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Bifunctionality of the human CD8 molecule

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Case Western Reserve University (Health Sciences), 1990
BIFUNCTIONALITY OF THE HUMAN CD8 MOLECULE

by

JOHN EDWARD HAMBOR

Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

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John R. Hamil
BIFUNCTIONALITY OF THE HUMAN CD8 MOLECULE

Abstract

by

JOHN EDWARD HAMBOR

CD8 is expressed on the surface of a subset of T lymphocytes which have two broadly defined functions: 1) class I major histocompatibility complex (MHC)-restricted cytotoxicity against virally-infected, allogeneic, and other cellular targets; and 2) immunoregulation of other lymphocytes. In order to define the role of CD8 in mediating these two distinct T cell functions, an experimental approach utilizing gene transfection analysis to modulate CD8 surface expression in natural and foreign cellular settings was conceived. The development of an Epstein-Barr virus-based episomal expression system and a methodology for stably transfecting nontransformed human T cell clones and other human cell types provided the means for generating antisense and sense cellular pairs which were used to define the molecular functions of the CD8 molecule.
By antisense-mediated inhibition of CD8 expression in nontransformed human CD8\(^+\) T cell clones and expression of CD8 in non-T cell lines, several sets of CD8\(^+\)/CD8\(^-\) paired cells lines were produced. These cellular reagents were used to demonstrate that the CD8 molecule plays an important, but distinct, role in each of the two classes of CD8\(^+\) T cell function. First, the CD8 molecule plays an essential accessory role as a co-receptor to the T cell receptor in class I MHC-restricted recognition of antigen which is subsequently required to trigger a number of T cell functional responses such as cytotoxicity, lymphokine secretion, lymphokine receptor expression and proliferation. Second, CD8 can serve as a novel inhibitory ligand permitting cells which express CD8 to modulate immune responses. Analysis of a genetically engineered, glycoinositolphospholipid-modified variant of human CD8\(\alpha\) revealed that the inhibitory ligand function was found to reside in the extracellular domain of the CD8\(\alpha\) polypeptide. These data support a multifunctional perspective for the cell surface-associated CD8 glycoprotein, ascribing to it an inhibitory ligand function in addition to its conventionally recognized receptor function.
Dedication

I dedicate this work to my family - past, present and future; and especially to my mother who, to paraphrase George Bernard Shaw, taught me not only to see things as they are and ask why, but also to dreams things that never were and ask why not.
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I express my appreciation to Dr. David Kaplan and Dr. Mark Tykocinski for their supervision, guidance and patience during the course of these studies. You taught by example as well as by words and made my graduate years an interesting, challenging and educational experience.

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I acknowledge Harvey Werber, whose memory constantly inspires me to live life to the fullest.

Deepest thanks to my loving wife, Mary, for her continuous support during these best and worst of times. You gave me the greatest gift of life in Matthew; together you make everything worthwhile.
"There is something fascinating about science. One gets such a wholesale return of conjecture out of such a trifling investment of fact."

- Mark Twain
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\( \beta_2 \text{m} \) \hspace{1cm} \beta_2\text{-microglobulin}
bp \hspace{1cm} base pair
Ca^{2+} \hspace{1cm} Calcium
CAT \hspace{1cm} Chloramphenicol Acetyltransferase
CD \hspace{1cm} Cluster of Differentiation
cDNA \hspace{1cm} Complementary Deoxyribonucleic Acid
CNBr \hspace{1cm} Cyanogen Bromide
CO_2 \hspace{1cm} Carbon Dioxide
\text{^{51}Cr} \hspace{1cm} Chromium-51
CS \hspace{1cm} Culture Supernatant
CTL \hspace{1cm} Cytotoxic T Lymphocyte
DAF \hspace{1cm} Decay Accelerating Factor
DAG \hspace{1cm} Diacylglycerol
DNA \hspace{1cm} Deoxyribonucleic Acid
DTH \hspace{1cm} Delayed-type Hypersensitivity
EBNA-1 \hspace{1cm} Epstein-Barr Virus Nuclear Antigen 1
EBV \hspace{1cm} Epstein-Barr Virus
E/T \hspace{1cm} Effector:Target Cell ratio
FL \hspace{1cm} Fluorescein
GPL \hspace{1cm} Glycoinositolphospholipid
HIV \hspace{1cm} Human Immunodeficiency Virus
HLA \hspace{1cm} Human Leukocyte Antigen
HSV \hspace{1cm} Herpes Simplex Virus
Hyg^R \hspace{1cm} Hygromycin B-resistant
ICAM \hspace{1cm} Intercellular Cell Adhesion Molecule
IFN \hspace{1cm} Interferon
Ig \hspace{1cm} Immunoglobulin
IL \hspace{1cm} Interleukin

\( xii \)
IL 2R  Interleukin 2 Receptor
IP₁  Inositol Phosphate
IP₂  Inositol Bisphosphate
IP₃  Inositol Triphosphate
Kb  Kilobase
kDa  Kilodalton
LCL  Epstein-Barr Virus-transformed Lymphoblastoid Cell Line
LCMV  Lymphocytic Choriomeningitis Virus
LFA  Leukocyte Function-Associated Antigen
LPV  Lymphopapilloma Virus
LTR  Long Terminal Repeat
Mab  Monoclonal Antibody
MHC  Major Histocompatibility Complex
MLR  Mixed Leukocyte Culture
mRNA  Messenger Ribonucleic Acid
OriP  Origin of Replication
PBMC  Peripheral Blood Mononuclear Cells
PBS  Phosphate-buffered Saline
PCN  Peak Channel Number
pCTL  Precursor Cytotoxic T Lymphocyte
PHA  Phytohemagglutinin
PKC  Protein Kinase C
PIP₂  Phosphatidylinositol Bisphosphate
rIL 2  Recombinant Interleukin 2
Rep  Rous Sarcoma Virus-based Episomal Replicon
RNA  Ribonucleic Acid
RSV  Rous Sarcoma Virus
SP  p-sulfophenylldiazo-4-hydroxyphenyl Acetic Acid
SV₄₀  Simian Virus 40
TCR  T Cell Receptor
TK  Thymidine Kinase
I. Background

A. Introduction

The vertebrate immune response involves a complex interplay between different cell types. T lymphocytes play a central role in immune reactions by performing important regulatory and effector functions. Activation of T cells requires that antigen, presented on cell surfaces in association with gene products of the major histocompatibility complex (MHC), be recognized by a disulfide-linked glycoprotein found on T cell plasma membranes known as the T cell receptor (TCR). The specific interaction of the TCR with antigen and MHC results in signal transduction via the CD3 molecular complex which has been shown to be intimately associated with the TCR heterodimer (Allison and Lanier, 1987).

Perturbation of the TCR/CD3 complex results in activation of a phosphodiesterase that hydrolyzes phospholipid phosphatidylinositol bisphosphate (PIP$_2$) to generate inositol triphosphate (IP$_3$) and diacylglycerol (Weiss et al., 1986; Imboden and Stobo, 1985a; Imboden et al., 1985b). Subsequently, IP$_3$ causes increases in free cytoplasmic [Ca$^{2+}$] and is converted into inositol
bisphosphate (IP$_2$), inositol phosphate (IP$_1$), and inositol. Diacylglycerol activates protein kinase C which may act as a cofactor in T cell activation. This occurs in conjunction with other cofactors (e.g., interleukins 1 and 6) that may be required, depending on the metabolic state of the T cell (McKenzie, 1988; Holsti and Raulet, 1989; Houssiau et al., 1989; Weaver and Unanue, 1990). The generation of these intracellular second signals is believed to result in subsequent changes in gene expression, including rapid and sequential expression of the nuclear proto-oncogenes c-fos, c-myc, and c-myb (Reed et al., 1985; Cleveland et al., 1987) and induction of interleukin 2 (IL 2) and IL 2 receptor expression (Granelli-Piperno et al., 1986), followed by initiation of DNA synthesis, and eventually proliferation. The CD3 complex also mediates the signal(s) necessary for triggering cytolytic activity by cytotoxic T lymphocytes (Lancki et al., 1987).

Although the TCR/CD3 complex serves as the trigger for initiation of an antigen-specific immune response, several other cell surface molecules appear to be involved in cell-cell contact, recognition, activation and function (Bierer and Burakoff, 1988). One of these molecules, CD8, is a cell surface glycoprotein expressed
primarily by subsets of thymic and peripheral T cells as well as by a subset of natural killer cells (Littman, 1987; Bierer et al., 1989; Parnes, 1989; Bucy et al., 1989). This molecule, also known as T8/leu2 in the human, Ly-2/3 in the mouse, and OX8 in the rat, has been referred to as an "accessory molecule" because of its role in T cell recognition and activation, and as a "differentiation antigen" because of its pattern of expression during T cell development.

Initially, it was thought that CD8 was only expressed on a distinct T cell population which possesses cytolytic/suppressor function (Cantor and Boyse, 1976; Reinherz et al., 1979). However, as cloned T cells became available, the expression of CD8 was found to correlate more strictly with the specificity of T cells for class I MHC products on target cells than with T cell function (Swain, 1981a, 1983; Swain et al., 1981b; Engleman et al., 1981a,b; Meuer et al., 1982a,b). Studies with these T cell clones showed that, for the most part, CD8+ T cells recognize or are restricted by class I MHC proteins (HLA-A,B, or C in humans; H-2K, D, or L in mice). This correlation led to the hypothesis that CD8 might be a receptor for a nonpolymorphic region on class I MHC proteins (Meuer et al., 1982a,b; Spits et
al., 1982; Engleman et al., 1983; Swain, 1983). Recent data, demonstrating a direct and specific interaction between CD8 and class I MHC products, have provided extremely strong evidence in favor of this notion (Rosenstein et al., 1988, 1989; Norment and Littman, 1989a; Norment et al., 1989b; Connolly et al., 1988, 1990; Salter et al., 1990). However, the mechanism by which CD8 impacts upon T cell responsiveness extends beyond its role as an adhesion molecule (adhesin). The discovery that the CD8 glycoprotein may be in close physical proximity with the TCR/CD3 complex during T cell activation (Takada and Engleman, 1987) and that a member of the src-family of tyrosine protein kinases (p56^lck) is associated with CD8 (Bolen and Veillette, 1989) indicates that CD8 may not only increase the avidity with which a T cell interacts with its antigen presenting cell or target cell but may also promote the interaction of the TCR with its appropriate antigen and may play a role in signal transduction.
B. Structure of the CD8 Genes and Glycoproteins

The polypeptide structure of the CD8 molecule is dependent upon species and tissue. Mouse CD8 has been traditionally referred to as Ly-2,3. This molecular complex consists primarily of two forms of heterodimers on the surface of thymocytes: one Ly-2 chain, either α (38 kDa) or α' (34 kDa), is disulfide linked to a Ly-3 (also called β) chain (28-30 kDa) (Ledbetter et al., 1981; Ledbetter and Seaman, 1982; Jay et al., 1982; Walker et al., 1984a,b; Luescher et al., 1984; Naim et al., 1984). Some tetramers containing equimolar amounts of Ly-2 (α + α') and Ly-3 (β) chains are also present on thymocytes (Ledbetter and Seaman, 1982). For the purpose of consistency, the terms Ly-2 and Ly-3 will be used from here on to refer to the antigenic determinants on these polypeptides, while the polypeptides themselves will be referred to using the CD8 nomenclature (i.e., CD8α, CD8α', and CD8β). The amounts of CD8α and CD8α' chains immunoprecipitated from a total thymocyte population are close to equal. In contrast, if one examines peripheral T cells from spleen or lymph node, very little α' chain is detectable in immunoprecipitates of cell-surface-labeled CD8 complexes (Walker et al., 1984b; Tagawa et al., 1986; Zamoyska and Parnes, 1988).
As discussed below, the two chains bearing the Ly-2 determinants (CD8α and CD8α') are the products of alternatively spliced mRNA species from a single CD8α gene, and these two polypeptide chains differ only in their cytoplasmic tail (Zamoyska et al., 1985; Tagawa et al., 1986).

The polypeptide bearing the Ly-3 determinant is the product of a distinct but closely related CD8β gene (Itakura et al., 1972; Gorman et al., 1988). All three of these polypeptides are glycosylated, with three N-linked glycan units on α and α' and one on β (Rothenberg and Triglia, 1983; Luescher et al., 1985). Two alleles have been defined serologically for both CD8α (Ly-2.1 and Ly-2.2) and CD8β (Ly-3.1 and Ly-3.2). The rat CD8 (OX8) complex similarly consists of heterodimers between two chains (32 and 37 kDa) that have been shown by molecular genetics to be homologous to the mouse CD8α and CD8β chains, respectively (Johnson et al., 1985; Johnson and Williams, 1986). As a result of the glycosylation pattern, the rat β chain is the larger of the two. No equivalent of the mouse α' chain has been described in rat.

The structure of human CD8 (originally designated T8 or Leu-2) has appeared to be distinct from that of
rodents. Biochemical studies of CD8 on human peripheral blood T cells have indicated that the molecule consists of homodimers (and some higher homomultimers) of a single 34 kDa polypeptide chain (Snow and Terhorst, 1983a; Snow et al., 1983b). This chain, which is the polypeptide that is recognized by most anti-human CD8 Mabs (e.g. OKT8, anti-Leu-2a, anti-Leu-2b; DiSanto et al., 1987), has been shown to be the homolog of mouse CD8α (Zamoyska et al., 1985), and will therefore be referred to as human CD8α. Although the human CD8α polypeptide is glycosylated, the carbohydrate is all O-linked rather then N-linked (Snow and Terhorst, 1983a). On human thymocytes the same apparent homodimer has been identified, but higher multimers have been found to contain an additional disulfide-linked polypeptide of 46 kDa that is not found in the periphery (Snow and Terhorst, 1983a). This larger polypeptide has been identified as CD1, a protein of unknown function expressed on human cortical thymocytes and some tumor lines (Ledbetter et al., 1985; Snow et al., 1985; Blue et al., 1989). CD1 is related to class I MHC molecules, although it is encoded on a different chromosome (Calabi and Milstein, 1986), and like class I proteins, it was initially identified as a heterodimer noncovalently
associated with β₂-microglobulin (Ziegler and Milstein, 1979). However, the CD1 that is disulfide linked to CD8 to form heteromultimers in human thymus is devoid of β₂-microglobulin (Ledbetter et al., 1985; Snow et al., 1985). An association with CD1 has not been found for CD8 in mouse or rat thymus. Recently, a noncovalent association between CD8 and class I MHC proteins has been described on the surface of human T cell clones (Bushkin et al., 1988; Blue et al., 1988). The physiological significance of such an association is not known.

Complementary DNA (cDNA) clones encoding human CD8α were initially isolated by subtractive hybridization techniques using cDNA libraries constructed from total human genomic DNA and selected for cell surface expression of CD8 (Kavathas et al., 1984; Littman et al., 1985). The predicted amino acid sequence of human CD8α was then determined from the DNA sequence of these clones (Littman et al., 1985; Sukhatme et al., 1985a). The most striking conclusion to be drawn from the protein sequence was that CD8α is a member of the Ig gene superfamily by virtue of its possession of an amino-terminal domain that is homologous to Ig variable (V) regions, especially light chain V regions. This
region contains many of the conserved residues of members of the Ig gene superfamily, including the centrally placed disulfide loop that is so characteristic of Ig homology units. Furthermore, computer analyses of the structural characteristics of this V-like domain suggest that this region can fold in a manner similar to folding of Ig domains (Sukhatme et al, 1985a). CD8α was the first example of a T cell differentiation antigen that was found to be a member of the Ig gene superfamily, a finding that has now been extended to many other T cell surface markers.

The predicted CD8α protein has a signal peptide of 21 amino acids followed by a mature protein sequence of 214 amino acids (Littman et al, 1985; Sukhatme et al, 1985a). The external portion of the protein consists of the 96 amino acid V-like domain and a membrane-proximal, 65 amino acid hingelike region or connecting peptide. This is followed by a 24 amino acid hydrophobic transmembrane segment and a highly basic 29 amino acid cytoplasmic tail. Although the sequence suggests the possibility of one N-linked glycosylation site, (Asn-X-Ser or Asn-X-Thr), the presence of proline as the variable residue (Asn-Pro-Thr) most likely accounts for the lack of usage of this site. The protein is
predicted to contain nine cysteine residues: three in the V-like region (two of which form the Ig-like disulfide loop) and two each in the hinge, transmembrane region, and cytoplasmic tail. Biochemical studies of Snow and Terhorst (1983) demonstrated that the cysteines in the V-like region are not involved in interchain disulfide bridges. Fujimoto et al. (1983, 1984) found that CD8 is released from the surface of human T cell tumor lines as a 27 kDa monomer, indicating that the disulfide bridge(s) most likely involve cysteines within the cytoplasm, the plasma membrane or the hinge-like region (membrane-proximal). This conclusion is supported by a recent study in which a hybrid cdNA clone was constructed and transfected, resulting in the cell surface expression of human CD8 with its cytoplasmic tail, transmembrane region and a membrane-proximal portion of its extracellular domain (all of which contain cysteine residues) replaced by the carboxy-terminal domain of decay accelerating factor (DAF), including the portion specifying a glycolipid linkage to the plasma membrane (Tykocinski et al., 1988). Immunoprecipitation of this hybrid protein under nonreducing conditions was consistent with it being a monomer, again indicating the the dimers and higher
multimers of CD8 are formed by disulfide linkages involving cysteines in the cytoplasmic tail, transmembrane region and/or the membrane-proximal portion of the extracellular hinge-like domain. Furthermore, the α' chain of mouse CD8, which can also dimerize, contains no cysteine residues in its cytoplasmic tail, implying that it must use one or both of the cysteines in its transmembrane region or the membrane-proximal cysteine in the extracellular hinge-like domain for disulfide linkage to CD8β. Direct analysis of CNBr fragments of purified CD8 α/β heterodimers revealed that an interchain disulfide bond exists between cysteine residues located either in the membrane-proximal region of the extracellular domains or within the transmembrane domains of both the α and β chains (Kirszbaum et al., 1989).

Mouse cDNA clones homologous to human CD8α were isolated by cross-hybridization to human clones (Zamoyska et al, 1985; Nakauchi et al., 1985). Zamoyska et al. (1985) then isolated and transfected the corresponding mouse gene and could show that the mouse homolog of human CD8α encoded polypeptides bearing the Ly-2 and not the Ly-3 determinant. These results further demonstrated that the two forms of polypeptide
chain that were known to express the Ly-2 determinant, i.e., α and α', were products of the same (hence, CD8α) gene. In accordance with these findings, the isolated mouse cDNA clones could be shown to be of two types: one fully corresponded to human CD8α, while the other had a 31 base pair deletion in sequence encoding the cytoplasmic tail (Zamoyska et al., 1985). This deletion shifted the reading frame, resulting in early termination. The encoded protein would have only 4 rather than 29 amino acids in the cytoplasm, resulting in a size difference consistent with the shorter clone representing the α' chain. Zamoyska et al. (1985) found by S1 nuclease mapping that mRNA corresponding to both forms of cDNA was present in normal mouse lymphoid tissues and cell lines. Transfection of the two forms of cDNA clones in expression vectors confirmed that they indeed represented the α and α' polypeptides (Tagawa et al, 1986; Zamoyska and Parnes, 1988).

The mature polypeptide chain of mouse CD8α is slightly larger than its human counterpart (220 versus 214 amino acids), but the proteins are quite similar in overall structure and sequence (Zamoyska et al, 1985; Nakauchi et al., 1985). All of the cysteine residues are conserved between the two. The amino-terminal
V-like domains of these molecules are the least conserved portions, with only 42% identical residues. Computer analyses of the structural features of this domain, based largely on the position of the conserved cysteine residues, initially suggested the formation of an intrachain disulfide loop that was homologous to Ig V-domains (Sukhatme et al., 1985a; Nakauchi et al., 1985). However, direct sequence analysis of cysteine-containing peptides of murine CD8α revealed an unusual intrachain disulfide bond resulting in a short, unique intrachain loop unlike the canonical Ig V-domain structure (Kirszbaum et al., 1989). Despite its lack of precedent in other Ig-related structures, this unusual disulfide loop can be parsimoniously accommodated into a modified domain which has retained the major features of the Ig structural motif. Interspecies sequence conservation is also evident in other regions of the polypeptide; the connecting peptides contain 59%, the transmembrane regions 79%, and the cytoplasmic tail 55% identical residues. The sequence predicts three N-linked glycosylation sites, consistent with the results of biochemical analysis of the protein. The rat CD8α sequence has also been determined from cDNA clones and is very similar to that of the mouse (Johnson et
al., 1985).

The CD8α gene organization is consistent with that of other members of the Ig gene superfamily. The mouse CD8α gene consists of five exons which correlate roughly with the functional domains of the protein, although surprisingly there is no intron separating the leader (signal peptide) sequence from the sequence encoding the V-like domain (Liaw et al., 1986; Nakauchi et al., 1987b). Significantly, the entire V-like domain is encoded within a single exon (exon I). As in the other members of the Ig gene superfamily, the splicing junctions all split codons between the first and second nucleotides (1/2 codon split) with the exception of intron 4, which separates two exons (IV and V) encoding the cytoplasmic tail. The structure of the gene confirmed the hypothesis that the α and α' forms of mouse CD8 mRNA arise from alternative splicing, because the 31 nucleotides missing in α' mRNA constitute exon IV of the gene (Liaw et al., 1986). Therefore, inclusion of exon IV results in α mRNA, while splicing from exon III to exon V results in α' mRNA. Although the difference in size between the mRNA species encoding α and α' is too small to be distinguished by Northern blot analysis, two different sizes of mRNA (~1.7 and ~3.5 kb)
can be seen on Northern blots using a CD8α probe (Zamoyska et al., 1985; Nakauchi et al., 1985). These different species are believed to originate from usage of alternative polyadenylation sites. Nakauchi et al. (1987b) have mapped the start site of transcription of the mouse CD8α gene 334-335 base pairs upstream of the initiation codon. The sequence TATTAA is located 29 base pairs upstream of the transcription initiation site and may constitute a promoter region (Nakauchi et al., 1987b). The genomic sequence necessary for expression of the 1.7 kb mRNA (and both α and α' polypeptides) upon transfection is all contained within a 5.4 kb fragment of DNA (Zamoyska et al., 1985), but this fragment is not sufficient for expression of the 3.5 kb mRNA species.

The sequences of the two serologically described allelic forms (Ly-2.1 and Ly-2.2) of mouse CD8α have been determined (Liaw et al., 1986). These sequences show very few differences, and a single base pair substitution and consequent amino acid change at residue 78 (Val, Ly-2.1; Met, Ly-2.2) accounts for the difference in serological properties. The human CD8α gene is similar in structure to that of the mouse, with the exception that the human gene, like most other members of the Ig gene superfamily, does contain an
intron separating the sequence encoding the signal peptide from that encoding the V-like domain (Littman et al., 1985; Liaw et al., 1986). Although one predominant mRNA species (2.5 kb) is seen on Northern blots of CD8+ human cells (Kavathas et al., 1984; Littman et al., 1985), an alternative form of mRNA splicing has also been identified for human CD8α (Littman, 1987; Norment et al., 1989). However, it is not the cytoplasmic tail that is altered in this instance, but rather the transmembrane region. The inclusion or exclusion of exon IV sequence of the human CD8α gene (corresponding to mouse exon III) does not alter the reading frame of the resultant mRNA, but encodes a protein that either contains or is missing the transmembrane region. This transmembrane-minus form of human CD8α is physiologically secreted as a homodimer from activated T cells in vivo (found in the serum of patients with EBV-induced infectious mononucleosis) and by transfected cells in vitro (Littman, 1987; Norment et al., 1989; Tomkinson et al., 1989). Since the cysteine residues in the transmembrane region are absent in secreted human CD8 α/α homodimers, it seems most likely that the membrane-proximal cysteine residue in the extracellular hinge-like domain of the α chain is
involved in disulfide bond formation. This form of protein should be distinguished from the 27 kDa single chain fragment of CD8α that has been found to be enzymatically cleaved and released from the surface of human T cell tumor lines as a monomer (Fujimoto et al., 1983, 1984).

The mouse CD8α gene had been known for many years to map to chromosome 6, closely linked to the Ig K light chain locus (Itakura et al., 1972; Gottlieb, 1974; Claflin et al., 1978; Gibson et al., 1978). The isolation of human CD8α probes allowed the chromosomal mapping of that gene to the short arm of chromosome 2 (2p12) at a location closely linked to the human Ig K light chain locus (Sukhatme et al., 1985b). This close linkage to K in both mice and humans supports the hypothesis that CD8α and K derived from a common ancestral precursor. Despite their similarities to Ig genes, the mouse and human CD8α genes are both single copy and do not require rearrangement for expression (Zamoyska et al., 1985; Littman et al., 1985; Sukhatme et al., 1985a).

cDNA clones encoding CD8β were first isolated in the rat (OX8, 37 kDa) (Johnson and Williams, 1986). The nucleotide sequence predicted a protein of 187 amino
acids, with 141 amino acids external to the cell, a 27 amino acid transmembrane region, and a 19 amino acid highly basic cytoplasmic tail. As in the case of the \( \alpha \) chain, CD8\( \beta \) was shown to possess an amino-terminal external domain (101 amino acids) that is homologous to Ig and TCR V regions. Strikingly, this region of the protein is followed by a 12 amino acid segment that is extremely similar to Ig joining (J) segments, with as many as 10 out of 12 residues similar to a human \( \lambda J \) sequence. The Ig-like portions are joined to the membrane by a connecting peptide of 28 amino acids. The protein contains six cysteine residues: two in the V-like region (forming the Ig-like disulfide loop), one in the connecting peptide, two in the transmembrane region, and one in the cytoplasmic tail. Although CD8\( \alpha \) and CD8\( \beta \) are both members of the Ig gene superfamily and show similarities throughout, they are not very closely related to one another, with only 21% identical residues in the rat (Johnson and Williams, 1986).

The mouse CD8\( \beta \) cDNA has also been cloned and sequenced by several groups, and is very similar to the rat CD8\( \beta \) (78% identical residues) both in sequence and in organization (Nakauchi et al., 1987a; Panaccio et al., 1987; Gorman et al., 1988; Blanc et al., 1988).
All of the cysteine residues are conserved. The mouse sequence contains only one N-linked glycosylation site, in contrast to three in the rat. The CD8β gene organization is consistent with that of other members of the Ig gene superfamily, consisting of six exons and five introns. The exons correlate roughly with the putative functional domains, namely a leader exon, a variable and joining region-like exon, a hinge region-like exon, a transmembrane exon and two intracytoplasmic exons. There is no intervening sequence between V- and J-like gene segments, indicating that unlike Ig genes, rearrangement is not necessary for expression (Nakayama et al., 1989).

Two predominant mRNA species of mouse CD8β are evident on Northern blots of all mouse strains examined (Nakauchi et al., 1987a; Panaccio et al., 1987; Gorman et al., 1988; Blanc et al., 1988). As in the case of mouse CD8α, these result from usage of alternative polyadenylation sites and do not alter the encoded protein (Gorman et al., 1988). Gorman et al. (1988) examined a large panel of mice and noted that the sizes of these mRNA species differ among inbred strains in a striking way depending upon the particular combination of serologically defined alleles expressed at both the
CD8α and CD8β loci. Thus, the CD8β mRNA sizes for the three described serological phenotypes are 1.6 and 2.4 kb for mice that are Ly-2.1, Ly-3.1; 1.6 and 2.6 kb for mice that are Ly-2.1, Ly-3.2; and 1.2 and 2.6 kb for mice that are Ly-2.2, Ly-3.2. In transfection studies the mRNA size polymorphisms could be shown to be encoded within the structural gene for CD8β and not to be a function of the mouse strain in which the gene is expressed (Gorman et al., 1988). Consequently, these polymorphisms define three different allelic forms of the mouse CD8β gene. The sequences of the two serologically described allelic forms (Ly-3.1 and Ly-3.2) of mouse CD8β have been determined (Nakayama et al., 1989). These sequences show very few differences; a single nucleotide change leading to an amino acid substitution at residue 77 (Ser, Ly-3.1; Arg, Ly-3.2) in the extracellular domain accounts for the difference in serological properties. As in the case of human CD8α mRNA, there is also an alternative form of splicing of mouse CD8β mRNA that deletes the sequence encoding the transmembrane region (Gorman et al., 1988). Although it has been demonstrated that this mRNA results in a secreted form of CD8β, it is not yet known what the function of such a protein might be.
Classical genetic studies led to the early conclusion that the genes encoding mouse CD8α and CD8β are closely linked both to each other and, as discussed above, to the Ig K light chain locus on chromosome 6. Gorman et al. (1988) have recently linked the mouse CD8α and CD8β genes to each other at the molecular level by chromosomal walking. These two genes are in the same transcriptional orientation and are separated by 36 kb of DNA, with the CD8β gene located to the 5' side of the CD8α gene. The CD8β gene spans approximately 16 kb of DNA, and hence is much larger than the CD8α gene (Gorman et al., 1988; Blanc et al., 1988). Like the CD8α gene, the CD8β gene is single copy and does not require rearrangement for expression (Nakauchi et al., 1987a; Gorman et al., 1988). The distance between these genes and the K locus has not been determined.

As discussed above, human CD8 has been thought to consist of homodimers and homomultimers of a single polypeptide chain on peripheral T cells, and biochemical studies did not reveal the presence of a polypeptide equivalent to mouse and rat CD8β. Johnson (1987) used a rat cDNA probe to demonstrate that there is a human gene homologous to rat CD8β and that mRNA corresponding to this gene is present in human thymus. This gene was
further shown to be located on human chromosome 2, suggesting the possibility that the CD8α and CD8β genes might be linked in the human genome as they are in the mouse genome (Spurr et al., 1988). Shiue et al. (1988) have recently isolated and sequenced human thymocyte cDNA clones corresponding to CD8β. These clones are fully in frame (with respect to the mouse and rat cDNA sequences) and encode a mature protein of 189 amino acids with 143 residues external to the cell, and transmembrane and cytoplasmic domains of 27 and 19 residues, respectively. The predicted protein is very similar to mouse and rat CD8β, with approximately 56% identical amino acids. The mRNA corresponding to this protein was shown to be present not only in the thymus, but also in peripheral blood mononuclear cells (Shiue et al., 1988). A second form of human CD8β cDNA (CD8β') with a longer cytoplasmic tail (55 amino acids) could be shown to derive from use of an alternative splicing acceptor site for the last exon of the gene, resulting in a frame shift (Shiue et al., 1988). The CD8β cDNA was then inserted into an expression vector and transfected into mouse L cells either alone or together with a human CD8α construct. To determine whether a human CD8β protein was expressed by these transfectants,
the cells were stained with an anti-CD8 Mab (2ST8-5H7) which had been shown not to bind to L cells transfected with the CD8α gene (DiSanto et al., 1987). It was reasoned that this Mab might be specific for or require the presence of the CD8β chain. Indeed, 2ST8-5H7 only bound to cells that had been co-transfected with both CD8α and CD8β constructs, and not cells transfected with either alone (Shiue et al., 1988). This might be because the Mab is specific for a combinatorial determinant expressed only on heterodimers between the two chains, or because CD8β may need to be complexed with CD8α to be expressed on the cell surface. In either case, these findings demonstrate that human CD8β protein can be expressed on the cell surface. Shiue et al. (1988) further demonstrated that 2ST8-5H7 positively stains the same population of peripheral blood mononuclear cells as does the anti-CD8α Mab OKT8, indicating that human peripheral blood T cells that have been identified as CD8+ can express both CD8α and CD8β. Northern blot analysis of a panel of human T cell leukemias and normal T cells further demonstrated that CD8α and CD8β are not invariably co-transcribed. Phenotypic analysis of fresh and IL 2-expanded peripheral blood mononuclear cells confirmed that CD8α and CD8β chains are differentially
expressed at the cell surface (Terry et al., 1990). From these findings, four distinct subpopulations of CD8+ cells have been identified based on the expression of CD8α/α or CD8α/β complexes: (1) TCRαβ+ T cells which are CD8α+/β+; (2) TCRαβ+ T cells which are CD8α+/β-; (3) TCRγδ+ T cells which are CD8α+/β-, and (4) natural killer (NK) cells which are TCRαβ- and TCRγδ-, but CD8α+/β-.

Studies to date have shown that mRNA encoding CD8α is found only in cells that express the corresponding protein on the cell surface (Zamoyska et al., 1985; Nakauchi et al., 1985). This, however, is not the case for mouse CD8α' and CD8β. It is likely that the major level of regulation of CD8α expression is transcriptional, but this will need to be confirmed by nuclear run-on assays. Carbone et al. (1988) have shown that sequences at the 5' end of the mouse CD8α gene are more heavily methylated in cells that do not express mRNA and protein as compared to cells that do.

In contrast to the α chain, the α' chain of mouse CD8 undergoes a developmental alteration in the manner in which its expression is regulated. Although close to equal levels of α and α' have been demonstrated on the cell surface of mouse thymocytes, very little α' is found on the surface of mature peripheral T cells
(Walker et al., 1984a; Gallagher et al., 1986; Tagawa et al., 1986; Zamoyska and Parnes, 1988). This is not a result of differences in the way in which the mRNA transcript is spliced in the periphery as compared to the thymus, since the ratio of α to α' mRNA is essentially identical in thymus and peripheral T cells (Zamoyska et al., 1985). Similarly, the ratio of metabolically labeled α to α' polypeptide does not vary between the thymus and the periphery, and turnover of α' chain is not greater in peripheral T cells as compared to bulk thymocytes (Zamoyska and Parnes, 1988). The α' polypeptide found within peripheral T cells appears to be properly glycosylated and associated with the β chain (Zamoyska and Parnes, 1988). However, a novel sorting mechanism results in only (or predominantly) α/β and not α'/β heterodimers traversing from the golgi to the cell surface in peripheral T cells (Zamoyska and Parnes, 1988). In contrast, double-positive (CD4+8+) cells in the thymus, and those few single-positive CD8 thymocytes which have an immature phenotype (i.e., CD8+4−J11D+) (Crispe and Bevan, 1987), do not distinguish between the two forms of CD8 heterodimers, both of which have equal access to the cell surface. The population of CD8+ thymocytes which has a mature phenotype (i.e.,
CD8\(^{+}4^{-\text{J11D}^{-}}\) is similar to mature peripheral CD8\(^{+}\) cells in restricting cell surface expression to heterodimers containing \(\alpha\) as opposed to \(\alpha'\) chains (Zamoyska and Parnes, 1988). It is tempting to speculate that \(\alpha'/\beta\) heterodimers might have a function distinct from that of \(\alpha/\beta\) heterodimers. In this regard, the finding that CD8 \(\alpha/\beta\), but not \(\alpha'/\beta\), heterodimers are associated with the tyrosine-specific protein kinase p56\(^{\text{Lck}}\) (Zamoyska et al., 1989) suggests that the function of CD8 as a signal transduction molecule may be regulated during T cell maturation (Eichmann et al., 1989).

Intracellular expression of CD8\(\beta\) polypeptide chains is regulated at the mRNA level, as it is for CD8\(\alpha\), but cell surface expression of the polypeptide is regulated at a posttranslational level. Studies in the mouse system have demonstrated that cell surface expression of CD8\(\beta\) requires concomitant expression of CD8\(\alpha\), presumably to form heterodimers (Gorman et al., 1988; Blanc et al., 1988). Gorman et al. (1988) found that human CD8\(\alpha\) can substitute for the mouse chain to allow cell surface expression of mouse CD8\(\beta\). Cell surface expression of human CD8\(\beta\) also requires CD8\(\alpha\) expression (Shiue et al., 1988; DiSanto et al., 1988; Norment and Littman, 1988c)). In contrast, both mouse and human CD8\(\alpha\) (and
CD8a' in the mouse) can be expressed on the cell surface as homodimers in the absence of CD8β mRNA or protein (Kavathas et al., 1984; Littman et al., 1985; Zamoyska et al., 1985; Tagawa et al., 1986). Analysis of human T cells demonstrated that α and β chains are differentially expressed at the cell surface; some T cells express both α/α and α/β complexes on their cell surface while others express only α/α homodimers (Terry et al., 1990). The observation that α/β, but not α/α, complexes are down-modulated from the cell surface after T cell activation (Terry et al., 1990) demonstrates that these two complexes have distinct properties and suggests that CD8 α/α and α/β dimers may not subserve identical functions.
C. Function of CD8 in T Cell Activation

Initial indications that CD8 plays a functional role in T cell activation came from studies using antisera and subsequently monoclonal antibodies (Mabs) specific for this protein. Mature T cells which express CD8 and are specific for or restricted by class I MHC products are incapable of a number of antigen-driven T cell functions (e.g., cytotoxicity, proliferation, and lymphokine release) when treated with these antibodies. In addition, the generation of mature single positive CD8 thymocytes during thymic development was also inhibited (Shinohara and Sachs, 1979; Nakayama et al., 1979; Hollander et al., 1980; Evans et al., 1981; Reinherz et al., 1981, 1983; MacDonald et al., 1982; Landegren et al., 1982; Engleman et al., 1981a, 1983; Swain, 1983; Ramsdell and Fowlkes, 1989). However, more extensive analysis of a number of CD8+ T cell clones revealed heterogeneity in the ability of various T cell clones to be blocked (MacDonald et al., 1982; Moretta et al., 1984; Maryanski et al., 1989). The observed heterogeneity is believed to be a result of the degree to which CD8 is required to assist the TCR in antigen recognition and/or signal transduction resulting in T cell activation, and thus is explained by the assumption
that resistant T cells express high affinity TCRs that make an additional adhesion molecule (adhesin) such as CD8 unnecessary. Alternatively, a functional association of CD8 with other molecules besides the TCR/CD3 complex may be required for some T cells but not others.

The finding that anti-CD8 could block conjugate formation between MHC class I-specific cytotoxic T lymphocytes (CTLs) and target cells (Hollander, 1982; Bonavida et al., 1983; Tsoukas et al., 1982; Landegren et al., 1982; Platsoucas, 1984) suggested a role for CD8 at an early step in the interaction between CTLs and their targets, possibly during the recognition phase. This led to the hypothesis that the function of CD8 is to stabilize or increase the avidity of the interaction between T cells and target cells by binding to class I MHC molecules, and thus augment the affinity of TCRs (MacDonald et al., 1982; Swain, 1983; Calvo et al., 1988). As would be predicted by this model, T cells bearing TCR molecules with apparently low affinity for antigen/class I MHC would be more dependent on CD8 interactions (i.e., their function is more easily blocked by anti-CD8 Mabs) than are T cells with apparently higher affinity TCRs (Shimonkevitz et al.,
1985; van Seventer et al., 1986; Calvo et al., 1989). Thus, this hypothesis could also explain the observed heterogeneity in the ability of different T cell clones to be blocked by anti-CD8 Mabs, as well as the finding that bulk cultures of CTLs from primary antigen responses could be blocked more easily than those from secondary antigen responses (MacDonald et al., 1982; Moretta et al., 1984). The fact that anti-CD8 uninhibitable class I allospecific CTL clones, which may be highly enriched for cells bearing high affinity TCRs, can be generated in vitro (McCarthy et al., 1988, 1989) lends further credence to the concept that, for any given antigen, there exists precursor CTLs (pCTLs) with a spectrum of avidities ranging from low to moderate to high. Accordingly, low/moderate avidity pCTLs would require the contributions of a CD8/class I interaction for the initiation of TCR-mediated signaling, whereas very high avidity pCTLs could be efficiently stimulated through their TCRs alone. In further support of the notion of CD8 acting to increase cell-cell avidity, some studies have shown that the approximation of the CTLs and target cells by lectins could overcome inhibition of cytotoxicity by anti-CD8 Mabs (Meuer et al., 1982b; Spits et al., 1982).
Initially, functional studies provided strong evidence that CD8 could function as a cell adhesion molecule (adhesin) and that the ligand for CD8 is contained within class I MHC proteins (Ratnofsky et al., 1987; Mann et al., 1989). Direct proof that the α3 domain of MHC class I gene products is the ligand for CD8 has recently come from cell-cell binding studies (Rosenstein et al., 1988, 1989; Norment and Littman, 1989a; Norment et al., 1989b; Connolly et al., 1990; Salter et al., 1990). These studies clearly demonstrate that CD8, by binding in trans (i.e., on the surface of another cell) to MHC class I proteins, can function as an adhesin. Thus, one mechanism by which CD8 could serve as an accessory molecule is by increasing intercellular adhesion between a T cell and its antigen presenting cell or target cell.

Recently, it has been suggested that CD8 may not be simply a cell adhesion molecule, but may additionally be involved in pathways of signal transduction. In this regard, anti-CD8 Mabs have been shown to block cytotoxicity not only during the phase of conjugate formation, but also at a post binding step (van Seventer et al., 1986; Blanchard et al., 1987; Abrams et al., 1989). Mabs specific for CD8 can inhibit T cell
activation induced by lectins or by Mabs specific for the TCR, CD3, CD2, Tp103, and Tp44, despite the absence of the appropriate ligand for CD8 (i.e., class I MHC) in these systems (Welte et al., 1983; Hunig, 1984; Beckoff et al., 1985; Fleischer et al., 1986a; van Seventer et al., 1986, 1988; Schrezenmeier and Fleischer, 1988; Quinones et al., 1989). These findings led to the hypothesis that CD8 might deliver a negative signal to the T cell, thereby inhibiting activation. However, there is still no direct evidence for transmission of a negative signal via CD8. Furthermore, the mere absence of a positive signal does not imply the presence of a negative signal.

Since this model is supported by studies that involve Mab blocking as opposed to physiological conditions, other possible explanations for the observed results, such as steric hindrance, must be considered (Takayama et al., 1987). The possibility that anti-CD8 Mabs could interfere sterically with T cell activation has become increasingly credible based on the growing body of evidence that there may be a physical association between CD8 and the TCR/CD3 complex. The demonstration of antigen-induced comodulation of CD8 and the TCR/CD3 complex indeed suggests that CD8 may
associate in cis (i.e., on the same cell membrane) with the TCR/CD3 complex. Takada and Engleman (1987) found that incubation of a human T cell clone with the relevant target cell resulted in modulation of 20-30% of the surface CD3 and CD8. Modulation of CD3 could be blocked by soluble anti-CD8 Mab and, similarly, modulation of CD8 could be blocked by soluble anti-CD3 Mab. Anti-CD3 followed by goat antimouse Ig could also modulate both CD3 and CD8. Although comodulation of CD8 with the TCR/CD3 complex does not directly imply a physical association, it is consistent with one.

If CD8 can associate in cis with the TCR/CD3 complex, it seems plausible that CD8 might also be involved directly or indirectly in the transmission of positive signals to activate T cells. Studies demonstrating that anti-CD8 Mabs could inhibit a postbinding step in CTL triggering (Blanchard et al., 1987) and that the state of CD8 aggregation with the TCR/CD3 complex controls IL 2-dependent T cell growth (Jonsson et al., 1989) indicate that CD8 may influence signal transduction via the TCR/CD3 complex and suggest that CD8 may play a more complex regulatory role in activation of cytotoxic and proliferative T cells. Antibody blocking studies of cytotoxic/helper hybrid
clones, which co-express CD4 and CD8, provide further evidence that cytolysis is closely linked to the CTL TCR and that CD8 has more than a passive role as an accessory molecule on the surface of T lymphocytes (Havran and Fitch, 1988). Additional evidence for involvement of CD8 in signal transduction pathways derives from data that cross-linking CD8 to the TCR/CD3 complex with Mabs can activate resting T cells and induce proliferation under conditions in which anti-CD3 Mabs alone does not (Emmrich et al., 1986, 1988; Eichmann et al., 1987; Boyce et al., 1988; Samstag et al., 1988; Schmidberger et al., 1988). It is assumed that in the case of antigen-driven T cell activation, class I MHC molecules act as the physiologic cross-linking ligands for CD8 and the TCR. Even though these results do not imply direct transmission of a signal via CD8, they do suggest that CD8 may enhance signal transduction by its association with the TCR/CD3 complex.

While the molecular mechanisms underlying the signal transduction network which initiates the events that lead to expression of an activated T cell phenotype are largely unknown, some of the earliest events that can be documented involve phosphorylation of membrane
proteins (Samelson et al., 1985; Patel et al., 1987; Klausner et al., 1987; O'Shea et al., 1987). For example, the CD3-γ and CD3-ζ proteins are phosphorylated on serine residues probably through the action of one or more protein kinase C (PKC)-related enzymes and ζ is phosphorylated on one or more tyrosine residues. Since T cell mutants lacking ζ respond poorly following antigen recognition (Sussman et al., 1988), stimulation of tyrosine-specific protein kinases and subsequent phosphorylation of cellular proteins (including ζ) appear to be important for T cell activation-dependent signal coordination and signal transduction.

Recent studies indicate that at least one of the T cell tyrosine protein kinases capable of phosphorylating the TCR/CD3-ζ protein is p56^lck, a member of the src family of tyrosine kinases which is predominantly expressed on the internal plasma membrane of T cells as a consequence of post-translational myristoylation of its amino terminus (Marth et al., 1985; Veillette et al., 1988a,b; Barber et al., 1989; Veillette et al., 1989b). Co-immunoprecipitation and co-modulation studies of a panel of CD8^+ T cell clones found that p56^lck is physically associated with approximately 25% of surface CD8 molecules and that CD8-p56^lck
immunoprecipitates are capable of direct phosphorylation of CD3-γ, -δ and -ε proteins as well as the ζ protein on tyrosine residues in vitro (Veillette et al., 1988c; Barber et al., 1989). A short stretch of amino acids found in the cytoplasmic domain of CD8α, but not CD8β, is responsible for binding the amino-terminal domain of p56^{lck} (Shaw et al., 1990). This sequence contains a common motif (Cys-X-Cys-Pro-X-X-X-Lys) that is shared by CD8α and CD4, another protein shown to be associated with p56^{lck} (Rudd et al., 1988; Shaw et al., 1989a; Veillette et al., 1989a). Mutation of either cysteine eliminates binding of both CD4 and CD8α to p56^{lck}. Furthermore, p56^{lck} contains two cysteine residues within its amino terminus that are essential for binding CD4 and CD8α (Shaw et al., 1989, 1990). Since the interaction between p56^{lck} and either CD4 or CD8α does not involve disulfide bond formation (Rudd et al., 1988; Shaw et al., 1990), it has been proposed that a metal ion could stabilize the complex.

The emerging experimental evidence supports the concept that the CD8-p56^{lck} complex is structurally distinct, but functionally similar to the class of surface receptors which possess intrinsic protein kinase activity. A model summarizing the current understanding
of the potential function of p56\textsuperscript{ck} in the signaling events of CD8\textsuperscript{+} T cell proposes that binding of ligand (whether MHC class I molecules on the surface of antigen presenting cells or divalent anti-CD8 antibodies) to CD8 proteins externally cross-links CD8 on the cell surface which results in the internal interaction of CD8-associated p56\textsuperscript{ck} molecules. This interaction of p56\textsuperscript{ck} proteins would result in the activation of their tyrosine protein kinase activity through mechanisms perhaps similar to those described for the insulin, epidermal growth factor, platelet-derived growth factor, and colony stimulating factor-1 receptors (Boni-Schnezler et al., 1986, 1988; Heldin et al., 1989; Scherr, 1990). The activation of the p56\textsuperscript{ck} tyrosine kinase presumably is then followed by the phosphorylation of cellular substrates, one of which is CD3-\textgreek{z}. The capacity of extracellular regions of CD8 and the TCR/CD3 complex to bind the same class I MHC molecule on antigen presenting cells (Connolly et al., 1990; Salter et al., 1990) could facilitate the intracellular physical interaction between p56\textsuperscript{ck} (associated with CD8) and CD3-\textgreek{z} (a component of the TCR/CD3 complex). Thus, CD8-p56\textsuperscript{ck} might be considered a transient TCR/CD3 component which could provide an
important accessory signal(s) that contributes to the signal(s) generated by the TCR/CD3 complex which, in turn, initiate events leading to T cell activation. Alternatively, CD8-p56^1ck-mediated phosphorylation of components of the TCR/CD3 complex could allow T cells to attenuate their responses to antigenic stimuli; therefore, interactions of CD8-p56^1ck with the TCR/CD3 complex may play a negative regulatory role akin to that seen for classical receptor desensitization. This is the case for the β-adrenergic receptor-coupled adenylate cyclase that is phosphorylated by a cAMP-independent protein kinase after its translocation from the cytosol to the plasma membrane resulting in inactivation of this signal transduction complex (Fishman and Perkins, 1988).

Physiologic activation of CD8^+ T cell by antigen presenting cells results in rapid serine-specific phosphorylation of p56^1ck via a mechanism distinct from conventional protein kinase C activation (Marth et al., 1989; Danielian et al., 1989). Levels of lck mRNA also decline rapidly after T cell activation. The pattern of disappearance of lck transcripts is the perfect reciprocal of the pattern of lymphokine mRNA expression; both require stimuli that fully activate T cells and both exhibit the same time course of response. These
finding are consistent with the view that this membrane-associated protein tyrosine kinase regulates some aspect of the lymphocyte activation sequence.

CD8 itself has also been shown to be rapidly phosphorylated on serine residues and then dephosphorylated upon activation of human T cell clones by specific antigen in the context of MHC class I or by phorbol esters (Acres et al., 1987; Blue et al., 1987; DiSanto et al., 1989). Mabs specific for class I MHC proteins could inhibit antigen-induced phosphorylation of CD8. Conflicting results have been reported for changes in CD8 surface expression following T cell activation and subsequent phosphorylation. Initial studies reported that phosphorylation of CD8 was not accompanied by modulation from the cell surface (Acres et al., 1987; Blue et al., 1987). Subsequently, partial modulation of CD8 from CTL clones was observed following activation by both specific antigen and phorbol esters (DiSanto et al., 1989). A study of genetically-engineered CD8α/class I MHC hybrid molecules revealed that the intracytoplasmic domain of CD8α controls the kinetics of phosphorylation-induced cell-surface modulation (Boyer et al., 1989). Further analysis revealed that CD8 α/β heterodimers, but not α/α
homodimers, are preferentially down-regulated following PKC activation by phorbol esters (Terry et al., 1990). Thus, these contradictory reports may, in part, be explained by the observation that heterogeneity in the expression of α/β heterodimers and α/α homodimers exists not only at the population level but also on the surface of individual cells (Terry et al., 1990). An alternative, though not mutually exclusive, explanation could be that different activation signals may be required for down-regulation of α/β heterodimers versus α/α homodimers; a concept supported by the observation that CD8 is modulated from the surface of thymocytes after activation by cross-linking of CD3 but not by phorbol esters or anti-CD2 Mab combinations (Blue et al., 1989).

The phenomenon of cell-surface modulation following cellular activation is well documented for certain T cell molecules such as the TCR, CD4 and CD3 proteins (Cantrell et al., 1985; Weyand et al., 1987). These cell-surface components play a central role in T cell activation for recognition of specific antigen, adhesion and signal transduction across the membrane. Thus, the function of these molecules, including CD8, may be regulated by their removal from the cell surface.
The function of CD8 has been more directly explored in a series of gene transfer studies into functional T cell hybridomas. Dembic et al. (1986) transferred the genes encoding the α and β chains of the TCR from a mouse CD8⁺ CTL clone, BDFL1.1.3, specific for Dd plus the hapten fluorescein (FL), into the CD8⁻ mouse cytotoxic T cell hybridoma SPH1.3, specific for Kk plus the hapten p-sulfophenylidiazole-4-hydroxyphenyl acetic acid (SP). While killing by the donor cell (BDFL1.1.3) could be blocked by anti-CD8α Mab, killing by the nontransfected recipient (SPH1.3) could not. After the TCR gene transfer they found that the transfectedant, BD7-S17, could not kill targets expressing normal amounts of Dd + FL, but could kill fluoresceinolated L cell targets which had been selected for overexpression of a transfected Dd gene. These results suggested that the affinity of the transferred TCR for Dd + FL was low, and that the absence of CD8 in the recipient cells might be the explanation for failure to kill target cells expressing normal levels of Dd + FL. The mouse CD8α gene was then transfected into the recipient hybridoma cells, resulting in cell surface expression of the encoded protein(s) (Dembic et al., 1987). The ability to kill targets expressing normal amounts of Dd + Fl was
indeed restored in BD7-S17-Ly2(2), a cloned CD8α+ transflectant. Furthermore, this killing could be blocked by Mab specific for mouse CD8α to the same extent as killing by the original donor cell. These findings demonstrate that the product(s) of the transferred CD8α gene can either increase the avidity of the interaction between a cytotoxic T cell hybridoma and its target, or in some way enhance the reactivity of the T cell hybrid so that killing can be accomplished with a lower concentration of cell surface antigen. Dembic et al. (1987) also observed a new response to Rk alone in the transflectants that had received the CD8α gene. It was not determined whether this specificity involves usage of the TCR from SPH1.3, which typically recognizes Rk plus hapten SP, or whether it involves a hybrid TCR using some combination of the various TCR α and β chains within the transflectant. In either case, though, the response to isolated Rk was dependent upon expression of CD8α. These data illustrate the ability of CD8α not only to decrease the required density of antigen for T cell activation, but also to facilitate responses to antigens that cannot trigger the specific T cell in the absence of CD8α.

Similar findings have been obtained for enhancement
of T cell responses by gene transfer of human CD8α. Ratnofsky et al. (1987) studied a mouse T cell hybridoma, BY155.16, which produces IL 2 in response to human class II HLA-DR antigens. The human CD8α cDNA was transferred into this cell line by use of a retroviral expression vector, resulting in cell surface expression of human CD8α. All three resulting CD8α⁺ hybridomas lines responded to human JY (HLA-DR⁺) stimulator cells with enhanced IL 2 production (about 10-fold), and IL 2 secretion could be detected with 10-fold fewer stimulator cells. This enhanced response could be blocked by Mabs specific for human CD8α or for the class I MHC molecules expressed by JY stimulator cells. Furthermore, the response to Daudi cells, which express human HLA-DR but lack cell surface class I expression, was increased no more that 2-fold in hybridomas expressing CD8α, as compared to 10-fold when stimulators expressing class I proteins (i.e., JY) were used. Therefore, expression of class I MHC molecules by the stimulators is important for the enhancement effect mediated by CD8.

These conclusions have been extended to a mouse helper hybridoma system by Gabert et al. (1987). In these studies the α and β chain genes of the TCR from a
CTL clone (KB5-C20) specific for the allogeneic class I MHC molecule K\textsuperscript{b} were transfected into a CD4\textsuperscript{+} T cell hybridoma (DO-11.10) specific for Ia\textsuperscript{d} plus ovalbumin. As in the experiments described above, the donor CTL clone, KB5-C20, was CD8\textsuperscript{+} and its cytotoxic function was blocked by Mab specific for mouse CD8\textalpha, while the recipient hybridoma, DO-11.10, was CD8\textsuperscript{−}, and its normal function (as measured by IL 2 release) was not blocked by anti-mouse CD8 Mab. Transfer of the KB5-C20 TCR genes alone did not result in the transfer of the ability to respond to the antigen K\textsuperscript{b} as presented by either spleen cells or L cells expressing a transfected K\textsuperscript{b} gene. However, the transfected TCR genes were expressed in a functionally relevant manner in that an anti-idiotypic Mab, Desire-1, specific for the transfected TCR could stimulate release of IL 2 by the transfectants when bound to Sepharose beads. In contrast, when the CD8\textalpha gene was transfected into the recipient cells in addition to the TCR genes, the resulting cloned transfectants could respond to K\textsuperscript{b} presented either on spleen cells or on transfected L cells by releasing IL 2. Furthermore, this response could be blocked by Mab specific for CD8\textalpha, but not by Mabs specific for either Thy-1 or CD4. These results
extend the previous study by showing that the functional requirement for CD8α is independent of the particular type of T cell, but rather dependent on the specific TCR (and the antigen being recognized by this TCR). In this instance the KB5-C20 TCR could not functionally respond to antigen (Kb) in the absence of CD8α or when CD8α was blocked by Mab, independent of whether it was in a CTL or a helper hybridoma. The most likely explanation is that the affinity of this TCR is too low for it to be stimulated by its specific antigen in the absence of an interaction between CD8α and its ligand (class I MHC).

To determine which structural features of the CD8 molecules contribute to its putative trans-acting (binding) and cis-acting (signaling) functions, the mouse DO-11.10 T cell hybridoma expressing the KB5-C20 TCR (specific for the class I MHC product H-2Kb) was transfected with a set of CD8 α chain (Ly-2) and/or β chain (Ly-3) genes encoding peptides with carboxy-terminal truncations or substitutions (Letourneur et al., 1990). When challenged with Kb-positive splenocytes, transfectants expressing genetically-engineered CD8α homodimers that lacked cytoplasmic tails (thus resembling naturally-occurring CD8α') responded nearly as effectively as wild-type CD8α.
transfectants. However, in marked contrast to the wild-type CD8α transfectants, tailless CD8α transfectants were greatly impaired in their ability to respond to Kb-transfected L cells. Coexpression of the CD8β gene did not restore this impaired response. The unique functional property of the CD8α cytoplasmic segment was further supported by analysis of a chimeric CD8β subunit in which the cytoplasmic segment was replaced by the one from the CD8α subunit. When associated with a soluble CD8α subunit lacking a transmembrane segment, the chimeric CD8β/α polypeptide was indeed sufficient to restore the response to Kb-transfected L cells. Since the lateral mobility of the tailless CD8α molecules on the cell surface was nearly identical to that of the wild-type CD8α molecules, their partially impaired function may indicate that they have lost their cis-acting signaling properties (possibly through loss of association with p56^lk) but retained their ability to bind class I MHC gene products in trans.

Notably the findings in all four sets of experiments indicate a positive function for CD8α; expression of the gene either increases the magnitude of measured T cell responses, or results in new responses
that do not occur at measurable levels in its absence. Although one cannot conclude that a positive signal is being transmitted through the CD8α molecule, one can conclude that in these systems the function is clearly not to transmit a negative signal and turn off a response. In light of the fact that CD8α can associate with the same class I MHC molecule as the TCR/CD3 complex (Connolly et al., 1990; Salter et al., 1990) and can associate with p56^lck, a member of the src family of tyrosine kinases that have been implicated as positive regulators of cell growth (Cartwright et al., 1986; Kmicik and Shalloway, 1987; Piwnica-Worms et al., 1987; Reynolds et al., 1987; Amrein and Sefton, 1988; Marth et al., 1988; Ziegler et al., 1989), it seems possible that CD8 may provide a transient positive accessory signal to the TCR/CD3 complex which, if coordinated with the appropriate antigen-specific signals mediated by the TCR, could result in signal transduction culminating in transcriptional activation of T cell genes and other events that govern the mature phenotypic response of those cells.

Signal transduction by CD8 could be regulated by association of CD8 (and bound p56^lck) with other molecules such as CD1 and MHC class I proteins which, in
turn, might regulate p56\textsuperscript{lck} activity. Alternatively, different forms of CD8 (homodimers versus heterodimers; $\alpha$ versus $\alpha'$) may play a role in modulating signal transduction through CD8. Since co-expression of both CD8$\alpha$ and CD8$\beta$ subunits in one set of transfectants did not result in a dramatic change in responsiveness (Letourneur et al., 1990), the exact role of the $\beta$ chain in T cell activation, in particular the influence of CD8$\beta$'s cytoplasmic domain on the kinase activity of p56\textsuperscript{lck} or the association of the cytoplasmic domain of CD8$\alpha$ with p56\textsuperscript{lck}, is still uncertain. However, it seems plausible that regulation of the type of CD8 complexes present on a particular T cell may play an important role in determining the outcome of CD8-mediated signal transduction and thus suggests a mechanism for the generation of distinct signals through CD8 that may be utilized at different stages of T cell differentiation.
II. Use of an Epstein-Barr Virus Episomal Replicon for Antisense RNA-mediated Gene Inhibition in a Human Cytotoxic T Cell Clone

A. Summary

A methodology was developed for stable gene transfer into cloned nontransformed human T lymphocytes. Stable high-level gene expression was achieved in cloned human T cells by using a self-replicating Epstein-Barr virus (EBV) episome. A comparison of five eukaryotic promoters established that the Rous sarcoma virus 3' long terminal repeat (RSV 3' LTR) and the lymphopapilloma virus (LPV 5' LTR) are optimal for episome-based expression in T cells. Effective (>95%), selective, and reversible antisense RNA-mediated gene inhibition of a model T cell-associated molecule (CD8) was achieved in a cytotoxic human T cell clone by using an EBV episome-based, RSV 3' LTR-driven expression system. The linking of antisense RNA mutagenesis and T cell cloning technologies should contribute significantly to studies of human T cell function.
B. Introduction

Gene transfection offers a powerful experimental approach for defining the functional role of specific molecules in T lymphocytes. Stable gene transfer has been accomplished for T cell tumor lines (Gunter et al., 1986; Siebenlist et al., 1986; Fujita et al., 1986; Toneguzzo et al., 1986), T cell hybridomas (Dembic et al., 1986; Dembic et al., 1987), cord blood lymphocytes (Stevenson et al., 1986), and cloned murine T lymphocytes (Kondo et al., 1986; Young et al., 1986; Uchida et al., 1986) but has not been described for cloned human T lymphocytes. Unlike T cell tumor lines and hybridomas, T cell clones possess normal karyotypes; are regulated in their proliferation by antigen, lymphokine secretion, and lymphokine receptors; and readily mediate specific and nonspecific cytotoxicity (Garrison et al., 1983). Hence, the availability of transfection mutants of cloned, human T lymphocyte lines would contribute significantly to molecular studies of human T cell function.

Plus-sense transfection analysis has been applied to the study of a variety of T cell-associated molecules such as the T cell antigen receptor (Dembic et al., 1986), the interleukin 2 (IL 2) receptor (Kondo et al.,
1986), Thy-1.2 (Gunter et al., 1986), CD8 (Dembic et al., 1987), CD4 (Maddon et al., 1986), CD7 (Aruffo and Seed, 1987), and interferon-γ (Young et al., 1986). However, the antisense RNA mutational approach (Green et al., 1986) has not been used in studies of T lymphocytes. Antisense RNA technology, by enabling selective gene inhibition, provides an alternative transfection strategy in which deletional mutants can be used to determine whether specific molecules play obligatory roles in defined cellular functions.

Our goal in the present study was to develop an efficient system for generating antisense RNA mutants of cloned human T lymphocytes. To this end, we have explored the utility of a high-copy number Epstein-Barr virus (EBV) episomal replicon (Yates et al., 1985) to serve as a vector for high level stable expression of sense and antisense RNA transcripts. Episomal replicons are circular DNA elements designed to self-propagate extrachromosomally in eukaryotic cells. As expression vectors, episomal replicons offer a means for amplifying gene copy number in cells and, furthermore, circumvent complications that arise from chromosomal integration—e.g., position effects on levels of transcription and insertional mutagenesis of host cell sequences at the
integration site. The EBV origin of replication (oriP) and the EBV nuclear antigen 1 together confer an episomal replication capacity to circular DNA elements in an array of human cell types, including lymphoid cells (Yates et al., 1985). Here we describe a methodology for stable gene transfer into cloned nontransformed human T lymphocytes by using an EBV episomal replicon, and we identify promoters suitable for episome-based expression in these cells. In addition, we establish the utility of episomal vectors for antisense RNA-mediated gene inhibition of a model surface glycoprotein, CD8, on a human cytotoxic T cell clone.
C. Materials and Methods

Reagents. Complete medium consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), L-glutamine (2 mM), Hepes (10 mM), penicillin (5 units/ml), and streptomycin (5 μg/ml). Recombinant DNA enzymes were obtained from New England Biolabs, Boehringer Mannheim, and Pharmacia. Other reagents were obtained from the indicated sources: chloramphenicol and lysozyme (Sigma); polyethylene glycol 1540 (Koch Light); hygromycin B and A23187 (Calbiochem; A23187 stock solution: 0.7 mM in ethanol); \(^{14}\text{C}\)-chloramphenicol (New England Nuclear); acetyl CoA lithium salt (Pharmacia).

Cells. Two independently derived nontransformed (IL 2 dependent) human T cell clones, V1 (CD4\(^+\)CD8\(^-\)) and 8L2 (CD4\(^-\)CD8\(^+\)), were used for these studies. The derivation of the influenza hemagglutinin-specific HLA-DR5-restricted cytotoxic cell line V1 has been described (Kaplan et al., 1984a; Kaplan et al., 1984b). The 8L2 line was cloned by micromanipulation of single cells (Autoclone, EPICS V, Coulter) from mixed lymphocyte cultures in 96-well microtiter plates containing 200 μl of complete medium per well. To each well we added 20,000 γ-irradiated (5000 rads; 1 rad =
0.01 Gy) allogeneic human peripheral blood mononuclear cells (PBMC) and 10% MLA-144 culture supernatant (CS) as a source of IL 2. The growth cycle of both of these clones is regulated and characterized by transient expression of IL 2 receptors (Kaplan et al., 1984a). Clones were stimulated weekly with OKT3 (anti-CD3) monoclonal antibody (Mab; 1 ng/ml; Ortho Diagnostics), IL 2 (10% MLA-144 CS), and irradiated allogeneic human PBMC (0.75 x 10^6 cells per ml). After 3 days of stimulation, clones were washed and subcultured in fresh complete medium supplemented with IL 2 (10% MLA-144 CS) for 4 days prior to restimulation (Figure 1).

**Plasmid Constructions.** To assemble promoter-chloramphenicol acetyltransferase (CAT)/220.2 plasmids (Figure 2a), we obtained promoter-CAT plasmids from several investigators: SV2CAT (simian virus 40 early promoter; B. Howard; Gorman et al., 1982a); RSVCAT [Rous sarcoma virus 3' long terminal repeat (RSV 3' LTR); B. Howard; Gorman et al., 1982b]; LPV-5'-CAT [lymphopapilloma virus (LPV) 5' LTR; R. Miksicek; Mosthaf et al., 1985]; II0CAT (rat GR78 gene calcium ionophore-inducible promoter; A. Lee; Resendez et al., 1985); HS1CAT (phMT-IIА-CAT; human heavy metal-inducible
Figure 1. **Stable gene transfer into human T cell clones.**

A 7-day cycling scheme was used to derive and maintain cloned human T cell transfectants. Cells were washed and stimulated at the start of the cycle (day 0) with anti-CD3 (OKT3) Mab, IL 2 (10% MLA CS), and irradiated PBMC, and washed and restimulated (with exogenous IL 2 only) on day 3. Hygromycin B (0.15 mg/ml) was added to the culture medium on day 2 and again on day 3 (after the wash).
HYGROMYCIN B

DAY: 0 1 2 3 4 5 6 7 or 0

↑ anti-CD3

IL 2

↓ PBMC

↑ IL 2
metallothionein II<sub>A</sub> promoter region encompassing ~850 base pairs of sequence upstream of the hMTII<sub>A</sub> gene; M. Karin and A. Haslinger; Scholer et al., 1986); and SVOCAT (no eukaryotic promoter; B. Howard; Gorman et al., 1982a). p220.2 was generously provided to us by B. Sugden (Madison, WI). This 8.9-kilobase plasmid is a derivative of p201 (Yates et al., 1985) in which a polylinker (Bam HI, Xba I, Sal I, Pst I, Hind III) has been inserted at the Nar I site within the herpes simplex virus 1 thymidine kinase gene termination sequence (B. Sugden, personal communication; see Figure 2a). For construction of promoter-CAT/220.2 plasmids, a second Bam HI site was first introduced at unique restriction sites upstream of the promoter in promoter-CAT plasmids using Bam HI linkers (New England Biolabs), and promoter-CAT cartridges were then mobilized by Bam HI digestion and inserted in both orientations into the unique Bam HI site of p220.2 (Hauer et al., 1989).

pT8F1 (generously provided to us by R. Axel; Littman et al., 1985), a cDNA plasmid encompassing the complete coding sequence of human CD8, was the source for the CD8 DNA segment in the antisense CD8 construct α-CD8/REP1 (see Figure 3 and Results). RSVPAl, a
plasmid in which the RSV 3' LTR (derived from pRSVCAT), Eco RV and Bam HI subcloning sites, and the simian virus 40 late polyadenylation/termination sequence (derived from pcdv1, Okayama and Berg, 1983) are sequentially arrayed, was assembled by us in a multistep procedure (Groger et al., 1989).

Transfection Procedures. Electroporation (used here for promoter-CAT/220.2 episomes) was performed with a Promega Biotech X-Cell 450 electroporator at 200 V, 1100 μF, 0.7 sec time load, in phosphate-buffered saline (PBS) containing 20 mM Hepes (pH 7.2), 500 μg of sheared salmon sperm DNA as carrier, and 20 μg of plasmid DNA. The protocol for protoplast fusion (used here for the α-CD8/REP1 episome) has been described (Sandri-Goldin et al., 1983), and we have introduced modifications for suspension cells. Protoplasts were copelleted with $10^7$ prewashed (3 X PBS), logarithmically growing target cells (5000:1, protoplast/target cell ratio) at 1700 x g in a Du Pont-Sorvall HS-4 centrifuge for 20 min at 4°C. Fifty percent polyethylene glycol 1540 in PBS (1 ml) was added dropwise over 1 min, and cells were then immediately diluted with PBS (1 ml) over 1 min and more PBS (20 ml) over 3 min, pelleted, washed twice in PBS, and resuspended in 6-well plates (10^6 cells per ml) in
complete medium supplemented with gentamicin (200 µg/ml) and IL 2. Stable transfectant lines corresponding to individual wells were derived by the protocol described in Results, and transfectants were not recloned for these studies.

**CAT Enzymatic Assay.** Cells (5 x 10^6) were harvested, washed three times in PBS, resuspended in 25 mM Tris (pH 7.8) (100 µl) and lysed by five cycles of freeze-thawing. Crude cellular extracts (20 µl per reaction mixture) were assayed for chloramphenicol acetylating activity by a standard 1h assay as described (Gorman et al., 1982a). Enzymatic mixtures were extracted with ethyl acetate, and unacetylated and acetylated (1-acetate, 3-acetate, 1,3-diacetate) forms of [14C]chloramphenicol were separated by ascending chloroform/methanol (95:5) thin-layer chromatography on silica gel plates (20 x 20 cm; Whatman). Spots were visualized by overnight autoradiography (Kodak XAR film; -70ºC with intensifying screen) and quantitated by excision of spots and liquid scintillation counting.

**Flow Cytometry.** Cells were immunostained as described (Kaplan et al., 1984a) using OKT8 (anti-CD8), OKT3 (anti-CD3), OKT11 (anti-CD2; Ortho Diagnostics), or normal mouse IgG (Miles) as primary antibodies, and
fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody. Cells were analyzed on an EPICS V fluorescence-activated cell sorter.
D. Results

For these studies, we used the episomal replicon p220.2 (Yates et al., 1985), which contains the EBV sequences required for episomal replication in human cells, as well as the E. coli hph gene, which confers resistance to the eukaryocidal antibiotic hygromycin B. Since the human T cell clones require feeder cells (PBMC) which are hygromycin B sensitive, we developed a modified selection protocol to permit the derivation of stable hygromycin B-resistant (hygR) transfectants. After transfection by electroporation or protoplast fusion, cells were resuspended in complete medium supplemented with IL 2. Cells were stimulated with IL 2, anti-CD3 antibody, and irradiated PBMC 2 days posttransfection, and hygromycin B was added 2 days later. A 7-day cycling scheme (Figure 1) was then initiated in which hygromycin B was present in the culture medium only on days 2-7 after stimulation with fresh PBMC, IL 2, and anti-CD3 antibody. After 5 days of incubation with hygromycin B (0.15 mg/ml), control nontransfected cell cultures contained virtually no viable cells. Stable hygR transfectants have been reproducibly obtained (~50% of transfections) for several cloned T cell lines by this protocol.
A systematic investigation of promoter function in EBV episomal replicons has not been previously reported. To address this issue, as well as to specifically explore the utility of EBV-based episomal replicons for the expression of transfected genes in human T cells, we assessed episomal replicon-based promoter function in our cloned T cell line V1. A panel of eukaryotic promoters consisting of various constitutive (simian virus 40 early, RSV 3' LTR, LPV 5' LTR) and inducible (GRP78 gene, hMTIIₐ gene) promoters were examined. For these analyses, the prokaryotic CAT gene, which is absent in eukaryotic cells, served as a reporter gene for promoter-driven transcriptional activity (Gorman et al., 1982b; Ciliberto et al., 1985). Promoter-CAT cartridges were inserted, in both orientations, into the EBV-based replicon 220.2 at the unique Bam HI site, which lies just downstream of the EBV oriP (Figure 2a; see Materials and Methods). The alternative orientations for cartridges in promoter-CAT/220.2 plasmids have been arbitrarily designated α (promoter is proximal to EBV oriP) and β (promoter is distal to EBV oriP).

Stable hyg² V1 transfectedants for each of the promoter-CAT/220.2 plasmids were independently derived,
Figure 2. Stable CAT expression in a human T cell clone using different episome based eukaryotic promoter elements.

(a) Schematic representation of promoter-CAT/220.2 episomes, showing the EBV oriP, a functional segment of the EBV nuclear antigen 1 gene (EBNA-1), the Escherichia coli hph gene, herpes simplex virus thymidine kinase 1 (HSV 1 TK) promoter and termination sequences (solid bars), pUC12-derived multiple cloning site sequence (horizontal stripe), pBR322-derived sequences (narrow band), pBR322 origin of replication (pBRori), ampicillin-resistance gene (ampR), and α- and β-oriented promoter-CAT cartridges. Arrows indicate direction of transcription. B, Bam H1; H, Hind III.

(b and c) CAT activities for various promoter-CAT/220.2 V1 transflectants. TLC autoradiograms (b) and % acetylation (c) are shown. Numbers 1–16 in b correspond to promoter-CAT/220.2 constructs listed (top to bottom) in c, in that order. Numbers to rights of bars (c) indicate % acetylation [1-acetylated + 3-acetylated - 1.3-diacetylated cpm/total (unacetylated + acetylated) cpm].
and the CAT enzymatic activity in them was compared (Figure 2 b and c). The RSV 3' LTR-based episomes yielded maximal CAT activity, which, based on titration experiments (data not shown), was marginally higher than the strong activity seen with the LPV 5' LTR. In contrast, the episomes incorporating the simian virus 40 early promoter, a constitutive promoter commonly used in eukaryotic expression work (Okayama and Berg, 1983), were significantly less efficient in driving CAT expression. The inducible GRP78 (I10CAT/220.2) and hMTIIA (HS1CAT/220.2) gene promoters both displayed high levels of basal activity and showed 22% (7 μM A23187) and 93% (10 mM cadmium) induction for α-oriented promoters, respectively, and 112% and 308% induction for β-oriented promoters. However, the levels of CAT activity induced with these promoters were significantly below those for either of the LTR-based (RSV and LPV) constitutive promoters. The orientation dependence of promoter activity (α > β) seen with some promoters — e.g., the GRP78 gene promoter — may result from a transcriptional enhancer effect exerted by the EBV oriP (Reisman and Sugden, 1986). An episome analogous to RSVCATα/220.2, in which plus-sense CD8 instead of CAT is driven by the RSV 3' LTR, yielded high levels of surface
Figure 3. Assembly of α-CD8/REP1 (antisense CD8) episome.

Sources for pT8F1, P220.2, and RSVPAl are described in Materials and Methods. S, Sau 3A; RV, Eco RV; SI, Sal I. An x indicates that the restriction site has been eliminated. PA, simian virus 40 late polyadenylation/termination sequence; b.p., base pair.
pT8F1
(CD8 cDNA)

1. Sau3A + EcoRV
2. Small fragment
   (459 b.p.)
3. Ligate into RSVPA1

α-CD8/RSVPA1

1. Sal I
2. Small fragment
3. Ligate into p220.2

α-CD8/REP1

EBVori

EBNA-1
CD8 on transfectants (Tykocinski et al., 1988), establishing the utility of episome-based RSV 3' LTR-driven expression systems for genes encoding cell-surface proteins as well as for cytoplasmic proteins.

To determine whether EBV episomal replicons can be used effectively for stable antisense RNA-mediated gene inhibition in cloned T cells, we selected CD8 as a model T cell surface protein. To inhibit CD8 expression, we constructed the episome α-CD8/REP1 (Figure 3). A 459-base-pair CD8 coding segment, spanning amino acids 9-161 of the 214-amino-acid-long processed CD8 protein, was first inserted in an inverted orientation downstream of the RSV 3' LTR and upstream of the simian virus 40 polyadenylation specification sequence from the late region of the virus. This promoter/α-CD8/polyadenylation unit was subsequently cartridged in an α-orientation into the EBV episomal shuttle vector p220.2. α-CD8/REP1 was stably transfected into 8L2, a CD4^−CD8^+ T cell clone derived by nonspecific stimulation via anti-CD3 Mabs and irradiated allogeneic feeder cells. The antigenic specificity and major histocompatibility complex restriction of this clone are unknown.

The level of surface CD8 expression on the parental
and transfected 8L2 lines was determined 46 days posttransfection by flow cytometry using an anti-CD8 Mab (Figure 4). Whereas nontransfected 8L2 and RSVCATα/220.2-transfected 8L2 cells displayed equivalent levels of surface CD8, the α-CD8/REP1 8L2 transfectant demonstrated a marked decrease (>95%) in surface CD8 expression. The absence of alterations in the cell-surface expression of CD2 (sheep erythrocyte receptor complex) and CD3 (a component of the T cell αβ antigen receptor complex) in the α-CD8/REP1 transfectant (Figure 4) established the specificity of this inhibition. Neoexpression of CD4 and CD1, surface molecules that are not expressed on 8L2 cells, was not observed for the CD8-suppressed transfectant (data not shown).

To establish that the antisense RNA inhibition was indeed mediated by an EBV-based episome, we transferred an aliquot of α-CD8/REP1-transfected cells into hygromycin B-free medium 39 days posttransfection and followed the stability of the mutant phenotype by serial flow cytometric analysis (Figure 5). A gradual loss of EBV-based episomes is known to occur after removal of the selective agent (Yates et al., 1985). As expected, in the absence of hygromycin B, surface CD8 gradually
Figure 4. Selective antisense RNA-mediated inhibition of surface CD8 expression on 8L2 cells.

α-CD8/REP1 and RSVCATα/220.2 8L2 transfectants and parental 8L2 cells were stained on day 46 with OKT8 (anti-CD8), OKT3 (anti-CD3), and OKT11 (anti-CD2) (stippled areas) or normal mouse IgG (Miles; open areas) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody.
Figure 5. Reappearance of surface CD8 on the α-CD8/REPI hygromycin B-resistant 8L2 transfectant line after removal of hygromycin B.

Stable α-CD8/REPI (squares) and RSVCATα/220.2 (triangles) 8L2 transfectants were derived as described in the text. On day 39, a subculture of the α-CD8/REPI transfectant (open squares) was washed free of hygromycin B and then maintained in the absence of this selective agent. CD8 expression was assessed at the indicated time points by flow cytometric analysis. Relative expression was calculated by comparing the peak channel number (pcn) for each group with the pcn for nontransfected 8L2 (pcn range, 190-201) at each time point.
reemerged in a time-dependent fashion, suggesting a progressive loss of the α-CD8/REP1 episome. After 1 week in hygromycin B-free medium, no change in the level of CD8 expression was detectable; after 2 weeks, an increase in CD8 expression was observed; by 5 weeks, surface CD8 levels approached those of the control RSVCATα/220.2 8L2 transfectant. In contrast, when the transfectant was maintained in the continuous presence of hygromycin B, CD8 inhibition remained stable for the entire 3-month observation period. The finding that the CD8-suppressed phenotype is stable for 1 week out of hygromycin B means that this agent can be temporarily cleared from the cells prior to functional assays. Also, the incremental increases in CD8 expression that occur after removal of the selective agent represent an experimental means for exploiting episome-based expression systems for gene dosage analysis.

Functional studies were performed on the α-CD8/REP1 transfectant grown in the continuous presence of hygromycin B. Nonspecific cytotoxicity mediated by either anti-CD3 Mab or the lectin phytohemagglutinin (PHA) did not differ significantly between the CD8 antisense mutant and controls (Figure 6). Moreover, there were no significant differences in the expression
of IL 2 receptor (as detected by anti-Tac Mab) after anti-CD3 Mab stimulation, the proliferative response to either PHA or anti-CD3 Mab, or the response to phorbol diester as measured by increased IL 2 receptor expression (data not shown).
Figure 6. Anti-CD3- and PHA-mediated cytotoxicity of U937 targets by nontransfected 8L2 cells and stable 8L2 transfectants (RSVCAβ/220.2 and α-CD8/REP1).

Cytolysis of $^{51}$Cr-labeled U937 targets (5 x $10^3$ cells per well) was mediated by either PHA (0.1 μg/ml; open circles) or OKT3 (2 ng/ml; solid circles) at various effector:target cell ratios in a standard $^{51}$Cr-release assay (Kaplan et al., 1984b). Maximal release of $^{51}$Cr was achieved in 1% Triton X-100. Spontaneous release in these experiments was 16%. Specific lysis was calculated as described (Kaplan et al., 1984b). Stable transfectants were selected in hygromycin B for 62 days and washed free of the antibiotic immediately prior to their use in this assay.
E. Discussion

In this study, we have described a methodology for the derivation and maintenance of stably transfected cloned human T lymphocytes. This entails the use of a 7-day cycling scheme in which the eukaryocidal antibiotic serving as a selective agent is absent for the first 2 days after addition of irradiated antibiotic-sensitive feeder cells in each cycle. This protocol can be used in conjunction with electroporation or protoplast fusion as alternative transfection modalities and yields stable transfectants in a reproducible fashion.

Furthermore, we have shown that stable expression of transfected genes can be achieved in cloned human T cells by using an EBV-based episomal replicon. A comparison of five eukaryotic promoters demonstrated that the RSV 3' LTR and LPV 5' LTR are most suitable for high level EBV episome-based expression in T cells. Differences in CAT activity observed in this episomal expression system may reflect not only variation in promoter activity but also differences in episome copy numbers per cell, since it is conceivable that particular promoter sequence elements may influence EBV episomal replication capacity. EBV episomes have been
reported to range up to 90 copies per cell (Yates et al., 1985). Although both inducible promoters studied here (GRP78 and hMTII_A genes) were inducible when episome-based and expressed in T cells, the fact that they demonstrate significant basal activity in the absence of their respective inducers could limit their usefulness for expression studies.

Using CD8 as a model T cell-associated molecule, we have further demonstrated that episome-based, RSV 3' LTR-driven expression systems can be used for antisense RNA-mediated gene inhibition. This approach can now be extended to antisense RNA work in other human hematopoietic and nonhematopoietic cell types. The gene inhibition achieved here was effective (>95% inhibition), selective (vis a vis CD3 and CD2), and reversible (by removal of the selective agent). Our results further indicated that the transfection/selection scheme did not interfere with cellular functions such as proliferation. Notably, we found no effect of inhibition of CD8, a marker for class I major histocompatibility complex-restricted cytotoxic T cells, on nonspecific cytotoxicity in our system. The possibility that the small amount of residual surface CD8 (<5%) in the α-CD8/REP1 transfectant was exerting a
significant functional effect seems unlikely but cannot be definitively excluded. Since the antigenic specificity of this clone is unknown, studies of the effect of CD8 inhibition on antigen-mediated recognition await stable transfection of \( \alpha \)-CD8/REPI into a human T cell clone with known antigenic specificity and major histocompatibility complex restriction.

In summary, these results establish the feasibility of deriving stable functionally intact antisense mutants of nontransformed human T cell clones. Furthermore, this study demonstrates the utility of episomal expression vectors for achieving efficient antisense RNA-mediated gene inhibition in eukaryotic cells. An episome-based system not only offers an expeditious means for achieving amplification of transfected genes, but also permits the in vitro manipulation of transfected gene copy number by altering the selective pressure on the episomes. The selectivity of gene inhibition, with the possibility of confounding experimental artifacts minimized, is a distinct advantage of such an antisense RNA-mediated mutagenesis approach. In addition, successful transfection of human T cell clones allows for the initiation of studies of functions that are associated with nontransformed cells.
such as regulated growth and antigen-specific cytotoxicity. The linking of gene-directed mutagenesis and T cell cloning technologies should permit a more precise definition of the functional roles of a variety of specific molecules in human T lymphocytes.
III. Functional Consequences of Antisense RNA-mediated Inhibition of CD8 Surface Expression in a Human T Cell Clone.

A. Summary

An experimental approach for defining the function of CD8 has been developed by linking antisense RNA mutagenesis and T cell cloning technologies. We have transfected an antisense 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 2R 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.
B. Introduction

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 (Reinherz and Schlossman, 1980; Meuer et al., 1982; Swain, 1983). 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 (Swain, 1981; Landegren et al., 1982; Swain et al., 1984; Spits et al., 1985; Schimonkevitz et al., 1985; Goldstein and Mescher, 1987; Aparicio et al., 1987; Moldwin et al., 1987). 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 (Welte et al., 1983; Hunig, 1984; Fleischer et al., 1986; Van Seventer et al., 1986; Geppert and Lipsky, 1986), 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 (Dembic et al., 1986; Dembic et al., 1987; Gabert et al., 1987;), 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 antisense 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 antisense RNA expression system (Hamboh et al., 1988a). The antigen specificity of the T cell clones 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.
C. Materials and Methods

Lymphoblastoid Cell Lines (LCL). 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 (Kaplan et al., 1984a) 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 µl 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 x 10⁴ γ-irradiated (5,000 rad) autologous PBMC and 5,000 γ-irradiated (10⁴ rad) AR.LCL cells. The T cell clone JH.ARL.1 was stimulated weekly with irradiated AR.LCL cells (10⁵ cells/ml), irradiated autologous PBMC (7.5 x 10⁵ 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 (1ng/ml; Ortho Diagnostics Systems Inc., Westwood, MA), irradiated
allogeneic PBMC (7.5 x 10⁵ cells/ml), and fresh IL 2.
d3 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 RSVCATα/220.2 and the transfection/
selection procedures have been described previously
(Hambor et al., 1988a). Episomes were stably
transfected into JH.ARL.1 by electroporation, and stable
transfectants were selected in media containing
hygromycin B (150 μg/ml; Calbiochem-Behring Corp., La
Jolla, CA).

Flow Cytometry. Expression of cell surface
molecules was analyzed by indirect immunostaining as
described previously (Kaplan et al., 1984; Kaplan et
al., 1984b). 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
Diagnostics 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 4h $^{51}$Cr-release assay, as described previously (Kaplan et al., 1984a), 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 μg/ml) in quadruplicate round-bottom wells of a 96-well microtiter plate (200 μl/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 (Mentzer et al., 1987). $5 \times 10^5$ 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 each) were added to conical centrifuge tubes, centrifuged, and incubated at room temperature for 30 min. The cells were centrifuged again, then gently resuspended using a constant shear force (five cycles of a 50-μl 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 fluorescence cell bound to one or more nonfluorescent cells.

**Proliferation Assay.** A standard $[^3H]$thymidine incorporation assay (Kaplan et al., 1984a; Kaplan et al., 1984b) 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 µg/ml) in quadruplicate wells of a 96-well microtiter plate (100 µl/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 (Kaplan et al., 1988). 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 2R Expression. IL 2R expression was induced by incubating JH.ARL.1 cells (2 x 10^5 cells/200 μl/well; 7 d post-anti-CD3 stimulation) with irradiated LCL cells in 96-well plates. LCL cells (2 x 10^5 cells/200 μl/well) were fixed before addition of JH.ARL.1 cells by centrifuging the cells, removing the supernatants, and air-drying for 30 min at room temperature. After incubation at 37°C and 7% CO₂ for 20h, IL 2R expression was assessed by immunofluorescence staining using a murine anti-IL 2R IgG Mab (anti-CD25; Becton Dickinson & Co.) and flow cytometry.
D. Results

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 B-resistant transfectants of JH.ARL.1 were derived for the episomes α-CD8/REP1 (with a 459-bp segment of CD8 cartridge in an antisense orientation downstream of the Rous sarcoma virus 3' long terminal repeat [RSV 3' LTR]; Hambor et al., 1988a) and RSVCATα/220.2 (with the prokaryotic chloramphenicol acetyltransferase gene driven by the same promoter).

The α-CD8/REP1 transfectant, the RSVCATα/220.2 transfectant, and nontransfected JH.ARL.1 cells were analyzed for expression of T cell surface molecules by immunofluorescence staining and flow cytometry (Figure 7). Expression of CD8 by the α-CD8/REP1 transfectant was markedly inhibited (>97%) in comparison with nontransfected JH.ARL.1 cells and the RSVCATα/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 (Hambor et al., 1988a), and they demonstrate that
Figure 7. Selective inhibition of CD8 expression on JH.ARL.1.

Flow cytometric analysis of CD8, CD2, CD3 and TCR surface expression on transfected and nontransfected JH.ARL.1 cells. The α-CD8/REP1 transfectant, the RSVCATu/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 IgG (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 α-CD8/REP1-transfected cells, the anti-CD8 peak was less than two times more intense than the control peak.
efficient and selective inhibition of CD8 expression can be obtained in T cell clones using an EBV episome-based expression system.

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 (Figure 8). 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 RSVCATα/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
Figure 8. Requirement for CD8 in Alloantigen-specific cytotoxicity by JH.ARL.1.

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 4h $^{51}$Cr-release assay. Effector cells (nontransfected JH.ARL.1, solid circles; the RSVCATα/220.2 transfectant, solid squares; the α-CD8/REP1 transfectant, solid triangles) were used at various E/T ratios. PHA was included in assays with K562 targets.
lectin-coated targets (PHA-coated K562). Thus, antisense 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 (Spits et al., 1986; Shaw et al., 1986) in T cell-mediated target cell lysis. It seems likely that the decreased expression of CD8 inhibits antigen-specific recognition of a class I MHC product.

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
TABLE I
Proliferative Response of JH.ARL.1

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IL 2</th>
<th>Nontransfected</th>
<th>RSVCA/T220.2</th>
<th>α-CD8/REP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JH.ARL.1</td>
<td>JH.ARL.1</td>
<td>JH.ARL.1</td>
</tr>
<tr>
<td>---</td>
<td>-</td>
<td>83</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>AR.LCL</td>
<td>-</td>
<td>25,674</td>
<td>26,564</td>
<td>3,591</td>
</tr>
<tr>
<td>DK.LCL</td>
<td>-</td>
<td>168</td>
<td>223</td>
<td>197</td>
</tr>
<tr>
<td>PBMC</td>
<td>-</td>
<td>149</td>
<td>134</td>
<td>189</td>
</tr>
<tr>
<td>PBMC + PHA</td>
<td>-</td>
<td>36,121</td>
<td>32,008</td>
<td>39,202</td>
</tr>
<tr>
<td>PBMC + anti-CD3</td>
<td>-</td>
<td>88,769</td>
<td>78,062</td>
<td>89,997</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3,576</td>
<td>2,067</td>
<td>2,696</td>
</tr>
<tr>
<td>AR.LCL</td>
<td>+</td>
<td>36,574</td>
<td>35,667</td>
<td>4,532</td>
</tr>
<tr>
<td>DK.LCL</td>
<td>+</td>
<td>2,636</td>
<td>2,587</td>
<td>2,732</td>
</tr>
<tr>
<td>PBMC</td>
<td>+</td>
<td>1,827</td>
<td>1,966</td>
<td>1,889</td>
</tr>
<tr>
<td>PBMC + PHA</td>
<td>+</td>
<td>65,727</td>
<td>59,821</td>
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<tr>
<td>PBMC + anti-CD3</td>
<td>+</td>
<td>96,287</td>
<td>87,319</td>
<td>110,633</td>
</tr>
</tbody>
</table>

JH.ARL.1 cells (11 days post anti-CD3 stimulation) were co-cultured with irradiated LCL cells or with irradiated PBMC and PHA or irradiated PBMC and anti-CD3. Cultures were incubated in the absence (-) or presence (+) of exogenous IL 2 (10% MLA-144 CS) for 3 days. ³H-thymidine was added during the last 18 hr of the incubation period. Standard deviations of means greater than 1,000 dpm were less than 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.
and PHA was assessed using a \(^{3}H\)thymidine incorporation assay (Table I). Whereas nontransfected JH.ARL.1 cells and the RVSCAT\(\alpha/220.2\) transfectant proliferated in response to stimulation by AR.LCL cells (HLA-B35\(^{+}\)), the proliferative response of the \(\alpha\)-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 \(\alpha\)-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 2R expression, we measured IL 2 secretion (Table II) and IL 2R expression (data not shown) after alloantigenic stimulation of JH.ARL.1. Nontransfected JH.ARL.1 cells and the RVSCAT\(\alpha/220.2\) transfectant secreted IL 2 and expressed IL 2R when stimulated with AR.LCL cells.
(HLA-B35$^+$), whereas profound decreases in both IL 2 secretion and IL 2R expression were observed for the α-CD8/REP1 transfectant. These results indicate the dependence of antigen-stimulated responses, including secretion of the lymphokine IL 2 and acquisition of IL 2R, on the expression of the surface molecule CD8.
### TABLE II

**IL 2 Production by JH.ARL.1**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>JH.ARL.1 Nontransfected</th>
<th>JH.ARL.1 RSVCATα/220.2</th>
<th>JH.ARL.1 α-CD8/REP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>197</td>
<td>230</td>
<td>182</td>
</tr>
<tr>
<td>AR.LCL</td>
<td>8,327</td>
<td>7,815</td>
<td>976</td>
</tr>
<tr>
<td>DK.LCL</td>
<td>213</td>
<td>182</td>
<td>256</td>
</tr>
</tbody>
</table>

JH.ARL.1 cells (11 days post anti-CD3 stimulation) were co-cultured with irradiated LCL cells. Supernatants were harvested after incubation for 18 hr and assayed for IL 2 activity on an IL 2-dependent indicator cell line. Standard deviations of means greater than 1,000 dpm were less than 10% of the means and have been omitted. Background level of thymidine incorporation was 210 dpm, and optimal response at 2.5 U/ml of recombinant IL 2 was 8,986 dpm. AR.LCL is the specific target for JH.ARL.1, and DK.LCL is an unrelated target.
E. Discussion

We have used an antisense 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 stimulation, 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 (Norment and Littman, 1988; Rosenstein et al., 1988) in a fashion analogous to the direct binding of CD4 to class II MHC products (Doyle and Strominger, 1987). 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 (Emmrich et al., 1986; Takada and Engleman, 1987). 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 by CD8+ clones have suggested that a negative signal transduction event occurs by perturbation of surface CD8 or that anti-CD8 inhibits positive signal transduction (Welte et al., 1983; Hunig, 1984; Fleischer et al., 1986; Van Seventer et al., 1986; Geppert and Lipsky, 1986). 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 T cell clones, 8L2 (Hambor et al., 1988a) 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 (Landegren et al., 1982; Mentzer et al., 1987; Blanchard et al., 1987; Spits et al., 1986; Platsoucas, 1984). 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 (Springer et al., 1987; Marlin and Springer, 1987).

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 antisense 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 antisense 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.
IV. An Immunoregulatory Function for the CD8 Molecule.

A. Summary

The molecular details of immunoregulatory phenomena associated with CD8+ T lymphocytes have not been clearly elucidated. We tested the hypothesis that the cell surface glycoprotein CD8 is itself essential in mediating the inhibitory effects associated with CD8+ T cells. For this purpose, we utilized a T cell clonal pair, consisting of a human CD8+ T cell clone and a specific CD8- phenocopy of this clone obtained via antisense RNA mutagenesis, to modulate allogeneic responses in vitro. Our findings indicate that the expression of the CD8 molecule by the inhibitory cells is essential for down-regulation of both allogeneic proliferation and generation of cytotoxicity in mixed lymphocyte cultures. These results define an immunomodulatory function for the CD8 molecule and provide insights into the molecular basis of immunosuppression.
B. Introduction

CD8+ T lymphocytes have been implicated in an array of immunoregulatory phenomena such as antigen-specific immunosuppression (Green et al., 1983) and veto cell-induced tolerance (Fink et al., 1988); however, the mechanisms of down-regulation at a molecular level have not been described. The cell surface glycoprotein CD8 functions as an essential accessory molecule in class I major histocompatibility complex (MHC)-restricted antigenic recognition (Meuer et al., 1982; Hambor et al., 1988a; Hambor et al., 1988b). Since immune cells capable of diverse types of cellular inhibition share CD8 positivity as a common feature, we considered the possibility that CD8 might in fact have a second function in mediating immunoregulation. Specifically we conjectured that the CD8 molecule, which is known to physically associate with class I MHC molecules (Norment et al., 1988; Rosenstein et al., 1989), may itself serve as a cell surface-associated inhibitory ligand and thereby constitute an essential part of the immunoregulatory pathway.

In previous reports we describe the linking of antisense RNA mutagenesis and human T cell cloning technologies (Hambor et al., 1988a; Hambor et al.,
1988b), which has permitted us to derive T cell clonal phenocopies for null mutations corresponding to selected T cell-associated molecules. JH.ARL.1 is a CD8+ human T cell clone with allogeneic specificity for HLA-B35. To study the accessory function of CD8, we previously derived an antisense CD8 (α-CD8/REP1) transfectant of this line that is markedly and selectively inhibited in its expression of the CD8 molecule (Hambor et al., 1988a; Hambor et al., 1988b). Since CD8+ cells have also been associated with the capacity to inhibit immune responses in a variety of contexts (Green et al., 1983; Fink et al., 1988), we have used JH.ARL.1 and its CD8- variant to determine whether the CD8 surface molecule itself functions in an immunoregulatory capacity.
C. Materials and Methods

Human T Cell Clones and Transfectants.

JH.ARL.1 and its transfectants have been derived and maintained as described (Hambor et al., 1988a; Hambor et al., 1988b).

Proliferation Assays. Responder ($10^5$) and stimulator ($5 \times 10^4$) peripheral blood mononuclear cells (PBMC) or Epstein-Barr virus (EBV)-transformed B cells (lymphoblastoid cell line, LCL) were added to quadruplicate wells of a 96-well flat-bottom microtiter plate in RPMI 1640 medium with 10% fetal bovine serum. For allogeneic responses stimulator PBMC received 5000 rads (50 Gy), and for EBV-specific responses stimulator LCL received 15,000 rads prior to culture. Inhibitory cloned T cells ($10^4$ per well) received 5000 rads prior to addition to the culture. After incubation at $37^\circ$C for various periods of time, 1 μCi (37 kBq) of [methyl-$^3$H]thymidine was added to each well and the cultures were incubated for an additional 18 hr. The cells were harvested, and the radioactivity incorporated (dpm) was measured as described (Kaplan et al. 1984a).

Cytotoxicity. Allogeneic cultures were established in 24-well plates in a volume of 2 ml of RPMI 1640 containing 10% fetal bovine serum. One
million responder PBMC from donor MW [MW(PBMC)], 5 x 10^5 irradiated stimulator PBMC from donor JH [JH(PBMC)], and 10^5 irradiated cloned JH.ARL.1 cells were added per well. After incubation for 6 days at 37°C, cells were harvested, and dead cells were removed by Histopaque density gradient centrifugation. A ^{51}Cr-release assay was performed as described (Kaplan et al. 1984a) with JH(LCL) as targets. Spontaneous release was 30%.

**HLA Haplotypes.** The HLA haplotype of JH(PBMC), JH(LCL), and JH.ARL.1 and its transfectants is A2,3; B7,44; DR2,4. The HLA haplotype of DK(PBMC) is A2,24; B13,50; DR2,7. The HLA haplotype of MW(PBMC) is A1,31; B8; DR3.
D. Results

We established a model T cell immunoregulatory system for monitoring cellular inhibitory effects. This system involves the use of specific cloned T cells to inhibit mixed lymphocyte reactions (MLRs) between PBMC from healthy volunteer donors. For these studies the human CD8⁺ clone JH.ARL.1, its CD8⁻ phenocopy, or a control transfectant was added to MLR cultures, and their effects on allogeneic proliferation were assessed. The CD8⁻ variant of JH.ARL.1 had been derived by stable transfection with the antisense CD8 episomal replicon α-CD8/REP1; a control transfectant of JH.ARL.1 was derived with an analogous episomal replicon, RSVCATα/220.2, encoding an irrelevant prokaryotic gene, chloramphenicol acetyltransferase (Hambor et al., 1988b; Hauer et al., 1989). Flow cytometric analysis indicated that the antisense CD8 transfectant possessed no detectable CD8 and that the control transfectant levels of surface CD8 were comparable to those of the parental line (Figure 9).

JH.ARL.1 is an alloreactive clone with specificity for HLA-B35 (Hambor et al., 1988b). In order to ascertain that immunoregulatory activity of the clone was not mediated through the T cell antigen receptor,
Figure 9. Flow cytometric analysis of transfected and nontransfected JH.ARL.1 cloned cells.

The α-CD8/REP1 transfectant, the RSVCATα/220.2 transfectant, and nontransfected JH.ARL.1 cells were stained with anti-CD8 IgG (Ortho Diagnostics; solid lines) or control mouse IgG (dashed lines) as primary antibody and fluorescein-conjugated goat anti-mouse IgG (ICN) as secondary antibody. Analysis was performed on an EPICS V flow cytometer (Coulter).
HLA-B35+ donors were excluded from this study. Thus, recognition of the responding or stimulating cells in the MLR by the added cloned cells does not confound the interpretation of the results. Since the JH.ARL.1 clone was originally isolated from donor JH, PBMC from this individual were used as either responder or stimulator cells in the MLR cultures.

The results of the addition of the JH.ARL.1 clone or its transfectants to MLR cultures performed in both directions can be seen in a representative set of experiments shown in Figure 10. The addition of irradiated nontransfected or control transfected JH.ARL.1 cells to the DK(PBMC) anti-JH(PBMC) cultures resulted in significant inhibition of the proliferative response, whereas addition of the irradiated CD8- transfectant had no effect (Figure 10, top). In this culture the CD8+ JH.ARL.1 cells mediating inhibition were syngeneic with the stimulating JH(PBMC) and therefore were also recognized by the specific responding T cells from DK(PBMC).

We next determined whether the cloned cells mediating inhibition must be specifically recognized by the responder cells. In a MLR with JH(PBMC) responders and irradiated DK(PBMC) stimulators, the addition of the
JH.ARL.1 cloned cells or its transfectants did not affect the proliferative response (Figure 10, middle). Likewise, the antigenic response of autologous JH(PBMC) to an EBV-transformed syngeneic B cell line, JH(LCL), was not inhibited by the cloned cells, which do not possess EBV antigens (Figure 10, bottom). Thus, there was no inhibition by cloned cells of syngeneic JH responders in either allogeneic or virus-specific responses. Furthermore, a third-party allogeneic response with DK(PBMC) responding cells and stimulators possessing a MHC haplotype completely disparate from the cloned cells was not inhibited, and polyclonal JH(PBMC) responses to anti-CD3 monoclonal antibodies or to the mitogen phytohemagglutinin were also unaffected (data not shown). Although the inhibitory cloned cells were present in these cultures, no bystander effects were observed, suggesting that a specific recognition event between the responding cells and the inhibitory cells is necessary for inhibition.

Since allogeneic cytotoxicity is also generated in MLR cultures, the possibility of CD8-dependent inhibition of cytotoxic T cell generation was investigated. JH.ARL.1 or the transfected derivatives were added to MW(PBMC) anti-JH(PBMC) cultures, and their
Figure 10. Inhibition of proliferation in a MLR by addition of cloned CD8+ T cells.

Mixed lymphocyte cultures were established with either DK(PBMC) as responders and irradiated JH(PBMC) as stimulators (Top) or JH(PBMC) as responders and irradiated DK(PBMC) as stimulators (Middle). An antigen-specific stimulation was also established with JH(PBMC) as responders and irradiated EBV-transformed autologous B cells, JH(LCL), as stimulators (Bottom). For each panel cloned JH.ARL.1 T cells or its transfectants were added at the initiation of these cultures. The JH.ARL.1 cloned cells are autologous with JH(PBMC) and JH(LCL). Symbols: open circles, no cloned cells added; solid circles, nontransfected JH.ARL.1 cells added; open triangles, JH.ARL.1 cells transfected with RSVCATα/220.2 added; closed triangles, JH.ARL.1 cells transfected with α-CD8/REP1 added.
Figure 11. **Kinetic analysis of inhibition of the generation of allogeneic cytotoxicity mediated by cloned CD8+ T cells.**

Mixed lymphocyte cultures were established by culturing MW(PBMC) with irradiated (5000 rads) JH(PBMC). Irradiated (5000 rads) cloned JH.ARL.1 T cells (autologous with the stimulator cells) or transfectants of JH.ARL.1 were added on various days after the initiation of the culture. After 6 days of culture, allogeneic cytotoxicity was assessed by using $^{51}$Cr-labeled JH(LCL) as target cells. Data presented were obtained at an effector/target ratio of 20:1. Ratios of 5:1, 10:1, and 40:1 yielded similar results. Symbols: dotted line, no cloned cells added; solid circles, JH.ARL.1 cells added; open triangles, JH.ARL.1 transfected with RSVCATa/220.2 added; solid triangles, JH.ARL.1 transfected with α-CD8/REF1 added.
effect on allogeneic cytotoxicity was assessed after 6 days of coculture. Furthermore, the cloned cells were added either initially or on successive days after allogeneic activation to assess the kinetics of inhibition. Both the JH.ARL.1 cells and the control transfectant markedly inhibited the generation of cytotoxicity, whereas the antisense CD8 transfectant exerted only a minimal effect (Figure 11). Hence, expression of the CD8 molecule appears to be important in mediating inhibition of cytotoxic T cell generation as well as inhibition of the proliferative response. Moreover, maximal inhibition was achieved when the cloned CD8+ cells were added at the initiation of the cultures or by day 2. After that time the response increased; addition of the cells on day 4 of the culture did not result in any demonstrable inhibition of the response.

To further characterize the regulation obtained in these allogeneic cultures, we studied the effect of the relative numbers of added cloned T cells on the regulation of the proliferative response (Table III). Inhibition of responsiveness was apparent at a ratio of stimulator cells to CD8+ inhibitory T cells of 500:1; at a ratio of 5:1 inhibition was >75%. Consequently, the
cloned CD8\(^+\) cells were potent inhibitors of the allogeneic response in the mixed lymphocyte cultures. Again, the CD8\(^-\) phenocopy was not inhibitory for allogeneic proliferation.
TABLE III
Effect of the ratio of stimulators to cloned T cells (JH.ARL.1) on inhibition

<table>
<thead>
<tr>
<th>JH.ARL.1 cells added</th>
<th>Stimulator/ JH.ARL.1 ratio</th>
<th>[3H]Thymidine incorporation, dpm</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>---</td>
<td>89,893</td>
<td>---</td>
</tr>
<tr>
<td>Nontransfected</td>
<td>5000</td>
<td>94,786</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>60,234</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>28,522</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>19,905</td>
<td>78</td>
</tr>
<tr>
<td>RSVCATα/220.2 transfectant</td>
<td>5000</td>
<td>84,788</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>50,151</td>
<td>44</td>
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<tr>
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<td>50</td>
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<td>72</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14,137</td>
<td>84</td>
</tr>
<tr>
<td>α-CD8/REP1 transfectant</td>
<td>5000</td>
<td>87,314</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>84,003</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>93,928</td>
<td>0</td>
</tr>
</tbody>
</table>

An allogeneic culture was established with MW(PBMC) as responder cells and irradiated JH(PBMC) as stimulator cells. Cultures were initiated in the absence or presence of various numbers of nontransfected CD8+ JH.ARL.1 T cells or its sublines transfected with RSVCATα/220.2 (transfection control, CD8+) or α-CD8/REP1 (antisense CD8 transfectant, CD8-). Cell proliferation was determined 6 days after antigenic stimulation. Percent inhibition was determined by comparing [3H]thymidine incorporation measured in the presence of nontransfected JH.ARL.1 cells or its transfectants with incorporation measured in the absence of added cloned cells.
E. Discussion

The availability of a CD8\(^+\)/CD8\(^-\) T cell clonal pair and the use of a model immunoregulatory system in which cloned T cells are used to modulate allogeneic responses have enabled us to investigate the role of CD8 in T cell-mediated immunoregulation. Our results establish the importance of the CD8 molecule in inhibition mediated by cloned CD8\(^+\) T cells. Down-regulation of both allogeneic proliferation and generation of cytotoxicity in MLR cultures was mediated by CD8\(^+\) cells, but not their CD8\(^-\) phenocopies. The inhibition in this model system demonstrates the following characteristics: (i) inhibitory cells must express surface CD8; (ii) inhibition requires specific antigenic recognition of the CD8\(^+\) inhibitory cells by the responding cells; (iii) bystander effects are not observed; (iv) inhibition is a radioresistant activity of the CD8\(^+\) cells; (v) inhibition occurs only when inhibitory cells are present early in the allogeneic culture; (vi) relatively few inhibitory cells are required to mediate a substantial effect.

The explanation for CD8-dependent inhibition in our system remains to be clarified. To explore this issue, we have derived a transfectant of the human
erythroleukemia tumor line K562 that expresses CD8 on its cell surface (Tykocinski et al., 1988). This engineered CD8+ non-T cell line was able to mediate an inhibitory effect similar to that mediated by the T cell clone JH.ARL.1, whereas the nontransfected CD8- K562 control was not inhibitory (data not shown). Thus, it appears that CD8 expression on the surface of a cell is sufficient for inhibition in the absence of additional T cell-specific factors. Furthermore, appropriate CD8+ cells can be fixed and still retain their inhibitory activity (data not shown), indicating that CD8 is functioning in a mode that is independent of the physiological status of the cell. These data, together with the antisense mutational analysis presented in this report, demonstrate that the CD8 molecule functions in an immunoregulatory capacity as an inhibitory ligand.

CD8 has been shown to physically associate with class I MHC products (Norment et al., 1988; Rosenstein et al., 1989). Consequently, a possible inhibitory pathway might involve the ability of CD8 on inhibitory cells to bind to class I MHC products on the responding cells. It has been shown that antibodies specific for class I MHC products inhibit T cell proliferation activated by lectins, alloantigens, or anti-CD3
antibodies (Turco et al., 1985; O'Neill, 1986; Taylor et al., 1986; DeFelice et al., 1987; Schwab et al., 1988; Sharon et al., 1989). A recent report suggests that this inhibitory pathway involving class I MHC products might involve changes in expression of the interleukin 2 receptor, since the interleukin 2 receptor is associated with class I MHC products on the cell surface (Sharon et al., 1989). Similarly, CD8 on the immunoregulatory cloned T cells could bind to class I MHC products on the responder cells and thereby initiate inhibition through other secondary molecules.

The inhibition observed for CD8⁺ T cells in our in vitro model is similar to suppressive phenomena mediated by CD8⁺ veto cells. In both instances there is a requirement that the targeted responding cells specifically recognize the inhibitory cells. However, the inhibitory activity documented in this report is radioresistant, whereas veto cell activity is radiosensitive (Fink et al., 1988). The inhibition is our system is distinguishable from suppressor cell activity by virtue of the absence of inhibition when putative inhibitory cells are syngeneic with the responder cells (Green et al., 1988). Nevertheless, the finding that the CD8 molecule is important for
inhibitory activity in our immunoregulatory model suggests that the CD8 molecule may play a broader role in mediating inhibition in the immune system. Based on our observations, essential conditions that may be required for CD8-dependent inhibition include (i) specific cellular interaction involving the responding and inhibitory cells, (ii) CD8 expression on the inhibitory cells, and (iii) the presence of inhibitory cells during the early phase of the response.

Previous observations relating to diverse immunoregulatory phenomena could be reinterpreted in the light of our findings. For instance, in one well-characterized system suppressor CD8⁺ T cells interact with autoimmune T cells reactive to myelin basic protein via anti-idiotypic recognition (Lider et al., 1988). This model of suppression in experimental autoimmune encephalomyelitis had been demonstrated both in vitro and in vivo, although the mechanism of inhibition at a molecular level has not been elucidated. Our results suggest that subsequent to anti-idiotypic recognition, the CD8 molecule on the suppressor cell may play an essential role in mediating inhibition.

Our demonstration that allogeneic responses can be inhibited by CD8⁺ cells has significant implications for
the design of clinically immunosuppressive protocols involving transplantation. Potent, specific inhibition of an allogeneic response has been achieved in vitro with a potentially benign agent, an irradiated CD8\(^+\) cell of donor origin. Furthermore, these findings open the possibility that CD8\(^+\) cells could be used as vehicles for inducing specific tolerance in vivo in a variety of other clinical contexts.
V. Regulation of Allogeneic Responses by Expression of CD8α on Stimulator Cells.

A. Summary

By antisense-mediated inhibition of CD8 expression in T cell clones and expression of CD8 in non-T cell lines, we have produced several sets of CD8+/CD8− paired cell lines. These cellular reagents have allowed us to assess the effect of CD8 surface expression on the immunogenic potential of stimulator cells. We found that the presence of CD8 on stimulator cells markedly decreased their capacity to stimulate proliferative responses or to induce the generation of cytotoxic activity. The CD8 α chain, in the absence of the β chain, was sufficient to mediate this inhibitory effect. Our findings support the notion that CD8 functions as an immunoregulatory ligand and that auxiliary cell surface molecules on stimulator cells can have profound effects on the immunogenic capabilities of these cells.
B. Introduction

CD8 is expressed on the surface of a subset of T lymphocytes which have two broadly defined functions: 1) responsiveness to specific antigen; and 2) immunoregulation of other lymphocytes. In order to define the role of CD8 in mediating these two distinct T cell functions, we have developed an experimental approach based upon the modulation of CD8 surface expression. By transfection of a CD8 antisense vector, we have derived a CD8⁻ phenocopy of a HLA-B35-specific nontransformed CD8⁺ human T cell clone, JH.ARL.1. Utilizing this CD8⁺/CD8⁻ clonal pair, we have demonstrated that the CD8 molecule performs an important, but distinct, role in each of the two classes of CD8⁺ T cell function (Hambor et al., 1988; Kaplan et al., 1989).

First, the CD8 molecule plays an essential accessory role in the recognition of antigen by the T cell receptor (Hambor et al., 1988). Antisense inhibition of CD8 expression on an antigen-specific T cell clone resulted in a marked loss of antigen-stimulated functional responses (cytotoxicity, proliferation, lymphokine secretion, activation antigen expression); however, all of these functions could be
elicited with stimuli that do not require antigen recognition (anti-CD3, PHA). Hence, CD8 expression on the responding T cell plays an important role in T cell activation via specific antigen recognition.

Second, expression of the CD8 molecule is essential for the immunoregulatory function of CD8+ cells (Kaplan et al., 1989). The addition of a CD8+ T cell clone, but not its CD8- phenocopy, inhibited both proliferation and generation of cytolytic activity in cultures of allogeneic peripheral blood mononuclear cells (PBMC). CD8-mediated modulation of these allogeneic responses required specific antigenic recognition of the CD8+ inhibitory T cells by the responding cells. Since CD8 can physically associate with class I MHC products (Norment et al., 1988; Rosenstein et al., 1988), the inhibitory pathway may involve the ability of CD8 on inhibitory T cells to bind to class I MHC products on the responding cells.

These results suggested that the CD8 molecule may serve as an immunoregulatory ligand permitting cells which express CD8 to modulate immune responses. In order to further test this hypothesis, we have now examined the direct effect of CD8 expression on allogeneic stimulator cells. For this purpose, we used
both CD8\(^-\) variants of CD8\(^+\) T cell clones derived by antisense mutagenesis and CD8\(^+\) variants of CD8\(^-\) non-T cell lines derived by gene transfer of CD8 expression vectors. In this study, we report the abilities of these CD8\(^+\)/CD8\(^-\) paired cell lines to induce allogeneic responses. Our findings indicate that expression of CD8 on stimulator cells directly influences their allogeneic stimulatory potential.
C. Materials and Methods

Cells and Transfectants. Human CD8+ T cell clones, JH.ARL.1 and 8L2, have been derived and maintained as previously described (Hambor et al., 1988a; Hambor et al., 1988b). Using the pα-CD8/REP1 antisense CD8 expression vector, we have produced stable transfectants of these clones with more than 95% inhibition of CD8 surface expression. Transfection of the leukemic cell line K562 with the CD8 expression vector (pCD8/REP2) has been previously described (Tykocinski et al., 1988). The B5 subline of the human SV40 large T-immortalized bone marrow stromal cell line KM-102 (Harigaya and Handa, 1985; provided by Dr. Harigaya) was stably transfected with pCD8/REP2 (Weber et al., 1990). All of the transfected B5 cells were positive for CD8 expression as determined by immunofluorescence. The JH(LCL) line is an Epstein-Barr virus transformed B cell line syngeneic with the JH.ARL.1 T cell clone. PBMC were obtained from peripheral blood of healthy volunteers by discontinuous gradient centrifugation over a ficoll/hypaque cushion.

Immunofluorescence and Flow Cytometry. Surface expression of CD8 was determined by immunofluorescence and flow cytometric analysis as
previously described (Hambor et al., 1988b; Kaplan et al., 1988; Kaplan et al., 1984a; Kaplan et al., 1984b).
Anti-CD8α chain IgG (OKT8) was obtained from Ortho, Raritan, NJ. Anti-CD8β chain IgG (2ST8-5H7) was generously supplied by Dr. Ellis Reinherz. Flow cytometry was performed on an EPICS V cytometer (Coulter, Hialeah, FL). Relative intensities of fluorescence were determined by preparing a standard curve using beads with standardized fluorescent intensities (Coulter). Antibodies to class I and II MHC products were obtained from the Histocompatibility Laboratory of University Hospitals of Cleveland.

Proliferation Assays. Allogeneic responses were established in quadruplicate wells of a 96-well flat-bottom microtiter plate using PBMC (10^5/well) as responders and the following irradiated stimulator cells: human T cell clones (10^5/well; 5,000 rads), B5 cells (3 x 10^4/well; 15,000 rads), and K562 cells (2 x 10^4/well; 20,000 rads). Because the antigenic specificity of the JH.ARL.1 clone is HLA-B35, HLA-B35+ PBMC donors were excluded from this study.

In experiments demonstrating the inhibitory potential of CD8+ K562 cells, various numbers of CD8+ K562 cells (2 x 10^3, 2 x 10^2, 2 x 10^1) were co-cultured
with CD8⁻ K562 cells (2 x 10⁴) and responding PBMC (10⁵).

The medium for all proliferative assays was RPMI 1640 plus 10% fetal calf serum in a final volume of 0.2 ml. After incubation at 37°C for various periods of time, 0.5 μCi of [³H]thymidine was added to each well and the cultures were incubated an additional 18h. The cells were harvested onto glass fiber filters, and the radioactivity incorporated (dpm) was measured as described (Kaplan et al., 1984a; Kaplan et al., 1984b).

**Cytotoxicity.** Allogeneic cultures were established in 10 ml volumes with 5 x 10⁶ PBMC and either irradiated JH.ARL.1 or 10⁶ irradiated K562 cells. After 6 days at 37°C, viable cells were isolated by centrifugation over a ficoll/hypaque cushion, and these cells were used in a 4 hour ⁵¹Cr-release assay (Kaplan et al., 1984a). Effector cells generated in the PBMC vs. JH.ARL.1 cultures were assayed against JH(LCL) target cells. Effector cells generated in the PBMC vs. K562 cultures were assayed against K562 target cells. Spontaneous release was 18% with JH(LCL) targets and 6% with K562 targets.
D. Results

In order to explore the inhibitory role of the CD8 molecule, we established an in vitro model system using CD8\(^+\) or CD8\(^-\) cells to induce allogeneic responses by PBMC from healthy donors. For this purpose, we used CD8\(^-\) variants of CD8\(^+\) cells and CD8\(^+\) variants of CD8\(^-\) cells and assessed the ability of these CD8\(^+\)/CD8\(^-\) paired cell lines to function as stimulator cells in an allogeneic reaction.

We have previously generated stable α-CD8/REP1 transfectants of two CD8\(^+\) human T cell clones, JH.ARL.1 and 8L2 (Hambor et al., 1988a; Hambor et al., 1988b). Both transfected cell lines were greater than 95% inhibited in the surface expression of the CD8 molecule. Parental CD8\(^+\) cells and their CD8\(^-\) phenocopies were used as allogeneic stimulator cells to induce proliferative responses by PBMC responder cells (Figure 12). Whereas both of the CD8\(^-\) T cell phenocopies stimulated vigorous allogeneic proliferation, the CD8\(^+\) clones initiated only a minimal response. Each member of these two clonal pairs expressed comparable amounts of class I and II MHC products (data not shown). These results demonstrate that inhibition of CD8 surface expression on the stimulator cell has converted the status of these cells
Figure 12. Proliferative response of PBMC to CD8$^+$ human T cell clones and their CD8$^-$ phenocopies.

Allogeneic cultures were established with PBMC as responder cells and irradiated human T cell clones as stimulators. Two T cell clones were studied, JH.ARL.1 (Top) and 8L2 (Bottom). The proliferative response to the CD8$^+$ nontransfected clones (open squares) and to their CD8$^-$ pα-CD8/REP1 antisense transfectants (closed squares) is shown.
from a nonstimulatory to a strongly activating state.

To further probe the mechanism whereby the presence or absence of CD8 expression influences the stimulatory potential of cells, we assessed the ability of CD8− non-T cells or their pCD8/REP2-transfected CD8+ phenocopies to stimulate proliferation of PBMC in a mixed cell culture. CD8+ transfectants of two non-T cell lines, K562 (a human myeloid leukemia cell line) and B5 (an immortalized human bone marrow stromal cell line) were used. As stimulators in a mixed cell culture, the CD8− parental cell lines in each case were capable of stimulating a strong proliferative response by the PBMC; in contrast, the CD8+ transfectants were not stimulatory (Figure 13). This result demonstrates that CD8 expression on a non-T cell stimulator can alter its immunogenic status.

We next determined if generation of an effector function is also influenced by expression of CD8 on the stimulator cell. For this purpose, we measured the cytotoxicity generated in PBMC stimulated either with the CD8+ T cell clone JH.ARL.1 or its CD8− phenocopy or with the CD8− K562 cell line or its CD8+ phenocopy (Figure 14). Cytotoxic cells specific for alloantigens on JH.ARL.1 were successfully produced in cultures which
Figure 13. Proliferative response of PBMC to two non-T cell lines, K562 and B5, and their CD8+ phenocopies.

Allogeneic cultures were established with PBMC as responder cells and either irradiated K562 (Top) or B5 (Bottom) cells as stimulator cells. The response to nontransfected CD8- cells is represented by the open squares and the response to their CD8+ phenocopies (CD8/REP2 transfectants) is represented by the closed squares.
Figure 14. Generation of cytotoxic response to JH.ARL.1 and K562 CD8⁺/- paired lines.

To generate cytotoxic activity, we established mixed cell cultures with PBMC as responders and the following cells as stimulators: CD8⁺ JH.ARL.1 (nontransfected), CD8⁻ JH.ARL.1 (α-CD8/REP1 transfectants), CD8⁻ K562 (nontransfected), and CD8⁺ K562 (CD8/REP2 transfectant). Cytotoxicity was measured after 6 days of culture using JH(LCL) as targets (Top) or K562 as targets (Bottom).
PBMC vs. JH.ARL.1

PBMC vs. K562

% Cytotoxicity

E:T Ratio

Nontransfected
α-CD8/REP1 transfec tant

Nontransfected
CD8/REP2 transfec tant
were stimulated with the CD8- transfectants whereas the parental, nontransfected CD8+ JH.ARL.1 cells were not able to induce the generation of allospecific cytotoxic activity. Furthermore, parental CD8- K562 cells were also able to induce cytotoxicity; however, virtually no cytotoxicity was detected in cultures stimulated the CD8+ transfectants. While CD8+ K562 cells were poor stimulators for the generation of cytotoxicity, CD8+ K562 cells were recognized as target cells in an independent cytotoxicity assay (data not shown). This result demonstrates that the transfection and subsequent expression of CD8 did not affect structures necessary for recognition. Hence, both proliferation and the generation of cytotoxicity are influenced by expression of CD8 on the stimulator cells.

CD8 is expressed on the surface of T cells as both an α/β heterodimer and an α/α homodimer (Snow and Terhorst, 1983; Ledbetter et al., 1981; Walker et al., 1984; Dembic et al., 1987; Gabert et al., 1987). In establishing CD8 variant phenocopies, we had used only α chain-based vectors. Moreover, the antibodies that we has previously used to characterize CD8 expression were α chain-specific. To ascertain the status of the β chain in these transfectants, we analyzed β chain
Figure 15. Flow cytometric analysis of CD8α and CD8β surface expression on transfected and nontransfected JH.ARL.1 T cell clones and K562 cells.

Nontransfected JH.ARL.1 T cells, the JH.ARL.1 pα-CD8/REP1 transfectant, nontransfected K562 cells, and pCD8/REP2 transfected K562 cells were stained with anti-CD8α IgG (solid lines), anti-CD8β (dashed lines), and control IgG (dotted lines). Fluoresceinated goat anti-mouse IgG was used as a second antibody. The x-axis represents 250 channels logarithmically distributed over three decades. The intensity of fluorescence seen with anti-CD8α staining of K562 was greater than 200 times brighter than with control IgG staining. The intensity of fluorescence seen with anti-CD8α staining of JH.ARL.1 was greater than 200 times brighter than with control IgG staining. The intensity of fluorescence seen with anti-CD8β staining of JH.ARL.1 was 9 times brighter than with control IgG for one peak and 47 times brighter for the other peak.
surface expression by immunofluorescence and flow
cytometry (Figure 15). Whereas the parental JH.ARL.1
cells expressed both CD8α and β chains, the α-CD8/REP1
transfectants expression neither of these chains.
Likewise, the parental K562 cells expressed neither
chain, but the CD8/REP2 transfectants expressed the CD8α
chain. These results indicate that expression of CD8α
alone is sufficient for mediating the immunoregulatory
function.

In a previous study of mixed PBMC cultures, we
demonstrated that addition of a CD8+ T cell clone, but
not its CD8− phenocopy, resulted in significant
inhibition of allogeneic proliferation. The
availability of the CD8+/CD8− paired K562 lines allowed
us to extend these findings in a non-T cell setting.
Various numbers of irradiated CD8+ K562 transfectants
were added to cultures established with PBMC responder
cells (Figure 16). The proliferative response was
completely inhibited in cultures with a ratio of CD8− to
CD8+ K562 of 10:1 and still significantly inhibited in
cultures with a ratio of CD8− to CD8+ K562 cells of
100:1. These results demonstrate that a genetically
engineered CD8+ non-T cell line was able to mediate an
inhibitory effect similar to that mediated by a human
CD8$^+$ T cell clone. Thus, it appears that CD8 expression on the surface of a cell is sufficient for inhibition in the absence of additional T cell-specific factors. These findings demonstrate that the CD8 molecule alone can endow a cell with immunoregulatory capability.
Figure 16. Inhibition of proliferation to CD8\textsuperscript{−} K562 mediated by the addition of CD8\textsuperscript{+} K562.

Mixed cell cultures were established with PBMC as responders and irradiated K562 as stimulators. Various numbers of irradiated CD8\textsuperscript{+} K562 cells were added. The ratio of CD8\textsuperscript{−} to CD8\textsuperscript{+} K562 is shown.
E. Discussion

Using CD8\(^+\)/CD8\(^-\) paired cell lines, we have demonstrated a significant role for the CD8 molecule in regulating the immunogenic status of stimulator cells in allogeneic cultures. By antisense conversion of two CD8\(^+\) T cell clones to a CD8\(^-\) phenocopy, the immunogenic capability of the cells was markedly enhanced. Likewise, surface expression of the CD8 molecule on two non-T cell lines markedly decreased their ability to stimulate an immunologic response. The combined use of antisense and sense transfection technologies in a complementary way has allowed us to demonstrate that the CD8 molecule can function as an immunoregulatory molecule in both native (T cell) and foreign (non-T cell) cellular settings. Hence, together our findings firmly establish that the CD8 molecule on the stimulator cells has the ability to regulate the activation of the responding cells.

We have previously described an immunoregulatory function for the CD8 molecule on the surface of T cells (Kaplan et al., 1989). CD8\(^+\) cloned cells, but not their CD8\(^-\) phenocopies, inhibited an allogeneic response directed against PBMC syngeneic with the immunoregulatory cloned cells. We have extended these
findings using K562 cells transfected with a CD8 expression vector. CD8⁺ K562 cells could inhibit the proliferative response of PBMC to the parental CD8⁻ K562 cells. Thus, inhibition of the response was demonstrated in the absence of any additional T cell-specific factors. These findings support our interpretation that the CD8 molecule itself acts as an immunoregulatory ligand in mediating inhibition.

Because the CD8 molecular complex consists of both α/β heterodimers and α/α homodimers (Snow and Terhorst, 1983; Ledbetter et al., 1981; Walker et al., 1984), we sought to assess the contribution of the α and β chains in the immunoregulatory function of CD8. Expression of the CD8 α chain alone in both the K562 and B5 lines was sufficient for transferring inhibitory function. In previous studies, investigators had also found that the α chain is sufficient in reconstituting the function of CD8 as an accessory molecule for T cell antigen recognition (Dembic et al., 1987; Gabert et al., 1987). Neither in these studies of the recognition role of CD8 nor in our own previous work defining the immunoregulatory function of the molecule was the contribution of the β chain directly assessed. The absence of its expression on the transfectants in the
present study indicates that it is clearly not essential for the immunoregulatory function. Furthermore, it is interesting to note that antisense inhibition of CD8 α chain resulted in a concomitant inhibition of CD8 β chain expression. This result confirms the requirement of α chain for β chain surface expression (Shiue et al., 1988; Norment and Littman, 1988; DiSanto et al., 1988).

Factors that affect the immunogenic capability of stimulator cells include the level of surface expression of MHC products, the capacity to secrete T cell activating cytokines such as interleukin 1, the inherent phagocytic activity, and the capacity to process proteins into appropriate fragments. We have now demonstrated the importance of another factor, auxiliary cell surface molecules. CD8 on the cell surface of the stimulators greatly influences the capacity of these cells to activate T lymphocytes. Inhibition of stimulatory capacity by the surface expression of the CD8 molecule represents an important step towards the molecular dissection of the activation event.
VI. CD8 Functions as an Inhibitory Ligand in Mediating the Immunoregulatory Activity of CD8-positive Cells.

A. Summary

Antisense and sense transfection technologies were employed in cellular coculture systems to investigate the physiological requirements for CD8-dependent immunoregulation. Our data indicate that cells bearing genetically engineered CD8 molecules incorporating a glycoinositolphospholipid membrane anchor, as well as fixed cells bearing natural CD8 molecules, retain specific, CD8-dependent immunoregulatory activity. These findings together support the novel concept that CD8, a molecule traditionally thought of as a receptor, can function as an inhibitory ligand. CD8-dependent inhibition was shown to induce nonresponsiveness, persisting for at least 24 hours, in antigen-specific responders. Moreover, only cells undergoing primary, but not secondary, antigenic stimulation through their T cell receptors were found to be susceptible to CD8-dependent inhibition. Both CD4+ and CD8+ responding T cells were inhibited by CD8+ modulatory cells. These functional analyses of inhibitory and responder cells in
CD8-dependent inhibition lay the groundwork for developing artificial CD8-based immunomodulatory peptides and deciphering CD8's role in natural immunoregulation.
B. Introduction

A diverse array of functional activities have been ascribed to cell surface-associated glycoproteins of eukaryotic cells. In the case of hematopoietic cells, for instance, cell surface molecules are known to function in signal recognition as "receptors", in signal presentation as "ligands", in the promotion of intracellular or acellular matrix adhesiveness as "adhesins", in the release of soluble glycoproteins as "biosynthetic intermediaries", in the catalysis of covalent modification reactions as "enzymes", and in allosteric induction of molecular complex association or dissociation as "regulators" (for examples, see Weiss and Imboden, 1987; Dinarello, 1989; Springer et al., 1987; Scherr, 1990; Low et al., 1987; Medof et al., 1987). Each cell surface-associated glycoprotein is conventionally thought of, and often had been named, in terms of the primary category of molecular activity first assigned to that particular molecule. However, such unidimensional functional categorizations may be misleading, and a multifunctional view may be more appropriate and less conceptually limiting for at least some cell surface molecules.

CD8 is a glycoprotein produced in cell surface-
associated and soluble forms by subsets of thymic and peripheral T lymphocytes (Zamoyska et al., 1985; Giblin et al., 1989; Norment et al., 1989). The cell surface-associated form of CD8 is known to consist of both α/α homodimers and multimers and α/β heterodimers in murine and human cells (Ledbetter and Seaman, 1982; Reilly et al., 1980; Snow and Terhorst, 1983; Ledbetter et al., 1981; Walker et al., 1984). CD8-positivity defines the subset of mature T lymphocytes in the periphery that mediate class I major histocompatibility complex (MHC)-restricted cytotoxicity against virally-infected, allogeneic, and other cellular targets. Early antibody blocking studies (Swain, 1981; Landegren et al., 1982; Swain et al., 1984; Spits et al., 1985; Schimonkevitz et al., 1985; Goldstein and Mescher, 1987; Aparicio et al., 1987; Moldwin et al., 1987) pointed to a role for the CD8 molecule in antigenic recognition and triggering of cytotoxicity in such CD8-positive (CD8⁺) cytotoxic T cells. While these antibody blocking studies were subsequently called into question due to the demonstration of nonspecific inhibitory effects mediated by anti-CD8 antibodies (Welte et al., 1983; Hunig, 1984; Fleischer et al., 1986; Van Seventer et al., 1986; Geppert and Lipsky,
1986), antisense (Hambor et al., 1988a; Hambor et al., 1988b) and sense (Dembic et al., 1986; Dembic et al., 1987; Galbert et al., 1987) CD8 transfection analysis have now definitively established an "accessory" molecular role, representing a receptor function, for this molecule in antigenic recognition. In this regard, the direct molecular links for CD8 are now thought to include class I MHC on the outside of the cell (Rosenstein et al., 1989; Norment et al., 1988) and the T cell-specific protein kinase (p56^ck) on the inside of the cell (Veillette et al., 1988; Barber et al., 1989; Zamoyska et al., 1980).

In addition to cytotoxicity triggered by antigen recognition, T lymphocytes with a CD8^+ phenotype are known to mediate other effector functions including an array of regulatory activities in the immune system. The molecular explanation for the various immunoregulatory phenomena that have been described for such cells has for a long time remained elusive. Our recent studies using antisense and sense transfection analysis (Kaplan et al., 1989; Hambor et al., 1990) have suggested that CD8, and specifically the CD8α chain, is a critical molecular determinant in the immunoregulatory activity mediated by CD8^+ lymphocytes. This insight had
led to a multifunctional perspective for the cell surface-associated CD8 glycoprotein, ascribing to it an inhibitory ligand function in addition to its conventionally recognized receptor function. Two major lines of evidence have supported this viewpoint. First we have used CD8−/CD8+ cellular pairs, generated by either antisense or sense transfection mutagenesis, to demonstrate that the proliferative response of responders to irradiated, allogeneic, cloned stimulators in a mixed cell culture is abrogated by the expression of CD8 on the stimulators (Hambor et al., 1990). Second, to determine whether this loss of response is in fact a consequence of inhibitory effects exerted upon the responders, we set up a cellular coculture system in which cloned, irradiated inhibitory cells are added, as third party cells, to a mixed cell culture including responders and irradiated, allogeneic stimulators. CD8-dependent inhibition of the proliferative response of responders was documented, and this inhibition was dependent upon coexpression of CD8 and specific allogeneic MHC (corresponding to that of the stimulators) on the inhibitory cells (Kaplan et al., 1989).

We now report our investigations which explore the
physiological requirements for the CD8+ regulatory cell and the responding T cell in CD8-mediated immunoregulation. Specifically, we have directly tested the concept of CD8 as inhibitory ligand, probing the physiological requirements for the CD8-bearing regulatory cell. Additionally, we have analyzed physiological aspects of the cell undergoing inhibition by CD8-mediated mechanisms. Our findings not only confirm the notion of CD8 as ligand, but also define the kinetics of CD8-induced nonresponsiveness for the responding cell and a window of susceptibility to CD8-mediated inhibition that is cell state-dependent.
C. Materials and Methods

Cells and Transfectants. JH-ARL.1, a CD8+ human T cell clone isolated from donor JH and specific for the alloantigen HLA-B35, was derived and maintained as described (Hambor et al., 1988). Using pα-CD8/REP1, an antisense CD8 expression vector, a stable transfectant of this clone was generated which exhibits greater than 95% reduction of CD8 surface expression. Stable transfectants of the nonadherent, human chronic myeloid leukemia cell line K562 were produced using a full-length CD8 expression vector (pCD8/REP2) and a chimeric CD8-DAF expression vector (pCD8-DAF/REP2; Tykocinski et al., 1988). Stable transfectants of KM-102, an adherent, human bone marrow stromal cell line previously derived by SV-40 large T transformation (Harigaya and Handa, 1985), were also generated using pCD8/REP2 (Weber et al., 1990). These cells were surface positive for CD8 expression as determined by immunofluorescence. CH.36 is a CD4+ human T cell clone isolated from a primary mixed lymphocyte culture consisting of human PBMC from donor CH (HLA haplotype: A24,28; B7,14; DR1,5) and irradiated, allogeneic human PBMC from donor JH (HLA haplotype: A2,3; B7,44; DR2,4). Cloning was performed by limiting dilution as described
(Hambor et al., 1988). Although the precise specificity of CH.36 has not been determined, this clone responds to alloantigens on JH.ARL.1 and PBMC from donor JH. PBMC were obtained from the peripheral blood of healthy volunteers by discontinuous gradient centrifugation over a ficoll/hypaque cushion. Because the antigenic specificity of the JH.ARL.1 clone is HLA-B35, HLA-B35+ PBMC donors were excluded from this study.

Proliferation Assay. All proliferation assays were performed in quadruplicate wells of a 96-well flat-bottom microtiter plate as described (Kaplan et al., 1984a; Kaplan et al., 1984b). Primary allogeneic responses were established using PBMC (10^5/well) as responders and the following irradiated stimulator cells: paraformaldehyde-fixed and non-fixed K562 cells (2 x 10^4/well; 20,000 rads) and paraformaldehyde-fixed KM-102 cells (3 x 10^4/well; 15,000 rads). Secondary allogeneic responses were established using viable responder cells isolated by discontinuous gradient centrifugation over ficoll/hypaque from primary allogeneic cultures. Specifically, a primary culture was established in a 25 cm^2 flask by coculturing 5 x 10^6 PBMC with 10^6 irradiated K562 cells for 10 days. Viable cells were isolated from these cultures and used as
responder cells (5 x 10^4/well) in a secondary response with irradiated, nontransfected and pCD8/REP2-transfected K562 stimulators (20,000 rads; 2 x 10^4/well). In addition, a primary culture was established by coculturing 5 x 10^6 PBMC with 5 x 10^6 irradiated PBMC from allogeneic donor JH. After 10 days, viable cells were isolated and used as responder cells (5 x 10^4/well) in a secondary response with irradiated, nontransfected and pα-CD8/REP1 transfected JH.ARL.1 stimulators (5,000 rads, 10^5/well). The proliferative response of the T cell clone CH.36 (5 x 10^4/well; day 10 post-OKT3 stimulation) was assessed using irradiated, nontransfected and pα-CD8/REP1-transfected JH.ARL.1 stimulators (5,000 rads; 10^5/well).

Cell Fixation. K562 Cells: Irradiated (20,000 rads), nonadherent K562 cells, suspended in PBS, were added to 96-well microtiter plates (2 x 10^4 cells/200 μl/well) or 25 cm² flasks (10^6 cells/flask) and centrifuged for 15 min. at 400 x g. The medium was aspirated, and the cells were air-dried for 1-2h at room temperature. Air-dried cells were treated with a 2% paraformaldehyde solution in PBS for 15 min. at room temperature, then washed 3 times with PBS and used
immediately. Cell ghosts could be microscopically observed at this stage.

**KM-102 Cells:** Irradiated (15,000 rads) KM-102 cells, suspended in RPMI 1640 with 10% FCS, were added to 96-well microtiter plates (3 x 10^4 cells/200 µl/well) and allowed to adhere by incubation at 37°C for 18h. The medium was aspirated, the cells were washed 2 times with PBS, then air-dried for 1-2h at room temperature. Air-dried cells were treated with a 2% paraformaldehyde solution in PBS for 15 min. at room temperature, then washed 3 times with PBS and used immediately.

**Preparation of T Lymphocyte Subsets.** Purified T cells were prepared by a modification of a described method (Julius et al., 1973). PBMC were incubated on a nylon wool column for 1h at 37°C and 7% CO₂. The cell population eluted off was >99% T lymphocytes as determined by fluorescent staining with an anti-CD3 monoclonal antibody (OKT3; Ortho Diagnostics) and flow cytometric analysis (EPICS V; Coulter Diagnostics). CD4⁺ and CD8⁺ lymphocyte populations were enriched using magnetic beads with anti-IgG antibodies attached (Collaborative Research, Inc.) according to a described method (Cruikshank et al., 1987). Nylon wool-purified T cells were first incubated with either anti-CD4 or
anti-CD8 monoclonal antibodies (OKT4 and OKT8, respectively; 500 ng monoclonal antibody/10^6 cells/ml) for 30 min. on ice, washed twice, then added to a slurry of pre-washed magnetic particles with goat anti-mouse IgG covalently attached. After incubation for 30 min. on ice, the bead/cell suspension was washed, then cell-monoclonal antibody-bead complexes were extracted from unlabeled cells in a strong magnetic field. Using this negative depletion method, the purity of CD4^+ and CD8^+ subsets (those depleted with anti-CD8 and anti-CD4 monoclonal antibodies, respectively) was >95% with 0% of the depleted cell population present as determined by immunofluorescent staining with anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies (OKT3, OKT4, and OKT8, respectively; Ortho Diagnostics).
D. Results

In our earlier studies, using antisense and sense transfection analysis, we have inferred a ligand function for the CD8 molecule (Kaplan et al., 1989; Hambor et al., 1990). This molecular activity was associated with the CD8α chain and found to be independent of additional T cell-specific factors. To more directly test the hypothesis that CD8 functions as inhibitory ligand, we determined whether ligand, unlike receptor, activity for the CD8 molecule, resides solely within the extracellular domain. We have previously reported a process, employing chimeric gene transfection technology, for producing a glycoinositolphospholipid (GPL)-modified variant of CD8α, in which the extracellular domain of CD8, a short 3'-end peptide segment of the complement regulatory protein decay accelerating factor (DAF), and a GPL moiety are sequentially linked (Tykocinski et al., 1988). The CD8 peptide derivative so engineered binds to membranes by the GPL membrane anchor and is missing the natural CD8 hydrophobic transmembrane and cytoplasmic domains, consisting of 25 and 28 amino acids, respectively. A mixed cell culture was set up in which the proliferative response of peripheral blood mononuclear cell (PBMC)
Figure 17. Inhibitory activity of the extracellular domain of CD8.

The proliferative response of PBMC to K562 cells with native-anchored and GPL-anchored CD8 was examined. Primary allogeneic cultures were established with PBMC as responders and irradiated K562 cells as stimulators. The proliferative response to CD8⁻ nontransfected K562 cells (closed triangles), pCD8/REP2 transfectants (open squares) and pCD8•DAF/REP2 transfectants (closed triangles) is shown.
responders to nontransfected K562 stimulators of transfected K562 stimulator cells bearing either GPL-modified CD8 or natural CD8 were determined. As shown in Figure 17, expression of GPL-anchored CD8 on transfectants resulted in a loss of stimulatory capacity that was equivalent to that seen for transfectants bearing natural (conventionally-anchored) CD8. As expected, cellular controls consisting of CD8-negative (CD8−) nontransfected K562 cells generated a potent proliferative response. Hence, the native CD8 transmembrane and cytoplasmic domains are not required, and the CD8 extracellular domain is sufficient, for abrogating the activating capacity of a stimulator cell.

To probe the physiological prerequisites for inhibitory cell activity, we determined whether CD8-dependent effects can be demonstrated with fixed, metabolically-inactivated cells. CD8+ and CD8− K562 cells were centrifuged to the bottom of tissue culture wells, air-dried and post-fixed with paraformaldehyde. PBMC responders were added to the well, and the proliferative response to the fixed stimulators was assessed. While fixed CD8− K562 cells induced a strong proliferative response, fixed CD8+ K562 cells were nonstimulatory (Figure 18). To establish the generality
Figure 18. Inhibitory activity of fixed, CD8-positive stimulators.

CD8-dependent inhibition of the proliferative response of PBMC by fixed stimulator cells was examined. Primary allogeneic cultures were established with PBMC as responder cells and either K562 (panel A) or KM-102 (panel B) cells as stimulator cells. Stimulator cells were irradiated, air-dried to the well and paraformaldehyde-fixed before use (see Materials and Methods). The response to nontransfected CD8⁻ cells is represented by the open squares and the response to the CD8⁺ phenocopies (CD8/REP2 transfectants) is represented by the closed squares.
PBMC vs. fixed K562

- None
- pCD8/REP2

PBMC vs. fixed KM-102

3H-Thymidine Incorporation (x 10^3 dpm)

DAYS

A

B
of this finding, we substituted KM-102 human bone marrow stromal cells for K562 cells as fixed stimulators in this coculture system. Again, CD8 expression on fixed stimulators abrogated the proliferative response (Figure 18). These findings establish that CD8-dependent inhibition can be mediated by metabolically-inactivated cells and that CD8 and specific alloantigen can deliver inhibitory signals as ligands in a fixed state.

Focusing upon the responding cell, we next determined the interval of time required for induction of nonresponsiveness. To address this kinetic aspect of CD8-induced nonresponsiveness, we asked whether inhibition of specific responders in the responder PBMC pool persists following removal of CD8+ inhibitory cells. Exploiting our previous demonstration that CD8 inhibitory ligand activity is retained post-fixation, we devised an experimental protocol whereby PBMC responders were exposed to fixed CD8+ K562 modulatory cells for various lengths of time, recovered free of the fixed modulatory cells, and reexposed to irradiated, CD8- K562 stimulators. When the PBMC responders were pre-treated with fixed CD8+ K562 cells for 24 h, the proliferative response to CD8- K562 stimulators presented immediately afterwards was markedly inhibited (Figure 19). Twelve
Figure 19. Kinetics of CD8-induced nonresponsiveness in responders.

A kinetic analysis of the specific anergic effect on PBMC responders by prior exposure to CD8-bearing cells was determined. PBMC responders were exposed to CD8+, paraformaldehyde-fixed, pCD8/REP2 transfectants of K562 cells for 6, 12, or 24 hours, then removed and cultured by themselves (speckled bars) or cocultured with either CD8-, nontransfected K562 cells (solid bars) or CD8-, nontransfected JY.LCL cells (cross-hatched bars). The proliferative response is shown on day 7 post initial exposure to the pCD8/REP2 transfectant.
hour pretreatment resulted in partial inhibition of the K562-directed response, whereas a shorter pretreatment period (6h) was ineffective in inducing nonresponsiveness that would persist following separation from the CD8+ inhibitory cells. In contrast to the proliferative response to K562 stimulators, the proliferative response to JY.LCL, an Epstein-Barr virus-transformed B cell line, was not affected in the CD8+ K562-treated responder cell population, clearly demonstrating antigenic specificity of the nonresponsive state and ruling out nonspecific bystander effects.

To assess the duration of the nonresponsive state, responders pretreated for 24h were stimulated after varying recovery intervals (Figure 20). Nonresponsiveness was still evident after a 24h recovery interval. Due to a nonspecific diminution of responsiveness with longer recovery intervals, as demonstrated by loss of responsiveness to JY.LCL at 72h, the longer-term persistence of the nonresponsive state could not be assessed.

To explore the physiological prerequisites for rendering a responder cell susceptible to CD8-mediated inhibition, we compared the relative susceptibility of
Figure 20. Duration of the CD8-induced nonresponsive state.

PBMC responder cells were either immediately stimulated with nontransfected (CD8⁻) K562 or nontransfected (CD8⁻) JY.LCL cells (0h pretreatment duration) or pretreated with CD8⁺, air dried and paraformaldehyde-fixed, pCD8/REP2 transfectants of K562 for 24h (24h pretreatment duration). After the pretreatment period, viable cells were isolated and either immediately stimulated with nontransfected (CD8⁻) K562 or nontransfected (CD8⁻) JY.LCL cells (0h posttreatment duration interval) or allowed to recover by culture in complete media for either 24 or 48h (24 and 48h posttreatment interval) prior to stimulation. As a control, PBMC were initially cultured by themselves in complete media for 72h (0h pretreatment time, 72h posttreatment interval), viable cells were isolated and stimulated with nontransfected (CD8⁻) K562 or nontransfected (CD8⁻) JY.LCL cells. The proliferative response is shown on day 7 (after initiation of the pretreatment period). Symbols: solid bars, response to nontransfected (CD8⁻) K562; cross-hatched bars, response to nontransfected (CD8⁻) JY.LCL.
3H-Thymidine Incorporation (x 10^3 cpm)

Pretreatment duration (hr)  0  0  24  24  24
Posttreatment interval (hr)  0  72  0  24  48

- K562
- JY.LCL
Figure 21. Refractoriness of secondary allogeneic responses and T cell clonal responses to inhibition by CD8-bearing stimulator cells.

The susceptibility of secondary and clonal responders to CD8-dependent inhibition was determined. Secondary allogeneic cultures were established with primed responder cells, isolated from 10 day cultures of PBMC with either irradiated nontransfected K562 cells (panel A) or irradiated allogeneic PBMC from donor JH (panel B), and either irradiated K562 cells (panel A) or JH.ARL.1 T cell clones (panel B) as stimulator cells. The proliferative response to CD8^- nontransfected K562 cells (open squares) and its CD8^+ phenocopy (pCD8/REP2 transfec tant, closed triangles) is shown in panel A. The proliferative response to CD8^+, nontransfected JH.ARL.1 T cell clones (open squares) and its CD8^- phenocopy (pα-CD8/REP1 transfec tant, closed squares) is shown in panel B. A clonal response (panel C), was established with CH.36 T cell clones as responders and either CD8^+, nontransfected JH.ARL.1 cells (closed squares) or its CD8^- phenocopy (pα-CD8/REP1 transfec tant, closed squares) as stimulators.
specific responders in primary versus secondary allogeneic responses to inhibition. Since CD8\(^-\) cells can elicit a primary allogeneic response, we set up a primary mixed cell culture using PBMC as responders and irradiated CD8\(^-\) K562 cells as allogeneic stimulators. After 10 days of coculture, viable cells were isolated by ficoll/hyphaque density gradient centrifugation and assessed for their ability to respond in a secondary mixed cell culture to stimulation by either member of the CD8\(^+\)/CD8\(^-\) K562 cellular pair. Whereas CD8-dependent inhibition could be demonstrated in a primary response (see Figure 17), no inhibitory effect was evident for either the CD8\(^+\) or CD8\(^-\) stimulators in a secondary response set up as described (Figure 21, panel A). Similar findings were observed when primed responders, generated in a primary MLR using JH(PBMC) stimulators, were stimulated in a secondary MLR with either member of the CD8\(^-\)/CD8\(^+\) JH.ARL.1 T cell clonal pair (Figure 21, panel B).

The absence of a CD8-mediated inhibitory effect in a secondary MLR suggested the possibility that a T cell that had undergone prior antigenic stimulation through its T cell receptor/CD3 complex is not susceptible to the CD8 ligand effect. To further test this notion, we
determined whether a long-term T cell clone, periodically stimulated via CD3, can respond to a CD8+ stimulator. A CD4+ T cell clone, CH.36, which is specific for alloantigens expressed by JH.ARL.1, was derived, and the proliferative response of these cloned CH.36 T cells to allogeneic stimulation by either member of the CD8+/CD8- JH.ARL.1 T cell clonal pair was assessed. As seen previously for secondary responders, no CD8-dependent inhibition was evident for the cloned T cell responders (Figure 21, panel C), lending support to the notion that only the primary response is susceptible to CD8-mediated immunoregulation.

To phenotypically characterize responders that are susceptible to CD8-dependent inhibition, we assessed the ability of isolated lymphoid subpopulations to proliferate in response to stimulation by either member of CD8-/CD8+ cellular pairs. Primary allogeneic cultures were set up with either PBMC, nylon wool-purified T cells or enriched fractions of CD4+ or CD8+ T cells as responders. Using either CD8+ K562 (Figure 22, panel A) or CD8+ KM-102 (Figure 22, panel B) transfectants as stimulators, marked CD8-dependent inhibition was evident for both CD4- and CD8-enriched responders. The overall proliferative response was
diminished in the enriched lymphoid subpopulations. The fact that the remixing of the CD4+ and CD8+ cells only partially restored the proliferative response seen with unmanipulated PBMC suggests that other "accessory" cells may be required for the full response. However, such additional cells are apparently not required for CD8-dependent inhibition.
Figure 22. Inhibition of both CD4⁺ and CD8⁺ responders by a CD8-dependent mechanism.

The susceptibility of isolated responder cell populations to CD8-mediated inhibition was assessed. Primary allogeneic cultures were established with either PBMC, nylon wool-purified T cells or enriched fractions of CD4⁺ or CD8⁺ T cells as responder cells and either irradiated K562 cells (panel A) or irradiated KM-102 cells (panel B) as stimulator cells. For both stimulator cell populations, the proliferative response to CD8⁻ nontransfected cells (cross-hatched bars) and CD8⁺ pCD8/REP2 transfectants (solid bars) is shown on day 7 after initiation of the mixed cell culture.
B. Discussion

Two of the fundamental classes of cellular effector functions that have been ascribed to CD8⁺ T lymphocytes in the immune systems of higher vertebrates are antigenic recognition, leading to the triggering of effector functions such as cytotoxicity, and immunoregulation. In this and previous investigations, we have applied antisense and sense transfection analyses to molecularly probe these T cell functions. Our experimental data support the notion that the CD8 molecule itself is a critical molecular determinant for both classes of cellular activities associated with CD8⁺ T cells and that this molecule contributes to each of them through two distinct functional modes. In the case of antigen recognition/cytotoxicity, the CD8 molecule functions on the T cell as a receptor in an accessory molecular role for the T cell antigen receptor, dictating responsiveness to antigen. In the case of immunoregulation, the CD8 molecule functions as an immunoregulatory ligand, inhibiting specific T cell receptor-bearing cells that are being specifically stimulated through antigenic cosignalling.

Two predictions based upon the CD8-as-ligand concept were confirmed in this study. First of all, we
demonstrated that the CD8 extracellular domain, when appended to a membrane via a covalently-linked glycoinositolphospholipid (GPL) moiety, retains the inhibitory activity of the native, conventionally-anchored molecule. Hence, the hydrophobic transmembrane and cytoplasmic domains, which are needed for CD8's receptor function, are not required for CD8's ligand function. Moreover, we observed that natural CD8 on a fixed cell maintains its function as a ligand, further distinguishing this particular role for CD8 from its receptor function. Taken together, these findings indicate that CD8-dependent inhibition stems from the ligand activity of the CD8 molecule itself and not from other secondarily expressed molecules or more complicated effector pathways.

Several natural molecular forms of CD8 have been described. In addition to the predominant cell surface-associated form that possesses a cytoplasmic domain, a variant cell surface-associated form has recently been reported (Zamoyska et al., 1989) that is lacking a cytoplasmic domain and hence cannot interact with the p56Lck protein kinase in a receptor mode. In light of our findings with an artificial GPL-modified (cytoplasmic domain minus) CD8 form, we would predict
that this natural CD8 variant retains ligand activity and may thereby participate in immunoregulation.

An important implication of our insight into CD8's ligand function is that CD8 peptides may be pharmaceutically exploited as immunomodulators. In this regard, the finding that a GPL-modified CD8 peptide retains the inhibitory ligand activity of the native molecule is particularly significant. A striking property of GPL-modified proteins is that they can be efficiently incorporated into biomembranes in the presence of low, nonlytic concentrations of detergents (Medof et al., 1984). Hence, biomembranes, such as those of cells or liposomes, could be readily coated with GPL-modified CD8 and used for immunotherapeutic purposes.

To explore CD8's role in immunoregulation, we have employed a series of complementary cellular coculture systems, schematized in Figure 23. These experimental systems encompass various permutations of responders, stimulators and inhibitors, and they were designed to address sequentially-related, but distinct, issues pertaining to CD8's effect upon immunological cell:cell interactions. Principal findings from this and our other recent investigations, can be summarized as
Figure 23. Schematic representation of cellular coculture systems used to examine CD8-mediated immunoregulatory phenomena.

Panel I: Nonresponsiveness of PBMC responders (a heterogeneous pool with regard to allospecificity of TCR) to cloned, CD8+ stimulator/inhibitor cells. Panel II: Refractoriness of secondary responders to CD8-mediated inhibition. Panel III: Refractoriness of cloned T cell responders to CD8-mediated inhibition. Panel IV: CD8+ cells function as inhibitors in responses of PBMC to CD8- stimulator cells (CD8+ inhibitors must express the same alloantigens as CD8- stimulators). Panel V: Induction of a nonresponsive state in PBMC responders by prior exposure to CD8+ cells.
follows:

(i) Primary responders are inhibited by CD8\(^+\) cells when the latter cells coexpress CD8 and specific alloantigen. This is most simply demonstrated in a simple "two-party" system, comprised of PBMC responders and irradiated, cloned stimulators (Figure 23, panel I). CD8 expression on a stimulator converts it into what can be termed a "stimulator/inhibitor", with this cell directing a CD8-mediated inhibitory signal against specific responders (Hambor et al., 1990). Both the proliferative response and generation of cytotoxicity, in such cultures, are abrogated by CD8-dependent mechanisms. The conclusion that responders are actually being inhibited by CD8, and that CD8 is not simply modulating the stimulator, is substantiated by a more complex "three-party" system comprised of PBMC responders, irradiated cloned (CD8\(^-\)) stimulators and third party inhibitors (Figure 23, panel IV). In this case, a third party cell, coexpressing CD8 and specific alloantigen, abrogates responsiveness to the stimulators.
(Kaplan et al., 1989). Both CD8⁺ T cells, and a variety of other non-T cell types converted to CD8-positivity by transfection, including K562, KM-102 and most recently JY.LCL cells (data not shown), mediate CD8-dependent immunoregulation in these coculture systems.

(ii) Secondary responders are not susceptible to CD8-dependent inhibition. Two distinct types of secondary responders were examined. In one case, PBMC responders were first stimulated with CD8⁻ stimulators and then secondarily stimulated with CD8⁺ variants of these cells (Figure 23, panel II). The second case entailed a highly simplified coculture comprised of cloned T cell responders and irradiated, cloned T cell stimulators (Figure 23, panel III). In both cases, the responders which are being secondarily stimulated are refractory to CD8's inhibitory effect.

(iii) Both CD4⁺ and CD8⁺ responders are inhabitable by CD8-dependent mechanisms, indicating that immunoregulation is dependent of the phenotype of the responders.

(iv) The inhibitory effect on specific responders
persists for at least 24h following removal of CD8\(^+\) inhibitors. This was demonstrated by first pretreating PBMC responders with fixed, plastic-adherent CD8\(^+\) stimulator/inhibitors, removing the treated PBMC responders from these plates, and subsequently stimulating with CD8\(^-\) stimulators after varying intervals (Figure 23, panel V). The short-term durability of nonresponsiveness was thereby demonstrated, but nonspecific loss of responsiveness at later time points prevented the analysis of long-term nonresponsiveness for establishing tolerance. Furthermore, the minimal exposure time for induction of nonresponsiveness was determined to be 12h.

Hence, this series of cellular coculture studies have defined some of the basic parameters which define the susceptibility of specific responders to CD8-mediated inhibition. There appears to be a window of susceptibility to inhibition, with primary, but not secondary, responders being susceptible to inhibition. The inhibition in the primary responders does not require the continuous presence of inhibitors and requires approximately 12h for the nonresponsive state
to be induced

The molecular mechanisms underlying CD8-mediated inhibitory signalling and the relationship of this to other forms of antigen-driven nonresponsiveness, remain to be determined. As it is known that CD8 physically associates with class I MHC (Rosenstein et al., 1989; Norment et al., 1988), it is tempting to speculate that the signal may be directed through class I MHC on the responder. In this regard, it is noteworthy that anti-class I MHC antibodies deliver inhibitory signals to responders (Santoli et al., 1988; Turco et al., 1985), and CD8 may mimic the antibody-mediated effect.

Our present findings for CD8 provide an example of a situation in which a cell's physiological state dictates its responsiveness to external molecular stimuli. The refractoriness of responders in secondary MLRs and of clones T cell responders to CD8-mediated inhibition suggests that in some way, a cell that has undergone prior specific or nonspecific stimulation via its T cell receptor loses its susceptibility to the inhibitory effects of the CD8 ligand. This may reflect some sort of "post-activation imprint" on the T cell. The precise molecular determinants that define the window of susceptibility to CD8-dependent inhibition are
unclear, and these could entail, for instance, loss or alteration of CD8 receptors or override mechanisms in the CD8 inhibitory pathway.

These insights into the immunoregulatory function of the CD8 molecule have relevance for understanding the phenomenology of immunological assays such as the classical mixed lymphocyte reaction (MLR). For instance, a conceivable explanation for the predominant class II MHC-specific proliferation in the MLR is that the response to allo-class I MHC is inhibited by the CD8/allo-class I MHC coexpressors in the stimulator population. In contrast, an unmanipulated stimulator population does not contain significant numbers of CD8/allo-class II MHC coexpressors.

The antigenic recognition/cytotoxicity and immunoregulatory effector functions of CD8$^+$ T cells have traditionally been thought of in distinct terms and considered to be mediated by distinct subsets of cells. However, it is interesting to speculate that CD8's inhibitory ligand function may simultaneously play a role in both killing and regulatory activities, and that cytotoxic T cells could in fact inhibit, and thereby regulate, target cells through CD8. In such a scenario, CD8's multifunctionality would be manifest in a single
cell. In other words, CD8 would serve both as an accessory molecule for specific cytotoxic T cell activation and as a ligand for target cell inactivation.

These studies have not addressed the issue of the relevance of CD8-dependent inhibition, as demonstrated in vitro, to natural immunoregulation in vivo. Nonetheless, the potency of the effects that we have documented in vitro make in vivo relevance seem highly likely. Of possible significance is this regard is that activated T cells express class II MHC and can function as antigen-presenting cells (Zier and Pickard, 1985). Hence an activated CD8+ cell acting in an antigen-presenting mode would serve to inhibit responsiveness to specific antigen. In addition to its potential role in peripheral immune mechanisms, it will be of considerable interest to ascertain whether CD8 plays a role in thymic regulatory events, and in particular, whether there is any significance in this regard to the fact that double positive T cells (CD4+8+) are the precursors for both CD4+8- and CD4-8+ T cells. It is at the double positive stage that both negative and positive thymic selection events transpire (Schwartz, 1989).

Our CD8 studies have established a general
experimental strategem for analysis of molecular function. This line of investigation has entailed the sequential application of antisense and sense transfection technologies to first define, and then probe, molecular function. The power of the antisense approach, as a subset of mutagenesis strategies, resides in the fact that in this case one is studying molecular function in the native cellular setting. Once a particular function is defined for a molecule, sense transfection technology can be applied in two principle contexts: (i) the native molecule, or engineered derivatives of the molecule, can be expressed in a foreign cellular setting; and (ii) engineered derivatives of the molecule can be expressed in a native cellular setting following the elimination of the natural form of that molecule in the cell. To enable the latter, we have recently developed dual episomal expression vectors, alternatively based upon Epstein-Barr virus and BK virus replicative signals and incorporating distinct selectable markers, which enable coupled antisense and sense transfection technologies in the same cell (Groger, R.K., and Tykocinski, M.L., unpublished observations). The combinatorial application of antisense and sense transfection
strategies, as applied to CD8, will serve as a paradigm for the analysis of other molecules, and in so doing, perhaps expand on the multifunctional perspective of individual molecules in the immune and other biological systems.
VII. Significance and Speculation

The application of antisense and sense transfection strategies provided a complementary approach for unravelling the functional repertoire of the human T cell-specific molecule, CD8. For this purpose, EBV-based episomal replicons containing the RSV 3' LTR promoter element (REP vectors) were developed for gene expression in human cells. In order to define those cellular functions for which CD8 is required in its natural cellular setting, antisense RNA mutagenesis and T cell cloning technologies were linked. The study of antisense CD8 transfectants, representing phenocopies of null CD8 mutations, for two human CD8+ T cell clones revealed that CD8 is obligatorily required as an accessory molecule to the TCR for antigen-specific, but not antigen-nonspecific (anti-CD3, lectin), activation of a variety of T cell functions: proliferation, lymphokine secretion, lymphokine receptor expression, and cytotoxicity. An additional line of investigation, initially using the CD8+/CD8- antisense-generated T cell clonal pairs in a model MLR-based cellular coculture system, suggested that the CD8 molecule itself may be a critical molecular determinant which mediates the...
immunoregulatory activity of CD8\(^+\) T cells. Exploration of CD8-dependent immunoregulation by transfection and expression of native and genetically engineered CD8 molecules in non-T cell lines, resulting in CD8\(^+\)/CD8\(^-\)-sense-generated nonlymphoid cell pairs, further defined CD8's inhibitory ligand function. Thus, the experimental evidence presented in this thesis indicates that CD8 is a bifunctional molecule with two distinct activities. On the one hand, CD8 functions in cis as an accessory molecule to the TCR that improves the specific recognition of MHC class I-restricted antigens on target cells or antigen presenting cells by CTLs; on the other hand, CD8 functions in trans as a novel inhibitory ligand that allows CD8\(^+\) cells to regulate the activities of other T cells in a cognate manner.

The functional analysis of a CD8\(^-\) variant of the human cytotoxic T cell clone, JH.ARL.1, clearly demonstrated that CD8 is required for antigen recognition but is not essential for the triggering of cytotoxicity, since this and other T cell functions can be elicited by antigen-nonspecific stimuli. This interpretation is supported by a related series of experiments (Munakata et al., 1988) using a number of variants of a long-term mouse CD8\(^+\) CTL clone that have
reduced or abolished expression of CD8, either alone or in combination with other deficiencies (CD3, LFA-1, cytolyisin). Further studies demonstrating that reexpression of CD8 by transfection of the CD8 gene restores specific cytotoxicity in these CD8-deficient variants provided more definitive proof that CD8 is required in recognition of MHC class I-restricted antigen (Eichmann et al., 1989).

In light of these studies on activated cytotoxic T cells, together with additional studies on thymocytes (Ramsdell et al., 1989) and resting virgin T cells (Emmrich et al., 1986, 1987; Eichmann et al., 1987; Boyce et al., 1988; Jonsson et al., 1989), it seems possible that the role of CD8 as a functional accessory molecule may change as T cells undergo maturation. During thymic development, the association of CD8 with the TCR causes positive selection and/or maturation from immature to mature thymocytes; however, CD8's exact role in this process is unclear. In resting, peripheral T cells, CD8 is obligatorily involved in MHC class I-restricted antigen recognition as well as in the activation from a resting state to growth and acquisition of effector activity. At this developmental stage, the essential role for CD8 lies not only in its
binding to MHC class I molecules in trans, but also in its association with the TCR in cis which has been shown to provide a signal (possibly through activation of p56^lck) especially directed towards the expression of IL 2 receptors and growth in IL 2 (Jonsson et al., 1989). However, in activated CTLs, CD8 may be involved merely as an auxiliary binding structure that improves the specific recognition of target cells through the TCR, but does not directly participate in the mechanism of cytotoxicity. Results from this work (Hambor et al., 1988a,b) and other studies (Munakata et al., 1988; Eichman et al., 1989), demonstrating that nonspecific induction of cytotoxicity and other T cell functions is maintained (i.e., neither enhanced nor diminished) in the CD8^- variants as well as the reconstituted CD8 sense transfectants, suggest that CD8 does not participate in T cell effector activities per se. At this more mature developmental stage, CD8 does not appear to be directly involved in signal transduction, but merely functions as a binding molecule which can associate with MHC class I molecules on target cells. In this manner, CD8 could assist in focusing the TCR towards MHC class I molecules (perhaps required only in cases of low TCR avidity) and thus aid in the recognition process without providing a
signaling activity. Although CD8 can associate in trans with MHC class I molecules on another cell and in cis with the TCR on the same cell, both activities are, in principle, independent, but may influence each other. Since CD8 exists in a variety of forms (α versus α'; homodimers versus heterodimers) which may differentially effect its association with p56<sub>1ck</sub>, and since expression of these isoforms may be differentially regulated during T cell maturation, it seems likely that changes in the composition of the CD8 molecule could regulate its associations with other molecules and thus in part account for the distinct cellular responses triggered through the TCR at different stages of T cell development.

In addition to its accessory function, these studies have identified the CD8 molecule as a critical molecular component of the regulatory machinery of CD8<sup>+</sup> T cells, ascribing to it a novel inhibitory ligand function. This represents the first step towards understanding the precise molecular mechanism(s) that mediate the immunoregulatory activities of CD8<sup>+</sup> T cells. Exploration of CD8's immunoregulatory role in a series of complementary coculture systems (Figure 23) resulted in a number of principle findings: (1) Cells which
coexpress CD8 and specific alloantigen are potent tolerogens which can induce an antigen-specific, nonresponsive state in naive, primary responder T cells that were in a resting or virgin state; (2) Both the proliferative response and the generation of effector function (cytotoxicity) are inhibited by CD8+ cells; (3) Mature, secondary or clonal responders, which have previously "seen" antigen and are in an activated state, are not susceptible to CD8-dependent inhibition; (4) both CD4+ and CD8+ resting, primary T cells are inhibitable by a CD8-mediated mechanism; and (5) supplementation with IL 2, phorbol ester (PMA), or calcium ionophore (A23187) does not overcome CD8-dependent inhibition (data not shown).

CD8, as an inhibitory ligand, endows cells with veto-like activity (Miller, 1987; Fink et al., 1988) in what can be more generally described as "cognate regulation". By recognizing an antigen on the surface of a CD8+ cell in a process that requires direct cell:cell contact, a resting T cell is induced in a nonfunctional manner, resulting in a state of tolerization for that T cell. Though specific nonresponsiveness has been demonstrated to persist for at least 24 hr following removal of CD8+ inhibitory
cells, it is unknown if this anergic state is permanent or reversible. The susceptibility of resting, but not activated, T cells to CD8-dependent cognate regulation parallels the observation that cloned CTL are insensitive to inactivation by veto (Miller, 1987; Fink et al., 1988), and suggests that some sort of post-activation imprint occurs in T cells which have been stimulated previously through the TCR/CD3 complex. If activated T cells are able to return to a resting state, it is unknown if these cells are also susceptible to CD8-mediated inhibition.

Control of lymphocyte proliferation has been proposed by others to occur, at least in part, at the level of the antigen presenting cell (Bretscher and Cohn, 1970; Miller, 1986; Fink et al., 1988; Mueller et al., 1989). In these models, the proliferation of T cells and development of effector T cell functions relies on the simultaneous occupancy of the TCR, resulting in signaling through the associated CD3 complex, and delivery of an accessory cell-derived costimulatory signal (Weaver et al., 1988; McKenzie, 1988; Holsti and Raulet, 1989; Houssiau et al., 1989; Weaver and Unanue, 1990). TCR occupancy and subsequent CD3-mediated signaling alone is interpreted by the T
cell as a signal to down-regulate further antigen responsiveness and possibly maintain self-tolerance (Lamb et al., 1983, 1987; Zanders et al., 1983; Feldmann et al., 1985; Roska and Lipsky, 1985; Geppert and Lipsky, 1987; Jenkins et al., 1987a,b, 1988; Jenkins and Schwartz, 1987c; Quill and Schwartz, 1987; Mueller et al., 1989a). At the biochemical level, TCR occupancy by peptide in the context of a MHC restricting element, independent of an accessory cell-induced costimulatory signal, is sufficient to induce hydrolysis of PIP$_2$ which sequentially leads to increases in [Ca$^{+2}$] and activation of PKC (as measured by phosphorylation of CD3-γ). Together these biochemical signals are insufficient to induce T cell proliferation; this requires the generation of a costimulatory signal acting independently on the IL 2 and IL 2 receptor genes (Schmidberger et al., 1988). Thus, T cell IL 2 and IL 2 receptor gene activation and subsequent proliferation are dependent on a number of distinct, coordinated biochemical signals. Furthermore, the generation of a [Ca$^{+2}$] or PKC signal in the absence of a costimulatory signal, induces a state of proliferative unresponsiveness (Mueller et al., 1989b, 1990). It now appears that a variety of factors, such as the cytokines
IL 1, 2, 4, and 6, as well as IFN-γ, are believed to fulfill the role of the essential costimulatory signal(s), and to act in synergy for induction of T cell activation, proliferation and differentiation (Miethke et al., 1988; German et al., 1988; McKenzie, 1988; Holsti and Raulet, 1989; Houssiau et al., 1989; Maraskovsky et al., 1989; Weaver and Unanue, 1990). Therefore, the innate ability of different cell types to provide costimulatory signal(s) may dictate if presentation of antigen by a given cell results in T cell responsiveness or nonresponsiveness.

The discovery of CD8 as an inhibitory ligand may represent a new category of factors that can influence the outcome of antigen presentation by a given cell type. In this case, expression of CD8 on a cell's surface confers veto-like activity upon that cell; i.e., if a resting T cell recognizes an alloantigen (or possibly processed endogenous or exogenous antigen) on the surface of a CD8⁺ cell through engagement of it's CD3/TCR complex, specific nonresponsiveness is induced in that T cell. Although the inhibitory ligand function has been identified to reside within the extracellular domain of the CD8α chain, the exact mechanism(s) by which CD8 can induce a nonresponsive state in resting T
cells still remains to be elucidated. In addition, the reasons for the refractoriness of activated T cells to CD8-dependent inhibition, are also unclear. However, the fact that cloned T cells can be induced into a nonresponsive state merely by the lack of an appropriate costimulatory signal from the antigen presenting cell (Mueller et al., 1989a) clearly distinguishes this mechanism of clonal inactivation from that mediated by CD8's inhibitory ligand activity which does not effect activated T cells.

Cell surface expression of CD8 may impact upon T cell:T cell cognate interactions at a number of levels. Since CD8 can associate in cis with MHC class I molecules on the membrane of the same cell, expression of CD8 could potentially conceal MHC class I alloantigens (or MHC class I-restricted antigens) on an antigen-presenting CD8+ T cell. This could occur either by CD8 directly binding to the α3 domain of MHC class I molecules, thus making this important site inaccessible, or by CD8 indirectly obscuring the α1 and α2 domains of MHC class I molecules, thus preventing recognition by specific TCR/CD3 complexes. In this manner, CD8+ cells whose MHC class I molecules are camouflaged by CD8 could go undetected by a potentially-reactive T cell.
However, this mechanism does not explain the inability of T cells (CD4+ T cells, for the most part) to recognize and respond to MHC class II molecules on CD8+ antigen presenting cells since CD8 has not been shown to associate in cis with, and thus could not disguise, MHC class II molecules. The finding that CD8+ cells which express both MHC class I and II molecules (human CD8+ T cell clones and the CD8 sense-transfectant of JY.LCL, data not shown) are incapable of eliciting a proliferative response in our model coculture systems indicates that CD8 expression on an antigen presenting cell induces T cell nonresponsiveness to both MHC class I and II molecules. In addition, the mere unrecognizability of cell surface antigens does not explain the potent inhibitory activity of these CD8+ immunoregulatory cells. Therefore, this mechanism alone does not completely account for all the experimental evidence encompassing CD8's inhibitory ligand activity.

As an alternative model to explain CD8-dependent inhibition, CD8 could directly modulate the generation of essential second messengers. Signal transduction by the TCR/CD3 complex involves activation of phospholipase C, a process generally regulated by guanine nucleotide binding (G)-proteins, which leads to generation of
inositolphosphates (IP) and diacylglycerol (DAG). Subsequently, IP$_3$ elevates intracellular [Ca$^{2+}$] levels by releasing calcium from internal stores and opening voltage-independent calcium channels, and DAG activates protein kinase C, a pivotal enzyme in the regulation of cellular functions and growth. By physically interfering with the TCR/CD3 complex and/or costimulatory factor receptor(s) on potentially-reactive T cells, CD8 could thus influence second messenger generation or action and, in turn, promote T cell nonresponsiveness. Alternatively, CD8 may directly prevent the expression or delivery of a costimulatory signal by an antigen-presenting T cell. By obscuring a costimulatory activity that is required by resting, but not activated, T cells, a nonresponsive state could be induced.

G-proteins transduce signals from surface receptors to intracellular target enzymes such as adenylate cyclase and phospholipase C, as well as regulate exocytosis and cell growth (Stryer and Bourne, 1986; Barrowman et al., 1986; Gilman, 1987; Burgoyne, 1987; Melancon et al., 1987; Barbacid, 1987; Bourne, 1988). Several G-proteins have been identified in T cells, yet the functional significance of these different isoforms
is unknown (Pessa-Morikawa et al., 1990). The finding that modulation of a TCR-associated G-protein leads to a general loss of transduction potential in T cells (Schrezenmeier et al., 1988), and that different receptors on a single cell type can transduce the same signal (phospholipase C activation) via at least two different G-proteins (Ashkenazi et al., 1989) suggests the possibility that CD8 could interfere with signal transduction through the TCR/CD3 complex by directly regulating either the TCR-associated G-protein or a related G-protein. In addition, since a change in the expression of some, but not all, G-proteins occurs during T cell activation, a means for the differential sensitivity of resting and activated T cells to CD8-mediated immunoregulation could also exist.

In lieu of the fact that prolonged stimulation of protein kinase C, resulting in translocation to the nuclear membrane, is necessary for activation of T cells (Berry et al., 1990; Szamel et al., 1990), it is possible that CD8 may directly or indirectly curtail this prolonged signal requirement. CD8 could induce rapid feedback inhibition of TCR/CD3-mediated DAG formation through the action of a protein kinase (Hunter, 1987), possibly by an effect on the G-protein,
which couples the TCR/CD3 complex to phospholipase C. The finding that heterogeneity in coupling of the TCR/CD3 complex to phospholipase C exists at various stages of T cell differentiation (Brattsand et al., 1990) could potentially have important implications for CD8-mediated antagonism of signal transduction pathways in resting versus activated T cells.

Another possible mechanism that might be attributable for CD8's inhibitory ligand activity is that CD8 may transmit a signal to the naive T cell that inactivates the signal transduced by either the costimulatory factor(s) or the TCR/CD3 complex. If this were the case, then the putative receptor for the CD8 ligand, which transduces this negative signal, would only be functional in resting, but not in activated, T cells.

Since CD8 has been shown to directly bind in trans to MHC class I molecules, this interaction could represent the putative ligand/receptor pair responsible for transduction of CD8's inhibitory ligand activity. The demonstration that MHC class I behave like receptor molecules (i.e., they are internalized via coated pits and coated vesicles upon binding ligand - in this case, anti-MHC class I antibodies) has led to the suggestion
that MHC class I molecules are involved in the regulation of T cell activation and differentiation (Dasgupta and Yunis, 1987; Dasgupta et al., 1988). In this regard, anti-class I antibodies (which could mimic the natural CD8 ligand) have been shown to have an inhibitory effect on the proliferation of resting, but not activated, T cells and on the development, but not the triggering, of T cell effector functions (Sterkers et al., 1983; Turco et al., 1985; Taylor et al., 1986; Spagnoli et al., 1987; DeFelice et al., 1987; O'Neill, 1988a,b; Santoli et al., 1988; Schwab et al., 1988).

Signals transduced via MHC class I receptors on resting T cells upon binding CD8 ligands could directly interfere with those transduced by either the TCR/CD3 complex or the costimulatory factor(s) and thus lead to induction of a nonresponsive state. Whereas cross-linking of MHC class I molecules on T cells leads to a slow, gradual and sustained rise in intracellular [Ca\(^{2+}\)] (Dissing et al., 1990) and does not result in cell proliferation, cross-linking of either CD2, CD3 or CD28 leads to a prompt, sharp and transient increase in intracellular [Ca\(^{2+}\)] and does lead to cell proliferation (Weiss et al., 1984; Brottier et al., 1985; June et al., 1986; Ledbetter et al., 1987; Moretta et al., 1987).
These findings probably reflect that different mechanisms of regulation of intracellular calcium levels are operating when T cells are activated via CD3 and MHC class I molecules, respectively, and suggest that anti-CD3 binding and MHC class I cross-linking results in activation of different arrays of intracellular second messangers. In light of the observation that T cells activated by MHC class I cross-linking are refractory to subsequent stimulation with anti-CD3 antibody or PHA (Davis et al., 1989; Dissing et al., 1990), it is tempting to speculate that CD8-mediated, MHC class I cross-linking might similarly induce a sustained elevation on intracellular [Ca\(^{2+}\)] resulting in a state of nonresponsiveness to any further T cell stimulation. In fact, the conformation of the TCR/CD3 complex, and possibly its signal-transducing function, is directly modified by calcium ions (Breittmayer et al., 1989). Thus, a noncoordinated rise in intracellular calcium might in some cases lead to induction of tolerance instead of T cell activation.

Besides production of an inhibitory effect as a result of direct CD8-mediated signal transduction through MHC class I molecules, indirect inhibitory effects of this interaction are also possible since MHC
class I molecules appear to associate in cis with receptors for peptide hormones such as IL 2 (Sharon et al., 1988), insulin (Due et al., 1986; Simonsen et al., 1985; Verland et al., 1989), epidermal growth factor (Schreiber et al., 1984), glucagon, γ-endorphin and thromboxane (Edidin, 1983), as well as with other molecules involved in signal transduction such as CD4/CD8 (Wacholtz et al., 1989), CD2 (Turco et al., 1988), MHC class II (Szollosi et al., 1989) and the TCR/CD3 complex (Bushkin et al., 1986; Brans et al., 1989). Recent experimental evidence suggests that, besides acting as signal-transducing molecules, MHC class I molecules may act as modulators of other signal-transducing molecular complexes in the membrane of T and B lymphocytes (Dasgupta et al., 1987; Southern and Dutton, 1989; Dissing et al., 1990). Therefore, the binding of CD8 in trans to MHC class I molecules may effect the signal-transmitting function of these cis-associated molecular complexes.

In contrast to resting T cells, Mab-mediated cross-linking of MHC class I molecules themselves or in combination with CD8 molecules appears to deliver a functionally important signal to activated T cells and suggests that MHC class I molecules may function to
transduce activation, rather than inhibitory, signals in activated T cells (Chouaib et al., 1988; Geppert et al., 1989; Bushkin et al., 1990; Dissing et al., 1990). This suggests that the outcome of signal transduction through MHC class I molecules may vary at different stages of T cell development; whereas MHC class I molecules could mediate a negative signal in resting T cells resulting in nonresponsiveness, MHC class I molecules could mediate a positive signal in activated T cells resulting in responsiveness. Thus, the correlation of structure and function or expression of MHC on resting and activated T cells could be different. In this regard, activated, but not resting, T cells express a considerable number of surface MHC class I molecules not associated with β2m (Schnabl et al., 1990). Since the conformational properties of MHC molecules unlinked to β2m are different from β2m-associated MHC class I molecules (Krangel et al., 1979; Lancet et al., 1979; Wilkinson et al., 1982), it is tempting to speculate that, upon T cell activation, the loss of β2m and subsequent conformational alteration of MHC class I proteins may have some important functional implications. Furthermore, the fact that expression of MHC class I molecules is differentially regulated
following T cell activation (Matsui, 1987), suggests that changes in MHC class I density on the surface of T cells may affect their role in cellular interactions.

Expression of a number of molecular changes occurs during T cell differentiation (Rettig and Old, 1989; Borst et al., 1989), including the expression of IL 1 and MHC class II molecules in activated, but not resting, T cells (Robbins et al., 1988; Bhardwaj et al., 1989; Tartakovsky et al., 1989; Diedrichs and Schendel, 1989). These more general phenotypic changes could also, in part, reflect the susceptibility of resting, but not activated, T cells to CD8-mediated inhibition. Furthermore, it is imaginable that the expression of an inducible costimulatory factor, such as IL 1, in addition to specific alloantigen (or MHC-restricted antigen) and CD8, is required for a CD8$^+$ cell to function as an immunoregulatory cell. If this were the case, then only activated CD8$^+$ T cells could regulate cognate interactions between T cells. This seems reasonable since resting CD8$^+$ T cells found in a normal mixed lymphocyte culture do not appear to have inhibitory activity, whereas activated CD8$^+$ T cells have been shown to have both veto and suppressor activity. Although activated CD8$^+$ cells can function as
immunoregulatory effector cells, they are incapable of regulating the activities of other activated T cells. Maybe another immunoregulatory mechanism is operable in this instance.

Since expression of the CD8 molecule is also inducible on mature CD4+ T cells (Blue et al., 1986; Paliard et al., 1988; Melchers and Rzepka, 1988, Melchers et al., 1988) and on thymocytes (Blue et al. 1989, Tatsumi et al., 1990), the capacity to transiently generate needed immunoregulatory cells on a temporary basis is possible. This ability lends further credence to the concept that T cell function is plastic.

The possibility that both CD8's accessory and inhibitory ligand functions may themselves be regulated is suggested by the fact that CD8 can associate with MHC class I molecules both in cis and in trans. Specifically, prior to a trans interaction between CD8 and MHC class I molecules, cis-associated disulfide bonds between CD8 and MHC class I molecules would have to be broken. Although the significance of this cis-trans conversion is unclear, given that MHC class I molecules are expressed on most somatic cells, it is conceivable that if CD8 were not complexed in cis with MHC class I molecules, CD8+ immunoregulatory T cells
might adhere via a trans CD8/MHC class I interaction to all cells.

These proposed mechanisms for CD8-dependent inhibition of naive, but not mature, T cells are not mutually exclusive. Clearly, elaboration of the mechanism(s) by which CD8 functions as an inhibitory ligand awaits both the identification of the molecule through which CD8's inhibitory ligand activity is conveyed, as well as a greater understanding of the exact requirements for T cell activation at different stages of development.

T cells play a pivotal role in the development of proliferative, helper (via lymphokines), delayed-type hypersensitivity (DTH) and cytotoxic responses to foreign antigens; however, T lymphocyte proliferation must be tightly regulated to protect against the development of inappropriate immune responses to self antigens, or exaggerated, pathologic responses to foreign antigens. Deletion of autoreactive T cell clones during maturation in the thymus, and induction of regulatory or suppressive T cell populations in the periphery both appear to represent control mechanisms capable of modifying the potential for T cell responsiveness to antigen. By posing as regulatory
antigen presenting cells, CD8⁺ T cells could participate in these control mechanisms for self-nonself discrimination and tolerance induction. In this regard, T cells have been shown to function as antigen presenting cells (Zier and Pickard, 1985; Kabelitz et al., 1987; Vitiello et al., 1989; Hewitt and Feldman, 1989).

The continued synthesis and presentation of self-antigens by CD8⁺ T cells could regulate autoreactive T cells, by either clonal deletion or clonal anergy, in both the thymus or in the periphery. The fact that CD8⁺ regulatory T cells could efficiently produce, process and present endogenous self antigen, but inefficiently process and present exogeneous foreign (nonself) antigen, makes them ideal candidates for discriminators of self and nonself. While self antigen is widely distributed and continually present, foreign antigen is narrowly distributed (located within lymphatic tissue in a very precise manner) and rapidly removed by efficient phagocytic cells (such as monocytes, macrophages and reticular dendritic cells in germinal centers), as well as by other professional antigen presenting cells (such as B cells). Thus, the innate ability of CD8⁺ regulatory T cells to distinguish
between self and nonself may, in part, be dictated by the location and duration of antigen.

Such self-nonself discrimination is subject to two forms of malfunction. First, some self antigens that are limited in their distribution in the body may gain access to stimulatory antigen presenting cells and induce immune responses. As in the initiation of an immune response to a limited foreign antigen, the enhanced ability of professional antigen presenting cells to outmaneuver CD8\(^+\) regulatory T cells in the capture of these released self antigens (available in limited quantities) allows for the initiation of an autoimmune response by specific pathogenic T cells. This is the case in a number of transient or permanent autoimmune conditions, such as sympathetic ophthalmia. Second, the wide distribution of foreign antigens may inactivate the immune response, such as in septicemia or metastatic tumor growth. As in the case of continued exposure to self antigens, chronic or excessive exposure of foreign antigen would allow inefficient CD8\(^+\) regulatory T cells to capture, process and present foreign antigen, and, in turn, inactivate any potentially-reactive T cell with specificity for that foreign antigen.

An imperfection of this immunoregulatory apparatus
involving CD8\(^+\) T cells might entail the exploitation of these inhibitory cells as havens for parasites. Viruses and other intracellular microorganisms could elude recognition by the host's immune system by specifically infecting CD8\(^+\) T cells. Indeed, persistent viruses such as lymphocytic choriomeningitis virus (LCMV) and human immunodeficiency virus (HIV, causative agent of AIDS) are harbored by CD8\(^+\) T cells and are inefficiently recognized by host defense mechanisms (McCheyne and Oldstone, 1987; Leist et al., 1988; Joly et al., 1989; Tsubota et al., 1989).

The extent of CD8-dependent immunoregulation in vivo is uncertain at present. Definition of the in vivo niche for CD8-mediated inhibition will require the development of animal model systems. Further investigations of the mechanisms underlying this novel inhibitory phenomenon using both in vitro and in vivo models should allow for the potential exploitation of the CD8 molecule in immunotherapeutic contexts, such as for facilitating tissue allograft survival and for inactivating pathogenic T cells which arise in various autoimmune diseases. Clearly, this thesis merely represents the first steps towards understanding the complex immunological principles behind this
immunoregulatory mechanism.
VIII. Literature Cited


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