

FUNCTIONAL CONSEQUENCES OF ANTI-SENSE
RNA-MEDIATED INHIBITION OF CD8 SURFACE EXPRESSION
IN A HUMAN T CELL CLONE

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CD8 is one of several cell surface glycoproteins implicated in various T cell activation and effector functions. The subset of T cells that express CD8 are restricted in their antigen recognition by class I MHC molecules (1-3). The participation of CD8 in MHC-restricted antigen recognition by T cells was suggested by findings from several groups that anti-CD8 antibodies inhibited T cell antigen recognition (4-11). This inhibition was attributed to antibody-mediated interference with the binding of CD8 to its ligand, thought to be nonpolymorphic determinants on class I MHC molecules. However, recent experiments have indicated that anti-CD8 antibodies may block T cell function by transducing a negative signal to the effector T cell (12-16), an observation that has necessitated a reinterpretation of earlier antibody blocking data. In view of the complexities of anti-CD8 antibody blocking studies, alternative approaches are required to define the function of CD8.

Gene transfer technology offers a more direct approach for characterizing the function of T cell surface molecules. In two independent transfection studies of CD8 (17-19), reconstitution of antigen specificity and class I MHC restriction was achieved in murine CD8⁻ T cell hybridomas by stable transfer of the genes encoding CD8 and the TCR α and β chains. The use of T cell hybridomas in these studies limited the number of functions that could be evaluated. Transfection studies with human T cell clones are advantageous because they allow for a more complete range of functional responses to be assessed.

In a recent study, we have linked anti-sense RNA mutagenesis and human T cell cloning technologies. Specifically, we demonstrated that surface expression of CD8 was selectively inhibited by >95% using an EBV episome-based anti-sense RNA expression system (20). The antigen specificity of the T cell clone used in that study was unknown, and therefore, effects on antigen recognition could not be assessed. In this report, we describe the functional consequences of inhibiting CD8 expression in a human T cell clone with known antigenic specificity.

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Materials and Methods

*Lymphoblastoid Cell Lines (LCL)*¹. Two human EBV-transformed lymphoblastoid cell lines, AR.LCL (HLA haplotype: A28,32; B27,35; DR1,5) and DK.LCL (HLA haplotype: A2,24; B13,50; DR2,7), were derived by a standard procedure (21) and used for these studies.

Derivation of the Human T Cell Clone JH.ARL.1. An HLA-B35-specific, CD8⁺ human T cell clone, JH.ARL.1, was derived from a primary MLC of human PBMC (HLA haplotype: A2,3; B7,44; DR2,4) and allogeneic AR.LCL cells. Cloning was performed by limiting dilution in 96-well microtiter plates with 200 μ l of complete medium per well supplemented with 10% MLA-144 culture supernatant (CS) as a source of IL-2. To each well, we added 2×10^4 γ -irradiated (5,000 rad) autologous PBMC and 5,000 γ -irradiated (10^4 rad) AR.LCL cells. The T cell clone JH.ARL.1 was stimulated weekly with irradiated AR.LCL cells (10^5 cells/ml), irradiated autologous PBMC (7.5×10^5 cells/ml), and IL-2 (10% MLA-144 CS). This stimulation protocol was followed for 4 wk after the cloning procedure; then JH.ARL.1 cells were stimulated weekly with OKT3 mAb (1 ng/ml; Ortho Diagnostic Systems Inc., Westwood, MA), irradiated allogeneic PBMC (7.5×10^5 cells/ml), and IL-2. 3 d after stimulation, JH.ARL.1 cells were washed and subcultured in fresh complete medium supplemented with IL-2 (10% MLA-144 CS) for 4 d before restimulation. JH.ARL.1 was specific for HLA-B35 as assessed by cytotoxicity against a panel of LCL targets.

Transfection and Selection of Stable Transfectants. Construction of the plasmids α -CD8/REP1 and RSV α /220.2 and the transfection/selection procedures have been described previously (20). Episomes were stably transfected into JH.ARL.1 by electroporation, and stable transfectants were selected in media containing hygromycin B (150 μ g/ml; Calbiochem-Behring Corp., La Jolla, CA).

Flow Cytometry. Expression of cell surface molecules was analyzed by indirect immunostaining as described previously (21, 22). The mAb WT31 (anti-TCR β chain) was obtained from Becton Dickinson & Co., Mountain View, CA, and OKT8, OKT11, and OKT3 mAbs were from Ortho Diagnostic Systems Inc. Immunofluorescence of stained cells was assessed on a flow cytometer (EPICS V; Coulter Electronics Inc., Hialeah, FL). Relative intensities of fluorescence were determined by preparing a standard curve using beads with standardized fluorescent intensities (Coulter Electronics Inc.).

Cytotoxicity Assay. A standard 4-h ⁵¹Cr-release assay, as described previously (21), was used to assess the ability of JH.ARL.1 cells to lyse alloantigen-specific, nonspecific, and lectin-coated targets. JH.ARL.1 effector cells were cocultured with LCL targets or with K562 targets and PHA (1 μ g/ml) in quadruplicate round-bottom wells of a 96-well microtiter plate (200 μ l/well). Spontaneous release of LCL and K562 targets was <26% and <8%, respectively.

Conjugate Formation Assay. The adhesion of JH.ARL.1 T cell clones to fluorescein-labeled LCL cells was assessed by a published method (23). 5×10^5 LCL cells/ml were incubated for 10 min at room temperature in 0.1 mg/ml fluorescein diacetate, and were washed three times. An equal number of labeled LCL cells and nonlabeled JH.ARL.1 cells (10^5 cells each) were added to conical centrifuge tubes, centrifuged, and incubated at room temperature for 30 min. The cells were centrifuged again, then gently resuspended by using a constant shear force (five cycles of a 50- μ l Eppendorf pipette). Assays were coded and 250–350 fluorescence cells were counted for each sample using a fluorescence microscope. A conjugate was defined as a fluorescent cell bound to one or more nonfluorescent cells.

Proliferation Assay. A standard [³H]thymidine incorporation assay (21, 22) was used to assess the proliferative response of JH.ARL.1 cells to antigen-specific and nonspecific stimuli. An equal number of JH.ARL.1 cells (10^5 cells/well; 11 d post-anti-CD3 stimulation) were cultured with irradiated LCL cells or with autologous PBMC and OKT3 (2 ng/ml) or PHA (1 μ g/ml) in quadruplicate wells of a 96-well microtiter plate (100 μ l/well). Cells were cultured both in the presence and absence of exogenous IL-2 (10% MLA-144 CS). Cells were pulsed with [³H]thymidine during the last 18 h of the 3-d culture period, then harvested, and the amount of incorporated radioactivity was measured.

IL-2 Secretion Assay. Secretion of IL-2 by JH.ARL.1 was assessed as described previously

¹ Abbreviations used in this paper: CS, culture supernatant; LCL, lymphoblastoid cell line; RSV 3' LTR, Rous sarcoma virus 3' long terminal repeat.

(24). Briefly, IL-2 secretion was induced by incubating an equal number of JH.ARL.1 cells (10^6 cells/ml; 11 d post-anti-CD3 stimulation) with irradiated LCL cells for 18 h at 37°C and 7% CO_2 . The supernatants from these cultures were collected and assayed for IL-2 activity using a cloned human T cell line (5 d post-anti-CD3 stimulation) that requires IL-2 for proliferation. rIL-2 (Collaborative Research, Lexington, MA) was used to standardize the assay.

Stimulation of IL-2-R Expression. IL-2-R expression was induced by incubating JH.ARL.1 cells (2×10^5 cells/200 μl /well; 7 d post-anti-CD3 stimulation) with irradiated LCL cells in 96-well plates. LCL cells (2×10^5 cells/200 μl /well) were fixed before addition of JH.ARL.1 cells by centrifuging the cells, removing the supernatant, and air-drying for 30 min at room temperature. After incubation at 37°C and 7% CO_2 for 20 h, IL-2-R expression was assessed by immunofluorescence staining using a murine anti-IL-2-R IgG mAb (anti-CD25; Becton Dickinson & Co.) and flow cytometry.

Results

Selective Inhibition of CD8 Expression on JH.ARL.1. JH.ARL.1, a human CD8^+ -alloreactive T cell clone specific for HLA-B35, was derived from a primary mixed lymphocyte culture using the human EBV-transformed B cell line AR.LCL as stimulator. Stable hygromycin-resistant transfectants of JH.ARL.1 were derived for the episomes $\alpha\text{-CD8/REP1}$ (with a 459-bp segment of CD8 cartridged in an anti-sense orientation downstream of the Rous sarcoma virus 3' long terminal repeat [RSV 3' LTR]; reference 20) and $\text{RSVCAT}\alpha/220.2$ (with the prokaryotic chloramphenicol acetyltransferase gene driven by the same promoter).

The $\alpha\text{-CD8/REP1}$ transfectant, the $\text{RSVCAT}\alpha/220.2$ transfectant, and nontransfected JH.ARL.1 cells were analyzed for expression of T cell surface molecules by immunofluorescence staining and flow cytometry (Fig. 1). Expression of CD8 by

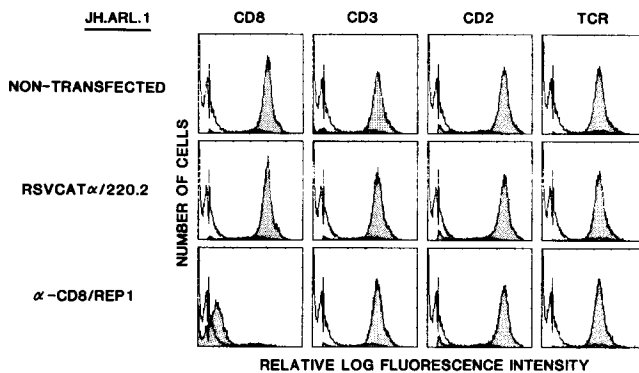


FIGURE 1. Flow cytometric analyses of CD8, CD2, CD3, and TCR surface expression on transfected and nontransfected JH.ARL.1 cells. The $\alpha\text{-CD8/REP1}$ transfectant, the $\text{RSVCAT}\alpha/220.2$ transfectant, and nontransfected JH.ARL.1 cells were stained with OKT11 (anti-CD2), OKT3 (anti-CD3), OKT8 (anti-CD8), WT31 (anti-TCR β chain) (shaded areas), or normal mouse Ig (open areas) as primary antibodies; FITC-conjugated goat anti-mouse IgG was used as the secondary antibody. The x-axis represents 256 channels logarithmically distributed over three decades. The intensity of the peak for nontransfected cells stained with OKT8 was ~ 50 times brighter than the cells stained with control IgG. For the $\alpha\text{-CD8/REP1}$ -transfected cells, the anti-CD8 peak was less than two times more intense than the control peak.

the α -CD8/REP1 transfectant was markedly inhibited (>97%) in comparison with nontransfected JH.ARL.1 cells and the RSVCAT α /220.2 transfectant. Expression of CD2, CD3, and TCR was not altered in either of the transfected cell lines compared with the nontransfected parent. These results parallel those previously reported for the transfected clone 8L2 (20), and they demonstrate that efficient and selective inhibition of CD8 expression can be obtained in T cell clones using an EBV episome-based expression system.

Requirement for CD8 in Alloantigen-specific Cytotoxicity by JH.ARL.1. The effects on T cell function of selectively inhibiting cell surface CD8 expression were investigated. The ability of the α -CD8/REP1 transfectant to mediate lysis of antigen-specific, antigen-nonspecific, and lectin-coated targets was determined using a standard ^{51}Cr -release assay (Fig. 2). Lysis of an antigen-specific target (AR.LCL) by the α -CD8/REP1 transfectant was inhibited at all E/T ratios examined in comparison with lysis by nontransfected JH.ARL.1 cells or the RSVCAT α /220.2 transfectant. Even at the highest E/T ratio (20:1), antigen-specific cytotoxicity mediated by the α -CD8/REP1 transfectant was less than that mediated by controls at an E/T ratio of 2.5:1. None of the effector cells were capable of killing a nonspecific target (DK.LCL; HLA-B35⁻), demonstrating that cytotoxicity was not inappropriately expressed in the transfected cell lines. To determine whether the cytotoxic machinery of JH.ARL.1 was affected by loss of CD8 surface expression, we studied lectin-dependent cytotoxicity. The parental cloned line and both transfectants could efficiently lyse lectin-coated targets (PHA-coated K562). Thus, anti-sense RNA-mediated inhibition of CD8 expression in an antigen-specific T cell clone markedly inhibited lysis of antigen-specific targets without affecting lectin-mediated killing.

Since cytolytic activity requires conjugate formation between effector and target cells, we next examined the ability of α -CD8/REP1 transfectant cells to form conjugates. Inhibition of CD8 expression on the JH.ARL.1 clone did not affect the ability of these cells to conjugate with either HLA-B35⁺ or HLA-B35⁻ cells (data not shown). This result suggests that the inhibition of antigen-specific cytotoxicity in the CD8⁻ variant is not explained by interference with the initial nonspecific adhesion events (25, 26) in T cell-mediated target cell lysis. It seems likely that the de-

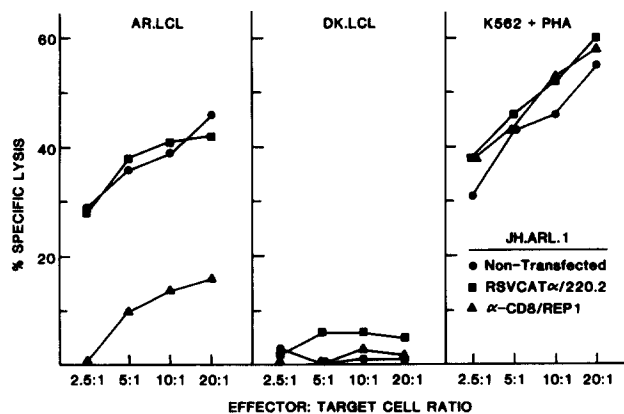


FIGURE 2. Cytolytic activity of the α -CD8/REP1 transfectant and control JH.ARL.1 cells against antigen-specific, antigen-nonspecific, and lectin-coated targets. AR.LCL, DK.LCL, and K562 cells were labeled with ^{51}Cr and used as targets in a standard 4-h ^{51}Cr -release assay. Effector cells (nontransfected JH.ARL.1, \bullet ; the RSVCAT α /220.2 transfectant, \blacksquare ; the α -CD8/REP1 transfectant, \blacktriangle) were used at various E/T ratios. PHA was included in assays with K562 targets.

creased expression of CD8 inhibits antigen-specific recognition of a class I MHC product.

Role of CD8 in Alloantigen-triggered Proliferation. Another event associated with TCR-transduced antigen-specific recognition is proliferation. The ability of the α -CD8/REP1 transfectant to proliferate in response to stimulation by irradiated LCL cells, irradiated PBMC, and anti-CD3 mAbs, or irradiated PBMC and PHA was assessed using a [³H]thymidine incorporation assay (Table I). Whereas nontransfected JH.ARL.1 cells and the RSVCA α /220.2 transfectant proliferated in response to stimulation by AR.LCL cells (HLA-B35⁺), the proliferative response of the α -CD8/REP1 transfectant to stimulation by AR.LCL was significantly decreased. This decrease was still evident even with exogenous IL-2 added to the culture. In contrast to the marked decrease in antigen-specific stimulation, anti-CD3- or PHA-mediated proliferative responses were unaffected in the α -CD8/REP1 transfectant. This result demonstrates that inhibition of CD8 expression on an alloantigen-specific T cell clone decreases its ability to proliferate in response to alloantigenic stimulation without affecting proliferation induced by stimuli that do not involve antigenic recognition (anti-CD3 or PHA plus PBMC).

Since proliferation of T cell clones is mediated by IL-2 and is dependent upon induction of IL-2-R expression, we measured IL-2 secretion (Table II) and IL-2-R expression (data not shown) after alloantigenic stimulation of JH.ARL.1. Nontransfected JH.ARL.1 cells and the RSVCA α /220.2 transfectant secreted IL-2 and expressed IL-2-R when stimulated with AR.LCL cells (HLA-B35⁺), whereas profound decreases in both IL-2 secretion and IL-2-R expression were observed for the α -CD8/REP1 transfectant. These results indicate the dependence of antigen-

TABLE I
Proliferative Response of JH.ARL.1

Stimulus	IL-2	[³ H]Thymidine incorporation		
		JH.ARL.1 nontransfected	JH.ARL.1 RSVCA α /220.2	JH.ARL.1 α -CD8/REP1
			<i>dpm</i>	
-	-	83	65	65
AR.LCL	-	25,674	25,564	3,591
DK.LCL	-	168	223	197
PBMC	-	149	134	189
PBMC + anti-CD3	-	88,769	78,062	89,997
PBMC + PHA	-	36,121	32,008	39,202
-	+	3,576	2,067	2,696
AR.LCL	+	36,574	35,667	4,532
DK.LCL	+	2,636	2,587	2,732
PBMC	+	1,827	1,966	1,889
PBMC + anti-CD3	+	96,287	87,319	110,633
PBMC + PHA	+	65,727	59,821	66,803

JH.ARL.1 cells (11 days post-anti-CD3 stimulation) were cocultured with irradiated LCL cells or with irradiated PBMC and anti-CD3 or irradiated PBMC and PHA. Cultures were incubated in the absence (-) or presence (+) of exogenous IL-2 (10% MLA-144 CS) for 3 d. [³H]Thymidine was added during the last 18 h of the incubation period. SD of means >1,000 dpm were <10% of the means and have been omitted. AR.LCL is the specific target for JH.ARL.1, and DK.LCL is an unrelated cell line.

TABLE II
IL-2 Production by JH.ARL.1

Stimulus	³ H]Thymidine incorporation into IL-2-dependent indicator cell line		
	JH.ARL.1 nontransfected	JH.ARL.1 RSVCA/Tα/220.2	JH.ARL.1 α-CD8/REP1
		<i>dpm</i>	
-	197	230	182
AR.LCL	8,327	7,815	976
DK.LCL	213	182	256

JH.ARL.1 cells (11 d post-anti-CD3 stimulation) were cocultured with irradiated LCL cells. Supernatants were harvested after incubation for 18 h and assayed for IL-2 activity on an IL-2-dependent indicator cell line. SD of means >1,000 dpm were <10% of the means and have been omitted. Background level of thymidine incorporation was 210 dpm, and optimal responses at 2.5 U/ml of rIL-2 was 8,986 dpm. AR.LCL is the specific target for JH.ARL.1, and DK.LCL is an unrelated cell line.

stimulated responses, including secretion of the lymphokine IL-2 and acquisition of IL-2-R, on the expression of the surface molecule CD8.

Discussion

We have used an anti-sense CD8 episomal expression construct to derive a CD8⁻ variant of an allospecific, nontransformed human T cell clone. This stably transfected clone was markedly inhibited in a number of T cell functional responses to alloantigenic stimulator, including cytotoxicity, proliferation, lymphokine secretion, and lymphokine receptor expression. In contrast to TCR-dependent activation, this CD8⁻ T cell variant could still mediate functions elicited by lectin or anti-CD3.

CD8 on T cells is thought to directly bind to class I MHC products on target cells (27, 28) in a fashion analogous to the direct binding of CD4 to class II MHC products (29). Along with this *trans* interaction, CD8 is postulated to associate in a *cis* interaction with the TCR/CD3 complex on the same T cell. The TCR/CD3 complex and CD8 have been shown to comodulate from the cell surface after specific antigenic stimulation (30, 31). By decreasing surface expression of CD8, we have eliminated the interactions that are essential for optimal activation of T cell functional responses requiring antigen recognition through the TCR/CD3 complex.

Previous findings that anti-CD8 antibodies inhibit anti-CD3- or lectin-mediated cytotoxic and proliferative responses of CD8⁺ clones have suggested that a negative signal transduction event occurs by perturbation of surface CD8 or that anti-CD8 inhibits positive signal transduction (12-16). Our data do not directly address this important issue, but it is interesting to note that marked inhibition of CD8 surface expression did not either positively or negatively affect effector functions elicited by anti-CD3 or lectin for two distinct human T cell clones, 8L2 (20) and JH.ARL.1. If signal transduction via CD8 is significant in the absence of the addition of anti-CD8 mAbs, some alteration would be expected in the CD8⁻ variants in anti-CD3 or lectin-mediated responses. To date we have not found any evidence for this type of alteration.

The finding that conjugation was not altered by the loss of surface CD8 suggests that effects on conjugation do not explain the observed functional defects in the CD8⁻ variant. Studies of the effects of anti-CD8 mAbs on conjugate formation have

reported contradictory results (5, 23, 32-34). Our findings using mutational analysis demonstrate that CD8 is not necessary for nonspecific conjugation, an interaction that is presumably mediated by LFA-1/ICAM-1 and CD2/LFA-3 ligand-receptor pairs (35, 36).

In no case were the functional deficits in the CD8⁻ variant reduced to background levels. This finding might be explained by the presence of residual surface CD8, or alternatively, there could be a low level of non-CD8-dependent stimulation occurring in the antigen-specific functional assays that could be mediated by the TCR alone. Nonetheless, it is clear that a >97% decrease in CD8 surface expression dramatically inhibited antigenic stimulation of all of the effector functions tested.

We have developed an alternative approach for defining the function of T cell molecules by applying anti-sense RNA mutagenesis to the analysis of human T cell clones. The use of a nontransformed human T cell clone has allowed us to assess an array of functions requiring antigen-specific recognition that could not be assessed with murine hybridomas. Our procedure does not require the use of exogenous antibodies, thereby obviating the difficulties in interpretation of such studies. The transfection of anti-sense expression vectors into human T cell clones represents a different approach for analyzing the functions of T cell surface molecules. In this case, we have demonstrated that CD8 is necessary for antigen recognition of a class I MHC product by an allogeneic human T cell clone.

Summary

An experimental approach for defining the function of CD8 has been developed by linking anti-sense RNA mutagenesis and T cell cloning technologies. We have transfected an anti-sense CD8 episomal expression vector into a CD8⁺ nontransformed human T cell clone that is specific for the human class I alloantigen HLA-B35. Expression of CD8 on this T cell clone, JH.ARL.1, was selectively and efficiently inhibited. Stimulation of this CD8⁻ variant with specific alloantigen resulted in a marked loss of a number of functional responses, including cytotoxicity, proliferation, IL-2 secretion, and IL-2-R expression. However, these same functional responses could be elicited with stimuli that do not require antigen recognition to activate the T cell (anti-CD3 mAbs, PHA). The results of our study support the hypothesis that CD8 is required for recognition of class I MHC alloantigens that results in activation of T cell functional responses.

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