Brief Definitive Report

Cell-Cell Adhesion Mediated by CD8 and Human Histocompatibility Leukocyte Antigen G, a Nonclassical Major Histocompatibility Complex Class 1 Molecule on Cytotrophoblasts

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Summary

The lymphocyte differentiation marker CD8 acts as a coreceptor with the T cell receptor (TCR) during recognition of peptide presented by major histocompatibility complex (MHC) class I molecules. The functions of CD8 in the TCR complex are thought to be signaling through the association of CD8 with the protein tyrosine kinase $p56^{lck}$ and adhesion to MHC class I through the $\alpha 3$ domain. While the ability of the CD8 α/α homodimer to bind to classical MHC class I molecules has been shown, it is unclear whether CD8 can also bind nonclassical molecules. Of particular interest is human histocompatibility leukocyte antigen (HLA)-G which is expressed on placental cytotrophoblast cells. These cells do not express HLA-A, -B and -C molecules. In this report, we demonstrate that CD8 can bind to HLA-G. It is possible, therefore, that a cell bearing CD8 may interact with HLA-G–expressing cells.

In humans three nonclassical MHC class I molecules have been described human histocompatibility leukocyte antigen (HLA)-E (1, 2), -F (3), and -G (4) which map to the MHC complex and which encode expressible proteins. While mRNA for all three genes has been detected in a number of cell lines (5), only expression of HLA-G on the cell surface was reported (6, 7). Because there are no specific antibodies against HLA-E, -F, and -G, specific expression of HLA-G can only be identified by analysis of labeled cell surface proteins on two-dimensional polyacrylamide gels (6). By this approach, HLA-G was the only HLA molecule expressed on fetal cytotrophoblast cells (6). Cytotrophoblasts invade the maternal decidualized endometrium and are directly exposed to maternal lymphoid cells yet the placenta is not normally rejected by the mother as an allograft. Thus, the ability of HLA-G to be recognized by immune cells is unclear.

Because CD8 can bind to MHC class I molecules including HLA-A2, we decided to determine whether CD8 could recognize and bind to HLA-G as well. Salter et al. (8) recently identified a conserved negatively charged loop on the MHC class I $\alpha 3$ domain that is important for binding to CD8. Single amino acid substitutions at each position in this loop from residues 223-229 severely reduced binding to CD8 in a cell-cell adhesion assay. At position 228 within this loop HLA-G has a valine instead of the conserved threonine found in HLA-A, B, and C. Based on this difference as well as other differences, it would not be predicted from sequence comparisons that CD8 would bind to HLA-G. To determine whether CD8 could bind to HLA-G, we obtained a cell line, LCL 221, that was null for expression of classical HLA class I molecules HLA-A, -B, -C and transferents of this cell line that expressed either HLA-G or HLA-A2 (6). Using a cell-cell adhesion assay, we found that CD8 could bind to HLA-G and that the binding was similar to the binding to HLA-A2.

Materials and Methods

Cell Lines. The lymphoblastoid cell lines: the HLA class I null mutant 221 and HLA-A2 and HLA-G transfectants of 221 were obtained from Dr. Robert DeMars. The HLA-G and HLA-A2 line were created by transfecting a genomic gene for HLA-G or HLA-A2 respectively in a pHeBo vector into the HLA class I null mutant 721.221 (9). The HLA-G revertants were grown in the absence of hygromycin B for 3–4 mo.

Cell-cell Adhesion Assay. The COS7 transient adhesion assay was performed as described (10). Briefly, 3×10^5 COS7 cells were plated in 35 mm dishes and transfected with SRα296 or SRα296 containing the cDNA for CD8 by lipofection. Lipofectin remained on the COS7 cells for 18 h. Transfected COS7 cells were used 48 h later in the cell-cell adhesion assay. 10^7 ^{35}S-cysteine-labeled UC, 221 or transferents were added to each 35 mm dish in 1.0 ml PBS supplemented with 10% heat-inactivated FCS. The cells were incubated for 1 h at 37°C. The B cells were poured off and the dishes were washed seven times. Cells were removed from each dish by adding 0.6 ml PBS containing 1% NP-40. Then 50 μl was placed in aqueous scintillation fluor and counted to determine the number of cells bound to the transfected COS7 cells.

For each experiment, the level of expression of CD8 on the trans-

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fected COS7 cells was determined by FACS analysis using an anti-CD8 antibody. Expression levels were consistently high (10).

^{35}S-Cysteine Labeling of B Cells. 10^8 B cells were labeled with 1 mCi ^{35}S-cysteine (Amersham Corp., Arlington Heights, IL) for 4 h at 37°C in cysteine-free RPMI containing 10% dialyzed FCS, washed two times and then used for binding as described above. After 4 h the specific activity ranged from 12 to 18 cpm per B cell.

FACS Analysis. Cells were washed with Ca^{2+}, Mg^{2+} free PBS and stained with saturating concentrations of anti-HLA class 1/1b mAb W6/32 (11) or an isotype matched antibody, S4B6, against murine IL-2, followed by staining with either a 1:50 or 1:25 dilution of FITC-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Propidium iodide was included to gate out dead cells. Analysis was performed on a FACScan (Becton-Dickinson and Co., Mountain View, CA).

Results and Discussion

The ability of CD8 to bind to HLA-G was determined using a transient cell-cell adhesion assay (14). Monkey kidney cells, COS7, expressing high levels of SV40 T antigen were transfected with the cDNA for CD8α in the pCDL-SRα296 expression vector or with vector alone. 70 h post-transfection the ability of the CD8$^+$ COS7 cells to bind the HLA-A, -B, -C class I-expressing human B lymphoblastoid cell line, UC, the HLA class I null line 221, or 221 transferents expressing HLA-G or HLA-A2 was determined. Because high levels of expression of HLA are required for sufficient adhesion, we cloned the 1% brightest expressing HLA-G transferents using a FACS. After sufficient growth the clones were analyzed and clones with homogenous staining patterns were observed (Fig. 1). An independent transferent of mutant 221 expressing HLA-A2 had high level expression after initial growth in hygromycin B (9).

The ratio of cells binding to CD8 transfected COS7 cells versus binding to vector alone transfected COS7 cells was determined for each cell line (Table 1, exp. 1 and 2). In two separate experiments the ratio of binding for the lymphoblastoid B cell line, UC was 5.25 and 8.32 whereas the ratio for 221 cells which do not express the HLA-A, -B, and -C genes was 0.91 and 0.92. CD8 binding ratios for the HLA-A2 transferent were 1.74 and 2.57. Similar transferents were previously shown to be positive for binding in a cell-cell adhesion assay using stable CD8$^+$ transferents of Chinese hamster ovary (CHO) cells (12). The HLA-G transferent ratios of 2.33 and 2.12 are well within the range of binding ratios observed for the HLA-A2 transferents. Thus, within an experiment the UC cells had higher binding than the 221 cells and the HLA-A2 and HLA-G transferents were intermediate. We obtained the same results in two experiments using the CHO cell-cell adhesion assay (data not shown).

To further substantiate that the expression of HLA-G was responsible for the binding, we grew the HLA-G transferents in medium containing no hygromycin B. Because HLA-G is contained in an EBV-based plasmid which autonomously replicates and lacks a centromere, there is a certain probability that a daughter cell will not contain the plasmid. By growing the cells in nonselectable medium, the proportion of cells that lose the plasmid can accumulate more rapidly

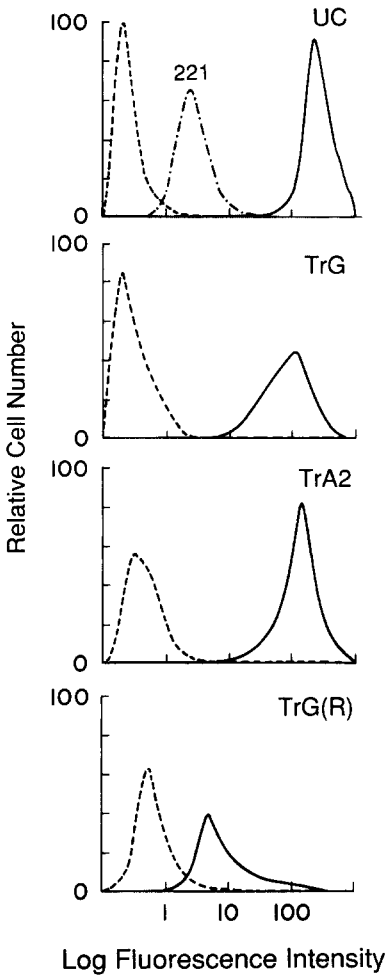

Figure 1. FACS assayed binding of HLA class I specific mAb W6/32 (11) to lymphoblastoid cell lines. The fluorescence intensity of the B lymphoblastoid lines UC and 221, HLA-G transferent (TrG), HLA-A2 transferent (TrA2) and HLA-G revertant (TrG(R)). Cells were stained either with the mouse MAb W6/32 (*solid line* or *dash/dot* for 221) or an irrelevant isotype matched antibody (*dotted line*) followed by second step staining with FITC-conjugated goat anti-mouse IgG.

resulting in the loss of HLA-G expression (13). These cells should be genetically identical to the parent line except for the loss of the HLA-G containing plasmid. After 8 wk of growth expression of HLA-G was greatly reduced (Fig. 1). The expression of HLA-DR, and the B cell antigen CD20 were unchanged (data not shown). These cells were tested twice in the COS assay. In both experiments the ratio of binding of the HLA-G revertants was similar to the 221 cells versus the ratio of binding for the parent HLA-G transferents (Table 1).

To determine if there was a statistically significant difference between UC and 221 and between HLA-G and 221, we analyzed the data from the four experiments using the nonparametric Wilcoxon two-sample test. There was a significant difference between UC and 221 and between HLA-G and 221 at the $p = 0.01$ level. Therefore, these results, in conjunction with the results from the HLA-G revertant

Table 1. *Binding of CD8 to MHC Class I and Ib Expressing Cells*

Experiment no.	Cell type	Cells bound per well		$CD8^+/CD8^-$ ratio
		$CD8^+$	$CD8^-$	
		($\times 10^5$)		
1	UC	13.0	2.47	5.25
	221	4.52	4.94	0.91
	HLA-A2 transferent	13.0	7.43	1.74
	HLA-G transferent	18.0	7.7	2.33
2	UC	11.9	1.43	8.32
	221	8.15	8.85	0.92
	HLA-A2 transferent	13.1	5.1	2.57
	HLA-G transferent	26.5	12.5	2.12
3	UC	25.6	1.9	13.2
	221	5.9	5.3	1.1
	HLA-G revertant*	9.0	9.7	0.9
	HLA-G transferent	17.8	5.7	3.1
4	UC	123.0	21.0	5.9
	221	20.0	18.0	1.1
	HLA-G revertant*	33.6	33.6	1.0
	HLA-G transferent	138.0	26.5	5.2

* The HLA-G revertant refers to HLA-G transferents grown in the absence of hygromycin B for 3–4 mo.

binding assays in which the loss of expression of HLA-G correlated with a lack of binding, demonstrate that CD8 does bind to HLA-G.

Whether the $CD8\alpha/\alpha$ homodimer binds to HLA-G in a manner similar to HLA class I molecules such as HLA-A2 is unclear. If the binding is similar, then the HLA-G substitution at position 228 in the conserved loop on the MHC class I $\alpha 3$ domain that is important for binding to CD8 may be tolerated whereas the change to alanine which was made by Salter et al. (8) that reduced binding may not. Interestingly, a methionine is present at this position in several mouse MHC class I alleles which are reported to bind to human CD8 (14). The significance of this change can be tested by substituting a valine for threonine in the HLA-A2 molecule, transfecting the mutated gene into cells, and determining if the transferent can bind to CD8. Alternatively, binding of HLA-G to $CD8\alpha/\alpha$ could be different from the binding of classical MHC class I to CD8.

The MHC class I molecules of some monkey species are most homologous to HLA-G and -F. In the cotton-top tamarin the expressed MHC class I genes are more closely related to HLA-G than to the HLA-A, -B, and -C genes (15). These animals can mount $CD8^+$ cytotoxic T cell responses (16). Our work would support the hypothesis that the CD8 molecule on these cells is likely to bind to the tamarin class I molecule.

While extensive studies of the function of classical HLA-A, -B and -C molecules have been performed, much less is known about the in vivo function of nonclassical MHC molecules. The expression of HLA-G on cytotrophoblasts suggests a potential in vivo role for this molecule. Although HLA-G is nonpolymorphic it may present peptides from proteins that are polymorphic and differ between mother and fetus. HLA-G counl serve as a recognition element by suppressor T cells which are $CD8^+$ and thus prevent "rejection" of the fetus. Wei and Orr (5) proposed that HLA-G may play a role in immune surveillance to recognize virally infected tissue, a target for $CD8^+$ cytotoxic T cells. Because a large proportion of the maternal cells in the decidua are $CD2^+$, $CD3^-$ large granular lymphocytes that express the CD56 antigen characteristic of NK cells (17), HLA-G may play a similar role in protecting cells from NK killing. Recent work (Kovats, S., P. Fisch, P. M. Sondel, and R. DeMars, manuscript submitted for publication) indicates that HLA-G expression can reduce lytic activity by IL-2 activated NK cells and $\gamma\delta$T cells. In humans, some NK cells express the $CD8\alpha$ homodimer. Therefore, CD8 could participate in recognition of HLA-G either by NK cells or by human T cells. Our finding that CD8 can recognize and bind to HLA-G supports and strengthens the hypothesis that expression of HLA-G is likely to be relevant for immune recognition of fetal tissue.

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