 2	10 ± 4	9 ± 1	5 ± 1
	B7.60-84	48 ± 3	32 ± 3	19 ± 2	42 ± 3	30 ± 1	17 ± 3	47 ± 3	36 ± 2	21 ± 2
	B27.60-84	10 ± 4	10 ± 2	6 ± 1	5 ± 1	3 ± 2	3 ± 1	10 ± 3	10 ± 3	4 ± 1
SWEIG007 (A2)	None	1 ± 5	0 ± 1	2 ± 1	−2 ± 1	2 ± 1	1 ± 2	−2 ± 1	−4 ± 1	−1 ± 1
	B7.60-84	59 ± 4	45 ± 1	30 ± 5	49 ± 2	42 ± 3	28 ± 3	56 ± 6	43 ± 5	27 ± 4
	B27.60-84	−3 ± 4	2 ± 1	1 ± 1	−1 ± 1	1 ± 1	2 ± 1	1 ± 1	1 ± 1	2 ± 1
JVM (B7)			8 + 1			8 + 2			4 + 1	
JVM (A2)			9 + 2			7 + 2			8 + 2	

* Cytotoxocity measured at E/T ratios of 20:1, 10:1, and 3:1. Results presented are means ± SD of triplicate cultures measured at an E/T of 20:1.

‡ Target cells were incubated with irradiated (20,000 rad) JY.X (HLA-A2, B7) or WT24.X (HLA-A2, B27) cells for 24 h before the cytotoxicity assay.

§ Cell lysates were prepared by sonicating 7 × 10^6 JY or WT24 cells for 30 min, followed by centrigugation at 2,000 rpm for 15 min to remove chromatin and particulates. Lysate from either JY cells (JY.Lys) or WT24 cells (WT24.Lys) was added to target cells at a 1:25 dilution for 48 h before assay.

‖ ^{51}Cr-labeled target cells (2 × 10^6) were resuspended in 0.5 ml of hypertonic medium containing 0.3 mg/ml of HLA-B7 molecules (B7) or A2 molecules (A2) at 37°C for 10 min. Hypotonic medium was then added, and the cells were incubated at 37°C for 3 min and then pelleted. The cells were resuspended in medium and tested in a 4-h cytotoxicity assay.

¶ E/T ratios.

riety of internalized antigens to T cells (10, 14, 28). Partial inhibition of the killing of SWEIG007 cells that had pinocytosed B7 molecules and been osmotically shocked was observed with Brefeldin A but not chloroquine, providing evidence in favor of an endogenous pathway for B7 presentation by HLA-DR11.1 (Table 2).

To test directly whether processing and presentation of B7 by DR11.1 in WT50 cells involves the endogenous or exogenous pathways of antigen presentation, the effects of the inhibitors Brefeldin A and chloroquine upon presentation were investigated. A unique aspect of this system, compared with previous studies of viral antigens (12–15), is that B7 is a constitutively synthesized and presented self protein. To deplete the levels of the B7/DR11.1 complex, before administration of the inhibitors, we preincubated WT50 cells with the α1 helix peptide of HLA-A1. This peptide is effective at replacing the endogenous B7 peptide bound to DR11.1 and is not recognized by CTL-AV (Fig. 3 *B*).

Treatment of WT50 cells with chloroquine did not inhibit killing of WT50 by CTL-AV, providing further evidence against the involvement of the exogenous pathway of antigen presentation. Positive evidence for the involvement of the endogenous pathway of antigen presentation was gained from experiments with Brefeldin A. Preincubation of WT50 cells with Brefeldin A and the HLA-A1 peptide reproducibly gave a partial inhibition (>50%) of lysis by CTL-AV. Pretreatment with the HLA-A1 peptide alone had no effect, showing that, in the absence of inhibitor, there was sufficient time after the preincubation for new complexes of B7 and DR11.1 to reach the surface. As expected, treatment with Brefeldin A alone had no effect, due to the pre-existence of complexes of B7 and DR11.1 at the cell surface. Further evidence for the specificity of the effect of Brefeldin A was that Brefeldin A–treated WT50 cells still presented synthetic B7 peptide, indicating that the inhibitor was not toxic for CTL-AV and did not affect the DR11.1 molecules already present on the

Table 3. *Effect of Brefeldin A and Chloroquine on Killing of HLA-B7$^+$, DR11.1$^+$ Target Cells*

Target cell	Peptide	Percent cytotoxicity of target cells:* Medium	Brefeldin A‡	Chloroquine‡
WT50 (A1)	None	25 ± 2	9 ± 2	23 ± 3
	B7.60-84	51 ± 3	43 ± 3	50 ± 4
	B27.60-84	21 ± 1	11 ± 3	22 ± 2
WT50	None	19 ± 1	20 ± 3	18 ± 2
	B7.60-84	50 ± 1	50 ± 3	51 ± 2
	B27.60-84	20 ± 3	25 ± 3	22 ± 2
SWEIG007 (A1)	None	4 ± 2	3 ± 3	3 ± 2
	B7.60-84	70 ± 1	60 ± 2	71 ± 3
	B27.60-84	7 ± 2	7 ± 1	6 ± 2
SWEIG007	None	8 ± 4	8 ± 2	6 ± 3
	B7.60-84	75 ± 2	70 ± 3	74 ± 2
	B27.60-84	6 ± 4	6 ± 2	6 ± 4

* Target cells were incubated with 20 μg/ml of HLA peptide A1.60-84 for 24 h, washed, and labeled with ^{51}Cr before testing in a cytotoxocity assay.
‡ Target cells were preincubated with Brefeldin A (15 μg/ml) and chloroquine (10 μM) for 30 min before the addition of effector cells. The inhibitors were present for the duration of the assay.

cell surface. In summary, the results are all consistent with the endogenous pathway being solely responsible for the presentation of HLA-B7 peptides by HLA-DR11.1 on the surface of WT50 cells.

Discussion

In general, it has been found that there is class discrimination in the processing and presentation of antigens to T cells (35). Thus, class I MHC molecules present peptides derived from endogenous proteins synthesized within the APC, whereas class II MHC molecules present peptides derived from exogenous proteins synthesized elsewhere that enter the APC by endocytosis. However, there are exceptions to these rules (12–15); for example, Nuchtern et al. (15) recently analyzed the recognition by CTL of an influenza peptide presented by class II MHC molecules and showed that presentation was via the endogenous pathway.

In the experiments reported here, we immunized human PBL in vitro with peptides having sequences derived from the highly variable α1 domain helices of allogeneic and syngeneic HLA-B,C molecules. As might have been expected from this exogenous route of antigen delivery, CTL that recognized a class I peptide presented by a class II MHC molecule were obtained. Thus, target cells that express HLA-DR11.1 could be rendered susceptible to specific lysis by CTL-AV by preincubating them with the peptide from HLA-B7 but not with the homologous peptides from 16 other HLA-A,B,C molecules. In these experiments, the antigen was provided to the APC in an already processed form. However, the lysis by CTL-AV of WT50, a cell line that expresses HLA-B7 and HLA-DR11.1, showed that these cells could process HLA-B7 and present it in the context of HLA-DR11.1. This processing and presentation could be inhibited by Brefeldin A, which blocks transport from the endoplasmic reticulum to the Golgi (29, 30), and was unaffected by chloroquine, an inhibitor of processing by the exogenous pathways (10, 14, 28). These results, indicating that HLA-B7 presentation by HLA-DR11.1 occurs via the endogenous pathway, are further supported by experiments showing that HLA-DR11.1$^+$, HLA-B7$^-$ cells could process and present exogenously added HLA-B if delivered to the cytoplasm, but not when delivered to endocytic vesicles.

This study thus provides a further exception to the rules of antigen presentation, and another example of class II presentation by the endogenous pathways. A significant difference in the exception described here and those previously reported (12–15) is that the antigen is a constitutively synthesized self protein rather than viral proteins only made subsequent to viral infection. Thus, B7 is continuously presented in WT50 cells. This property probably explains why Brefeldin A treatment produced only a partial inhibition of presentation of HLA-B7 in WT50 cells.

The antigen receptors of CTL-AV are highly specific for the combination of HLA-DR11.1 and the peptide from the HLA-B7 α1 helix; and they do not recognize HLA-DR11.2, which only differs at three amino acids from HLA-DR11.1 (32, 33). However, peptide competition experiments show that peptides from the α1 helix of various HLA-A and -B molecules, in addition to B7, also bind to the HLA-DR11.1 molecule.

Endogenous presentation of B7 by DR11.1 was only observed in one of two cell lines that coexpress these molecules. That it was the homozygous cell (WT50) that was lysed by CTL-AV and the heterozygous (ARB) cell line that was not, suggests that the concentration of endogenously produced B7 peptides is the limiting factor. This conclusion is consistent with the observation that lysis of WT50 was improved by addition of exogenous B7 peptide. Another factor that could contribute to the difference in B7 presentation by WT50 and ARB is the relative capacity of peptides derived from the other MHC molecules expressed by the two cells to compete with the B7 peptide for binding to HLA-DR11.1. Precedent for such cellular differences in antigen presentation exist. Bodmer et al. (36) have shown that two closely related HLA molecules, HLA-A2.1 and HLA-Aw69, can both present a synthetic influenza matrix peptide, M58-68, to A2.1-restricted CTL. However, on infection with influenza virus only, HLA-A2.1-expressing cells, and not HLA-Aw69-expressing cells, were lysed by specific CTL, indicating that naturally processed products of the influenza matrix protein failed to associate with Aw69 molecules.

A critical issue is whether the class II MHC presentation of endogenously processed self proteins described here, and of viral proteins described by others (12, 13, 15), represent curiosities or examples of a significant, if minor, pathway. As far as we know, class I and class II MHC molecules are both assembled in the same compartments of the endoplasmic reticulum and pre-Golgi. At this stage of biosynthesis, class I MHC molecules bind peptides, and a major question has in fact been to explain why class II MHC molecules do not generally bind and present peptides from this class I pool of peptides to which they are exposed. One model proposed is that invariant chain association blocks peptide access to the binding site until the class II MHC molecule is targeted to a cellular compartment containing peptides derived from the exogenous pathway of antigen processing. A second model is that endogenous processed peptides are picked up in the endoplasmic reticulum by class II MHC molecules, but that they are subsequently exchanged for exogenously processed peptides (14, 37, 38). There is nothing in the nature of these, or other, reasonable models that would ensure a total segregation of the peptides presented by class I and class II MHC molecules. For example, if the invariant chain acts to block the class II MHC binding site, then in the endoplasmic reticulum, a competition will exist between peptides and the invariant chain to gain the binding site first; and with some frequency, a peptide will win. Similarly if MHC class discrimination is achieved by a washing out of endogenous peptides from class II MHC molecules, it is unlikely that such a process will be 100% efficient and that certain endogenous peptides will be retained and presented by class II MHC molecules. Thus, it is both possible and reasonable that a spectrum of endogenously processed peptides is presented by class II MHC molecules and that this represents an intrinsic "leakiness" in the segregation of the endogenous and exogenous pathways of antigen presentation.

This study provides a further example of presentation of an MHC peptide by an MHC molecule (22–24, 39, 40). It is possible that such complexes may play a role in determination of the T cell repertoire. In certain combinations, the complexes of peptides derived from the product of one MHC locus with the intact product of another locus may confer either immunological advantage or disadvantage through selection, elimination, or inactivation of T cells with particular specificities. In such cases, there could be natural selection to maintain or eliminate certain combinations of HLA alleles, and this might have been a contributing factor to the linkage disequilibrium that is a characteristic feature of the HLA system. Alloreactive cytotoxic T cells directed against class II MHC molecules may be involved in the rejection of allogeneic transplants (39, 40), and these may include cells with composite class I/class II specificity of the type described here. For example, the induction of class II MHC molecules on activated human T cells (41, 42) might result in expression of composite targets at particular stages in the allogeneic response. In vitro immunization with synthetic peptides may contribute to the characterization and analysis of such alloantigens.

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