

DETERMINANTS RECOGNIZED BY HUMAN CYTOTOXIC T  
CELLS ON A NATURAL HYBRID CLASS I HLA MOLECULE

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The major histocompatibility complex (MHC) class I molecules are the primary determinants recognized by allogeneic cytotoxic T lymphocytes (CTL), and serve as restricting elements for CTL recognition of viral, chemical, or minor histocompatibility antigens (1). Our understanding of the manner in which these molecules participate in immune responses has been significantly advanced by the development of techniques for expressing MHC genes following DNA-mediated transfer into suitable recipient cells (2–4). More recently, transfections have been undertaken with genes that have been manipulated in vitro to produce novel structures. Results from such studies have shown that the major determinants recognized by murine CTL lie in the highly polymorphic  $\alpha_1$  and/or  $\alpha_2$  domains (5–9). However, similar analyses of human CTL recognition of transfected HLA molecules have been more difficult to interpret. A number of investigators (10, 11) have shown that human HLA-A2 and -B7 genes transfected into murine L cells are not recognized by human allogeneic CTL, suggesting that species-restricted factors may play a role in the cellular but not serological recognition of human MHC class I molecules. In contrast, others have reported (12, 13) that HLA-A3 and -Aw24 transfected into and expressed by murine L cells can be recognized by human CTL. Thus, it appears that properties of the recipient cell and/or a particular HLA molecule can affect the ability of human CTL to recognize transfected HLA genes.

We have previously shown (14)<sup>1</sup> that HLA-Aw69 is a naturally occurring hybrid class I molecule, in that the  $\alpha_1$  domain is identical to that of HLA-Aw68, and the  $\alpha_2$  and  $\alpha_3$  domains are identical to those of HLA-A2. To localize the functional determinants recognized by human allogeneic CTL, we used human B lymphoblastoid cell lines, which normally express these HLA molecules, as stimulators to generate CTL clones, and as targets to assess CTL specificity. We show here that human CTL clones can recognize determinants in either the  $\alpha_1$  or  $\alpha_2$  domains, and that interaction of the  $\alpha_1$  and  $\alpha_2$  domains can result in the formation of determinants or the loss of preexisting determinants.

**Materials and Methods**

*Cell Lines.* CTL clones were generated as described previously (15, 16). Briefly, peripheral blood lymphocytes from a normal donor (HLA-A3,-; B7,w38; C,-; DR6,6)

<sup>1</sup> N. Holmes and P. Parham. Exon shuffling in vivo can generate novel HLA class I molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.

were separated on Ficoll-Hypaque and stimulated in primary culture with irradiated (10,000 rad) B lymphoblastoid cells expressing HLA-A2, -Aw68, or -Aw69. After 6 d, cells were stimulated in secondary culture with a different irradiated B lymphoblastoid cell, and 6 d later were cloned by limiting dilution on a third irradiated B lymphoblastoid cell in medium supplemented with T cell growth factors, including interleukin 2. 18 different combinations were used to generate the clones tested. For example, some CTL in group 1 were stimulated in primary culture with JY, secondary culture with LB, and cloned on LCL-721. Clones, which arose at 1 cell/well, were expanded in microtiter wells and tested for cytotoxicity of appropriate target cells using a  $^{51}\text{Cr}$ -release assay. Clones exhibiting desired specificities were expanded and subcloned. Clones arising at 0.3 cells/well were expanded and retested for cytotoxicity against 10 lines expressing HLA-A2, 10 lines expressing HLA-Aw68, 6 lines expressing HLA-Aw69, and 3 lines expressing different HLA-A locus genes.

B cell lines expressing the HLA-Aw69 antigen were kindly provided by F. Ward (Duke University, Durham, NC) (JSM); P. Antonelli and J. Hansen (University of Washington, Seattle, WA) (BJ, ZM, HS, SR); and the American Type Culture Collection (Rockville, MD) (IDF). B cell lines expressing HLA-Aw68 were obtained from F. Bach and M. Segall, University of Minnesota.

The expression of HLA-Aw68, -Aw69, and -A2 was confirmed by radioimmune cell-binding assay with appropriate monoclonal antibodies (mAb).

**Cytotoxicity Assay.** Clones were incubated at a variety of effector/target ratios with a panel of  $^{51}\text{Cr}$ -labelled B lymphoblastoid cell lines. Cytotoxicity assays were carried out as described previously (15, 16). Percent specific release was calculated as  $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{Triton X-100 release} - \text{spontaneous release})]$ . Values are expressed as means of triplicate cultures.

### Results and Discussion

HLA-A2 and -Aw68 are highly homologous, as shown by the schematic of the protein sequences (14, 17)<sup>1</sup> (Fig. 1). There are six amino acid differences in the  $\alpha_1$  domain, six in the  $\alpha_2$  domain, and one in the  $\alpha_3$  domain. CTL specific for HLA-A2, -Aw68, and/or -Aw69 were generated by stimulating peripheral blood lymphocytes from a normal donor (HLA-A3,-; B7,w38; C,-; DR6,6) in primary culture with irradiated Epstein Barr virus-transformed B lymphoblastoid cell lines expressing HLA-A2, -Aw68, or -Aw69 (HLA shown in Table I). Cells were stimulated in secondary culture with a different B lymphoblastoid cell line, and were cloned by limiting dilution using a third B lymphoblastoid line. Clones arising at 1 cell/well were screened for cytotoxicity on a panel of cell lines

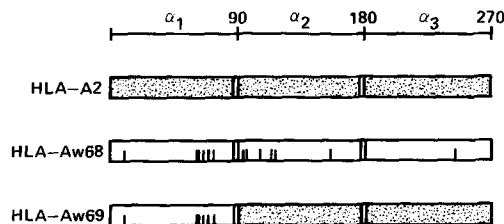


FIGURE 1. Schematic comparison of the deduced protein sequences of HLA-A2, -Aw68, and -Aw69. The amino acid sequences of the three external domains (residues 1-270) of the HLA molecules -A2, -Aw68, and -Aw69 are compared schematically. Each vertical bar represents a single amino acid difference with the prototype HLA-A2 sequence from LCL-721; the HLA-Aw68 sequence is from the cell line LB; the HLA-Aw69 from BJ. The HLA-A2-identical domains are shaded and the HLA-Aw68-identical domains unshaded. The transmembrane and cytoplasmic domains are not shown.

TABLE I  
Specificity of Anti-HLA-A2, -Aw68, and -Aw69 CTL

HLA	Target cells								
	JY	LCL-721	LB	LCL-100	JSM	IDF	BJ	PGF	K562
A	2	1, 2	w68	w68, 32	w69, w25	w69, 26	w69, w30	3	—
B	7	8, 5	40	44, 27	14, w22	38, 18	w35, w22	7	—
C	NA*	NA	3	2	w1, w8	NA	w6, w7	2	—
DR	4, 6	3, 1	6	2, 5	7	5	2	2	—
Group	Percent specific release								
1	59	62	7	1	0	1	0	0	3
2	56	21	25	59	0	0	4	2	3
3	70	53	71	76	80	81	69	3	0
4	1	9	39	43	56	59	47	6	3
5	3	5	9	4	44	43	29	6	4
6	77	56	3	0	23	54	47	4	2

Results are of a single clone representative of each group and tested at an effector/target ratio of 5:1.

\* NA, data not available.

expressing HLA-A2, -Aw68, -Aw69, and other HLA types. Clones exhibiting desired cytotoxic specificities were subcloned at 0.3 cells/well. No changes in specificity were observed following subcloning. In addition, mAb PA2.6 and TS1.16 were used to confirm the class I specificity of these CTL. In all cases, cytotoxicity was inhibited by PA2.6 (anti-class I) but was unaffected by TS1.16 (anti-class II). The cell surface phenotype of all CTL was 100% Leu-2<sup>+</sup>, Leu-3<sup>-</sup>, Leu-4<sup>+</sup>, as determined by fluorescence activated cell sorter analysis (not shown).

93 clones were initially assayed for cytotoxicity. 21 were not cytotoxic, and thus were not studied further. The remaining 72 clones were tested for cytotoxicity against 10 lines expressing HLA-A2, 10 lines expressing HLA-Aw68, 6 lines expressing HLA-Aw69, and 3 lines that do not express HLA-A2, -Aw68, or -Aw69. Representative results from such analyses are shown in Table I and are summarized in Table II.

CTL clones exhibiting six of the seven possible patterns of cytolysis were identified. Clones restricted largely by the  $\alpha_1$  domain (group 4) or by the  $\alpha_2$  domain (group 6) were identified. CTL in group 1 may be specific for the  $\alpha_1$  domain of HLA-A2 alone, or some combinational determinant comprised of  $\alpha_1$  plus  $\alpha_2$ . In addition, some CTL recognized determinants that were dependent on both the  $\alpha_1$  and  $\alpha_2$  domains. The determinant recognized by CTL in group 5 (HLA-Aw69 specific) is found only on the hybrid molecule. In contrast, CTL in group 2 lyse targets expressing HLA-A2 or -Aw68, and not those expressing HLA-Aw69. Their target determinant therefore is not present in the recombinant HLA-Aw69 molecule. CTL in group 3 recognize targets expressing HLA-A2, -Aw68, and -Aw69, suggesting that these CTL are restricted by a shared determinant. This determinant may be identical to that recognized by B cells, since many anti-HLA-A2 mAb bind to both HLA-Aw68 and -Aw69 (18). Finally, no CTL specific for HLA-Aw68 alone (group 7) were identified. Since other CTL specific for the  $\alpha_2$  domain were generated (group 6), this finding does not

TABLE II  
*Summary of Specificities of T Cell Clones Screened*

Group	HLA types lysed	Major restriction element(s)	Number of clones identified
1	A2	$\alpha_1$ or $\alpha_1 + \alpha_2$	19
2	A2, Aw68	$\alpha_1 + \alpha_2$	9
3	A2, Aw68, Aw69	$\alpha_1$ and/or $\alpha_2$	13
4	Aw68, Aw69	$\alpha_1$	1
5	Aw69	$\alpha_1 + \alpha_2$	7
6	A2, Aw69	$\alpha_2$	6
7	Aw68	$\alpha_2$ or $\alpha_1 + \alpha_2$	0
8	Unclassified	?	17
9	Noncytotoxic	?	21
Total:			93

Clones derived and tested as described in Table I were grouped by their HLA specificity. Only CTL clones that lysed all members of the specificity panel were included in groups 1–6; CTL that lysed one or two lines expressing a particular HLA type are grouped as unclassified, and are presumed to be specific for other antigens expressed on those target cells.

reflect an intrinsic lack of antigenicity of the  $\alpha_2$  domain. In our study, CTL were derived from peripheral blood lymphocytes that type as HLA-A3. Comparison of the protein sequences of HLA-A2, -Aw68, and -A3 (19) offers some insight into our inability to generate group 7 CTL. There are six amino acid differences between HLA-Aw68 and -A2 in the second domain, five of which occur in the region including residues 95–116. Interestingly, there is only one amino acid difference between HLA-A3 and -Aw68 in this region. It is therefore possible that lymphocytes from individuals expressing HLA-A3 cannot see this region as antigenic. It is also possible that our sample of 72 CTL clones was not large enough to include CTL specificities that occur in low frequency.

This is the first examination of human CTL recognition of a hybrid human class I molecule expressed in human cells; other investigations (4–9) have involved murine or xenogeneic systems. In this particular system, we avoid the two major problems of gene manipulation, transfection, and expression. By using the product of a natural exon shuffle, we are studying a functionally relevant hybrid gene that has survived evolutionary selection pressure, rather than hybrid genes produced by in vitro manipulation of DNA, which are determined by available restriction enzyme sites. Second, the hybrid HLA-Aw69 gene and the related -A2 and -Aw68 genes are naturally expressed in human B lymphoblastoid cells, the targets conventionally used for study of the human allogeneic response. In this way, we have avoided problems inherent in systems involving gene transfection and expression, such as the role of accessory molecules, glycosylation, cell surface density of the expressed antigen, and species differences (10, 11).

Our findings complement those of others (2–13) who have used gene manipulation, transfection, and expression to show that murine CTL are restricted by determinants in the  $\alpha_1$  and/or  $\alpha_2$  domains. More importantly, we have shown that the interaction of the  $\alpha_1$  and  $\alpha_2$  domains can result in either the formation or loss of determinants that cannot be directly correlated with the primary structure. Perhaps the most surprising finding is that some determinants expressed by both HLA-A2 and -Aw68 are lost in the recombinant HLA-Aw69 molecule. Since there are no sequences unique to HLA-Aw69, this finding

underscores the role of secondary and/or tertiary structure in the generation of functionally relevant epitopes, and points out the potential problem of creating or deleting determinants when hybrid genes are generated in vitro from genes that are even more disparate than HLA-A2 and -Aw68.

### Summary

The major histocompatibility complex class I HLA molecules are the primary determinants recognized by allogeneic cytotoxic T lymphocytes (CTL), and serve as restricting elements for CTL recognition of viral, chemical, or minor histocompatibility antigens. HLA-Aw69 is a naturally occurring hybrid class I molecule that we have used to investigate the regions of class I antigens involved in human CTL recognition. HLA-Aw69 appears to have resulted from an exon shuffle between two closely related class I genes: the  $\alpha_1$  domain of HLA-Aw69 is identical to that of HLA-Aw68, while the  $\alpha_2$  and  $\alpha_3$  domains are identical to HLA-A2. The determinants recognized by human allogeneic CTL clones specific for HLA-A2, -Aw68, and/or -Aw69 fall into three patterns: (a) CTL determinants are located on both the  $\alpha_1$  and  $\alpha_2$  domains; (b) interaction of the  $\alpha_1$  and  $\alpha_2$  domains results in new combinatorial determinants; (c) interaction of the  $\alpha_1$  and  $\alpha_2$  domains in the hybrid molecule results in the loss of CTL determinants that are present on both parental molecules. Thus, using human CTL clones, target cells, and HLA molecules, we show that the interaction of the  $\alpha_1$  and  $\alpha_2$  domains alters CTL determinants in ways not directly predictable from primary structure.

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