Immune Recognition of HLA Molecules Downmodulates CD8 Expression on Cytotoxic T Lymphocytes

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Summary

An HLA-A2$^+$ cytotoxic T lymphocyte (CTL) line restricted by HLA-A2 in recognition of an influenza B virus nucleoprotein (BNP) peptide uses the CD8 coreceptor in the recognition of this viral peptide. Incubation of these CTL with BNP peptide in the absence of antigen-presenting cells downmodulates CD8α and CD8β expression and reduces their ability to lyse target cells without inducing self-lysis. CD8 downmodulation was dependent on peptide concentration, time of exposure, and T cell receptor specificity. Another viral peptide from the influenza A virus matrix protein interacting with HLA-A2 had no effect on CD8 expression. Upon further investigation, an anti-HLA class I monoclonal antibody (mAb), anti-HLA class II mAb, and HLA alloantisera were found to downmodulate CD8α and CD8β expression and induce CTL nonresponsiveness without causing degranulation. When CD8α and CD8β expression was modulated by viral peptide or anti-HLA mAbs, other cell surface molecules were unchanged. Finally, incubation of peripheral blood lymphocytes with these anti-HLA mAbs induced no change in CD8 expression on resting cells but did downmodulate it on mitogen-activated cells. These results suggest that T cell recognition of the HLA-A2-BNP peptide complex on neighboring CTL may be the mechanism for CD8 downmodulation induced by the BNP viral peptide. This mechanism may be important in clonal anergy.

Activation of T lymphocytes occurs when the TCR recognizes HLA molecules and antigen on APC (1). In binding to this ligand, the TCR is assisted by CD8 or CD4 accessory molecules expressed on T lymphocytes (2, 3). In general, CD4 and CD8 molecules are expressed in a mutually exclusive manner on mature T cells and have therefore been used to define two T cell subsets (4, 5). The majority of CD8$^+$ cells with cytotoxic function recognize antigen with HLA class I molecules, whereas CD4$^+$ cells with helper function recognize antigen with HLA class II molecules (6, 7). In this T cell recognition, CD8 and CD4 molecules are thought to both strengthen the interaction between T cells and APC by binding to HLA molecules (8–10) and assist in activation through their association with a tyrosine protein kinase (11–13). When CD8 or CD4 expression is blocked with specific mAbs, T cells can not be activated (14, 15).

The importance of CD8 molecules in T cell responsiveness has also been established from studies using cloned TCR genes. When a transgene TCR restricted by H-2D^b in its specificity for fluorescein is expressed on CD8$^-$ cells, no lysis of lymphoblast target cells occurs unless a cotransfected CD8 gene is expressed along with the transgene TCR (16). Male mice carrying a transgene TCR specific for the HY male antigen and restricted by the class I molecule, H-2D^b, have peripheral T cells nonresponsive to the HY antigen (17). This peripheral T cell tolerance is apparently brought about by peripheral T cells expressing the transgene TCR on T cells with low expression of CD8. Downmodulation of CD8 molecules could explain why the transgene T cells have low CD8 expression, as downmodulation of IgM has been correlated with B cell nonresponsiveness (18). CD8 molecules have been found to be downmodulated on thymocytes incubated with anti-CD3 or PMA and on alloreactive CTL treated with PMA or alloantigen (19, 20), although neither the signal nor the effect of this downmodulation on T cell responsiveness have been determined.

In this study, a CTL line, from individual A.T., restricted by HLA-A2 and specific for a viral peptide from influenza B nucleoprotein (BNP),[1] 82–94, (21) has been used. Direct interaction of the viral peptide with these CTL was investigated since preincubation of helper T cells with their target viral peptide or of HLA-A2 alloreactive CTL with an HLA-A2 peptide has been reported to inhibit T cell responsiveness (22, 23). CTL incubated with the BNP peptide downmodulate CD8α and CD8β expression and decrease their ability

[1] *Abbreviation used in this paper:* BNP, influenza B nucleoprotein.

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to lyse target cells without displaying any self lysis of themselves. The effect appears to be specific to CD8α and CD8β, and not due to cocapping of several cell surface molecules, since incubation of CTL with the BNP peptide did not change CD3, HLA-A2, HLA-DR, or β_2-microgloblin expression. This effect is dependent on time of exposure to peptide, concentration of peptide, and TCR specificity, because another viral peptide from the influenza A matrix protein that interacts with HLA-A2 does not downmodulate CD8 expression on this CTL line. In determining how the viral peptide downmodulated CD8, two anti-HLA mAbs and HLA alloantisera were identified that also downmodulated CD8 expression. CTL incubated with anti-HLA-A2 (clone MA2.1) or anti-HLA-DR (clone L243) downmodulated CD8α and CD8β expression and lost responsiveness, without changing expression of other cell surface molecules. CD8 downmodulation by anti-HLA mAbs depends on mAb concentration, presence of antibody aggregates, and time of exposure. Both mAbs downmodulated CD8 on activated PBL without affecting CD8 expression on resting PBL. These results suggest that crosslinking HLA molecules on activated T cells downmodulates CD8 expression, thereby inducing T cell nonresponsiveness.

Materials and Methods

Cytotoxic T Lymphocytes. The A.T. CTL line grown from an influenza B virus-specific polyclonal culture and has been shown to be HLA-A2 restricted and specific for a peptide from the influenza B virus nucleoprotein, 82–94, as described (21).

Cytotoxic T Lymphocyte Assay. CTL killing was quantitated by a 5-h ^{51}Cr-release assay as described (24). Target cells were autologous EBV-transformed B lymphocytes labeled with sodium ^{51}Cr-chromate (Amersham International, Amersham, UK) and incubated for 1 h at 37°C with 100 μg/ml viral peptide in RPMI with 10% FCS or medium alone. Target cells were washed three times in RPMI with 10% FCS before addition to the assay. OKT3 and MA2.1 hybridoma cells used as target cells were radiolabeled, washed, and added to the CTL assay. CTL used as target cells were labeled with ^{51}Cr-chromate, incubated for 2 h with 100 μg/ml viral peptide, washed, and incubated for 5 h in a CTL assay. CTL prepared as targets were viable, and the spontaneous release in the absence of effectors was <20%. Where indicated in the text, CTL (10^6 cells/ml) used as effectors were preincubated for 10 h with anti-HLA mAb at 100 μg/ml in RPMI with 10% FCS, or preincubated for 2–10 h with 100 μg/ml viral peptide, washed, and added to the CTL assay at the indicated killer to target (K/T) ratio. This incubation had no effect on cell viability.

Antibodies, Purification, and Antibody Conjugates. Purified antibodies were obtained from ascites as described (25). Briefly, immunoglobulin was precipitated from ascites twice by 45% saturated ammonium sulfate, resuspended in PBS with 0.1% NaN_3, and purified by gel filtration using G-200 superfine or by affinity chromatography using recombinant protein G. Purity was determined by coomassie SDS-PAGE, and column fractions were pooled. Purified mAbs were stored in PBS with 0.1% NaN_3 at −70°C. Aggregate formation was facilitated by storage at 4°C. Anti-β_2-microglobulin (clone L368), FITC-conjugated anti-CD2, FITC-conjugated anti-IL-2R, PE-conjugated anti-CD3, and FITC-conjugated anti-CD3 were gifts from Becton Dickinson Immunocytometry Labs (Mountain View, CA). Other mAb clones used were anti-CD8α (clone B941), anti-CD8β (mAb 597; reference 26), anti-HLA-DR (clone L243), and anti-HLA-A2 (clones PA2.1 and MA2.1). FITC was conjugated to purified mAb (2 mg/ml) at 100 μg FITC per milligram of antibody overnight at 4°C, and purified over PD10 columns as described (27). HPLC profiles of purified antibody were obtained on 100-μl mAb aliquots loaded at different concentrations onto a Superose 12 column on FPLC system (Pharmacia Fine Chemicals, Piscataway, NJ) run at 0.25 ml/min collecting 250-μl fractions monitored by UV absorption.

Immunofluorescence. CTL (2×10^5 cells) and PBL (10^6 cells) were used for immunofluorescence staining. Cells were resuspended in 100 μl PBS with 0.10% NaN_3 containing 5 μg/ml PE-conjugated or 20 μg/ml (saturating concentrations) FITC-conjugated mAb. After a 15-min incubation at 4°C, cells were washed and resuspended in PBS containing 1% paraformaldehyde. Cells were protected from light and kept at 4°C until analysis by flow cytometry.

Flow Cytometry. Immunofluorescence data was detected on a FACScan, which distributes the fluorescence intensity over a four-decade log scale (Becton Dickinson Immunocytometry Systems). Each immunofluorescence profile shown in the text represents 5,000 live cells gated by forward-angle and 90° light scatter.

Results

T Cell Nonresponsiveness by Viral Peptide and Anti-CD8. The A.T. CTL line is specific for the influenza BNP peptide, 82–94, restricted by HLA-A2 (21). A.T. CTL are nonresponsive in the presence of anti-CD8 but not in the presence of another mAb of identical isotype and concentration, demonstrating the requirement of CD8 in recognition of the BNP peptide (Fig. 1 *A*). A.T. CTL are also less responsive to target cells after preincubation with the BNP peptide, in the absence of APC (Fig. 1 *B*). Because these CTL express HLA-A2, the decrease in responsiveness could have occurred from CTL lysis of neighboring CTL expressing HLA-A2 with the BNP peptide. However, A.T. CTL prepared as target cells were not lysed by A.T. CTL (Fig. 1 *B*). This observation was not unique to the A.T. CTL line, as similar results were obtained with another HLA-A2-restricted CD8$^+$ CTL line, from individual J.M. specific for the influenza A virus matrix peptide, 56–68 (28; Fig. 1 *C*). Thus, for these two CD8$^+$ CTL lines, a decrease in responsiveness was induced by direct interaction with their viral peptide, by a route apparently distinct from self-lysis. The ability of CTL to avoid lysis by themselves and by other CTL has been previously noted (29, 30).

CD8 Downmodulation by Viral Peptide. To determine whether incubation of CTL with the viral peptide induced changes in cell-surface glycoproteins, mAb binding to T cell antigens was examined. CTL were preincubated with 100 μg/ml BNP peptide or with medium for 10 h, washed, and then stained with FITC-conjugated mAbs. CTL preincubated with the BNP peptide had downmodulated CD8α and CD8β but not HLA-A2, β_2-microglobulin or HLA-DR expression (Fig. 2), suggesting that downmodulation was not due to cocapping of several cell surface molecules. CTL were next preincubated with either the BNP peptide or matrix peptide over a 10-h time period and examined for changes in CD8 and CD3 expression (Fig. 3). Both the matrix peptide and the BNP peptide bind HLA-A2, but only the BNP peptide is recognized by the A.T. CTL TCR (21, 28). Incubation

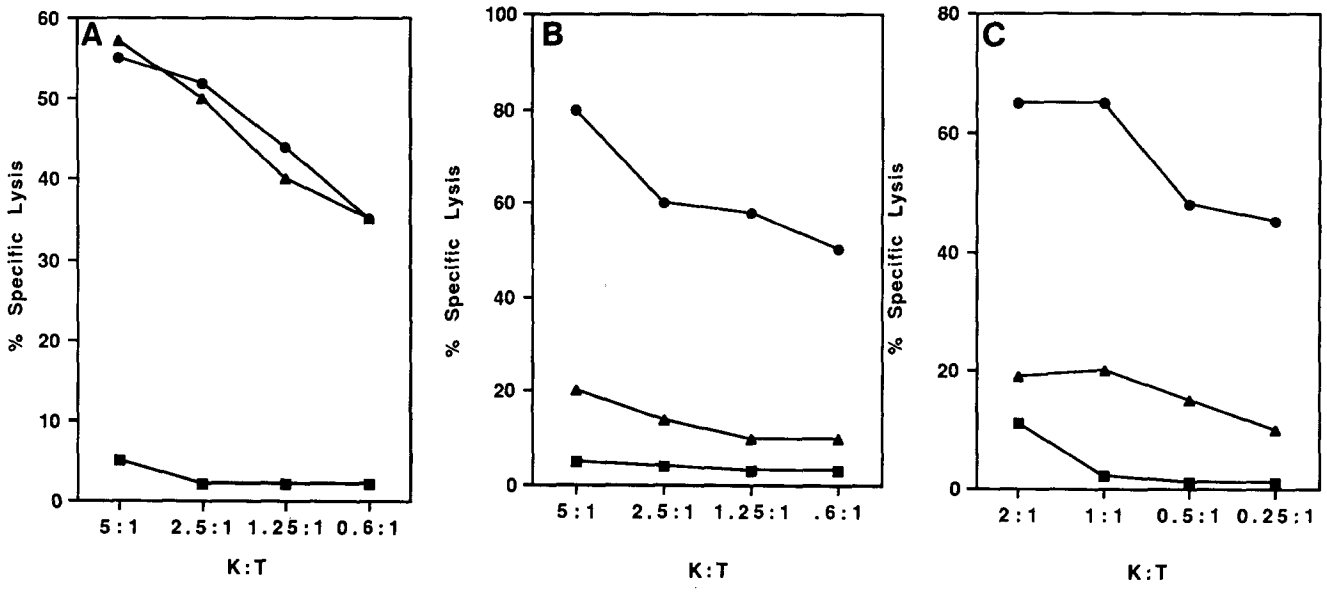

Figure 1. Responsiveness of CTL to viral peptides. (*A*) A.T. CTL lysis of autologous EBV-transformed B lymphoblasts preincubated in 100 μg/ml BNP peptide for 1 h in the presence of medium alone (●), 0.81 μg/ml IgG1 anti-CD8 mAb (■), or 0.81 μg/ml IgG1 anti-HLA-DQ mAb (▲). (*B*) A.T. CTL lysis of autologous B lymphoblasts preincubated in 100 μg/ml BNP peptide for 1 h (●), lysis of the same targets after A.T. CTL (10^6 cells/ml) were first preincubated for 2 h in 100 μg/ml BNP peptide followed by washing with centrifugation before addition to the CTL assay (▲), and lysis by A.T. CTL of ^{51}Cr-labeled A.T. CTL preincubated for 2 h in 100 μg/ml BNP peptide followed by washing with centrifugation before addition to the CTL assay (■). K:T refers to the ratio of CTL to target cells. (*C*) J.M. CTL lysis of autologous B lymphoblasts preincubated in 100 μg/ml matrix peptide for 1 h (●), lysis of the same targets after J.M. CTL (10^6 cells/ml) were first preincubated for 2 h in 100 μg/ml matrix as above (▲), and lysis by J.M. CTL of ^{51}Cr-labeled J.M. CTL preincubated for 2 h in 100 μg/ml matrix peptide as above (■).

with the BNP peptide downmodulated CD8 expression on a significant fraction of CTL within the line. Incubation of the A.T. CTL line with the matrix peptide had no effect on CD8 expression (Fig. 3 *A*), and neither peptide altered CD3 expression during this time period (data not shown). Similar results were obtained with the J.M. CTL line in that incubation with the matrix peptide but not the BNP peptide downmodulated CD8 expression (data not shown). CD8 downmodulation depended on the BNP peptide concentration (Fig. 3 *B*), which was most effective at 100 μg/ml. The BNP peptide is insoluble above 100 μg/ml, so higher concentrations could not be tested. Because downmodulation was peptide specific suggests that it might be due to CTL recognition of the HLA-peptide complex on neighboring CTL. That 100% of the cells were not downmodulated probably reflects the different TCR affinities within the CTL line. If the effect

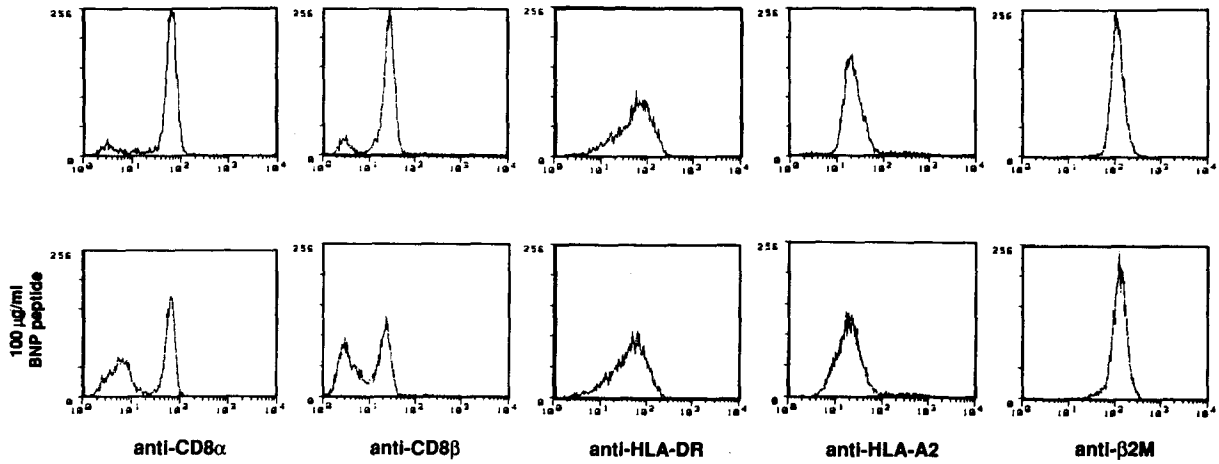

Figure 2. Expression of cell surface molecules on A.T. CTL preincubated with and without the BNP viral peptide. Antigen expression on CTL preincubated in medium alone (*top*) and in 100 μg/ml BNP peptide (*bottom*) for 10 h at 37°C. Cell surface molecules were detected by the FITC-conjugated mAb indicated at the bottom of each column.

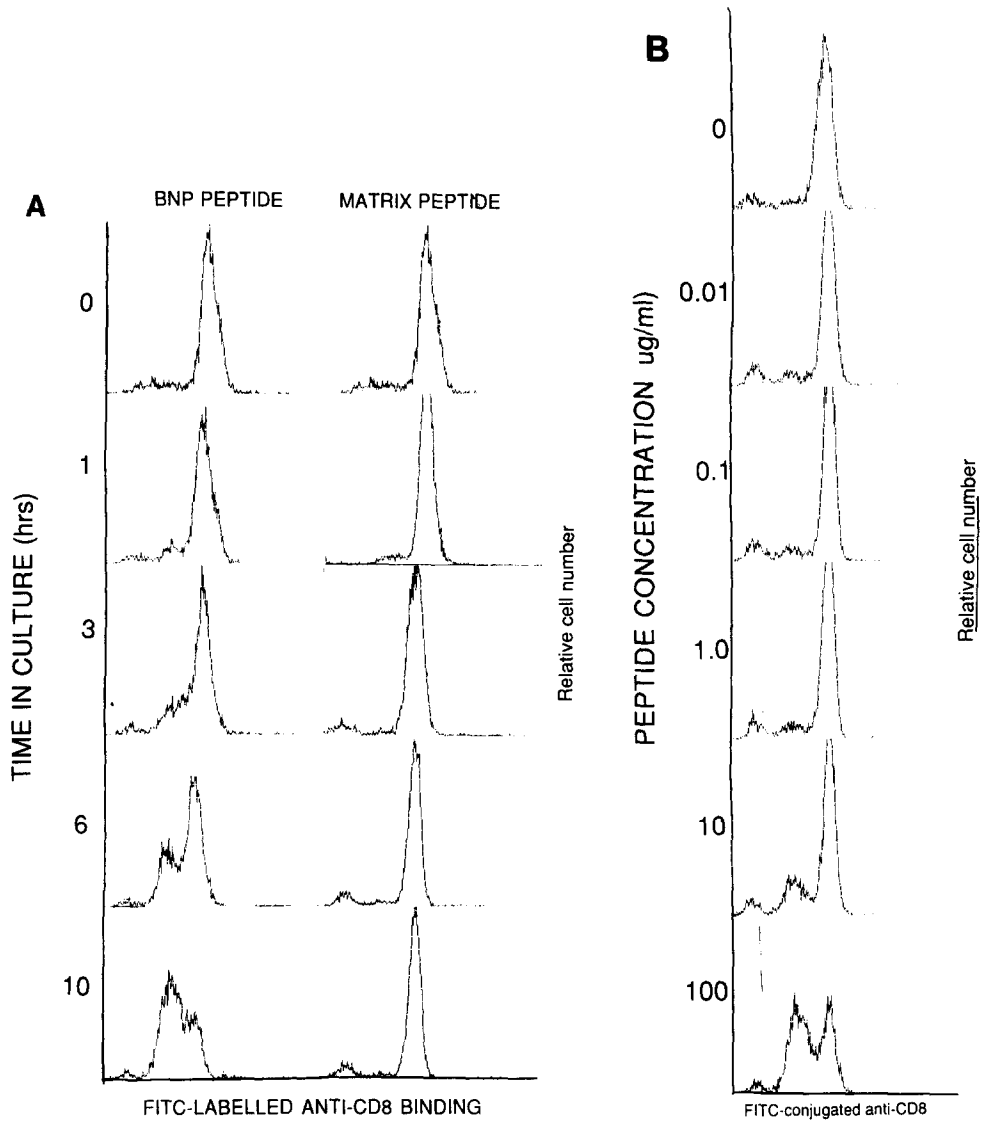

Figure 3. Kinetics, specificity, and peptide concentration of CD8 downmodulation. (*A*) Expression of CD8α detected by FITC-conjugated anti-CD8 mAb binding to A.T. CTL after preincubation of CTL (10^6 cells/ml) with 100 μg/ml BNP peptide (*left*) and with 100 μg/ml matrix peptide (*right*). Incubation with viral peptides was done over a 10-h time period indicated at the left. (*B*) Expression of CD8α detected by FITC-conjugated anti-CD8 binding to A.T. CTL preincubated for 10 h in the concentration of BNP peptide indicated at the left.

is due to T cell recognition of HLA-peptide complex, cells with downmodulated CD8 coreceptor will be impaired in their ability to recognize HLA-peptide complexes on remaining T cells.

Nonresponsiveness and CD8 Downmodulation by Anti-HLA Antibody. In the process of determining whether peptide was interacting with HLA-A2, incubation of CTL with 100 μg/ml MA2.1, an anti-HLA-A2 mAb interacting with residues 62–66 in the α1 domain of HLA-A2 (31, 32), was found to downmodulate CD8α and CD8β without changing CD3, CD2, IL-2R, or β_2-microglobulin expression (Fig. 4 *A*). Another anti-HLA-A2 mAb, PA2.1, which reacts with HLA-A2 residue 107 outside the TCR site (32, 33), had no effect on CD8 expression (data not shown). When CTL were incubated for 10 h with 100 μg/ml MA2.1 or PA2.1, and then tested in a 5-h CTL assay, cells incubated with MA2.1 but not PA2.1 were nonresponsive (Fig. 4 *B*). This downmodulation by anti-HLA-A2 mAb (clone MA2.1) depended both on the concentration of antibody (Fig. 4 *C*) and time of exposure (data not shown). This anti-HLA-A2 mAb also downmodulated CD8 expression and induced nonresponsiveness of a HLA-A2-positive HLA-B27-restricted CTL line to influenza A virus nucleoprotein peptide, 380–392 (34; data not shown). Hence, the effect of the mAb was not limited to the restricting HLA molecule.

To determine whether other HLA molecules expressed on A.T. CTL (HLA type: HLA-A2,11; B54,60; Cw1; DR4,12; DQw3) could alter CD8 expression, HLA alloantisera reacting with these molecules were tested for their effect on CD8 expression. By testing panels of antisera with binding activity to A.T. CTL, mono-specific serum to HLA-A2, HLA-A11, HLA-Cw1, HLA-DR12, and HLA-DQ3, and dispecific serum to HLA-B40/60, were identified that had a strong to moderate effect on CD8 expression (Fig. 5). CTL incubated with serum specific for HLA-A1, A24, A32, B8, B27, or Cw3 had no effect on CD8 expression, ruling out effects due to Fc binding

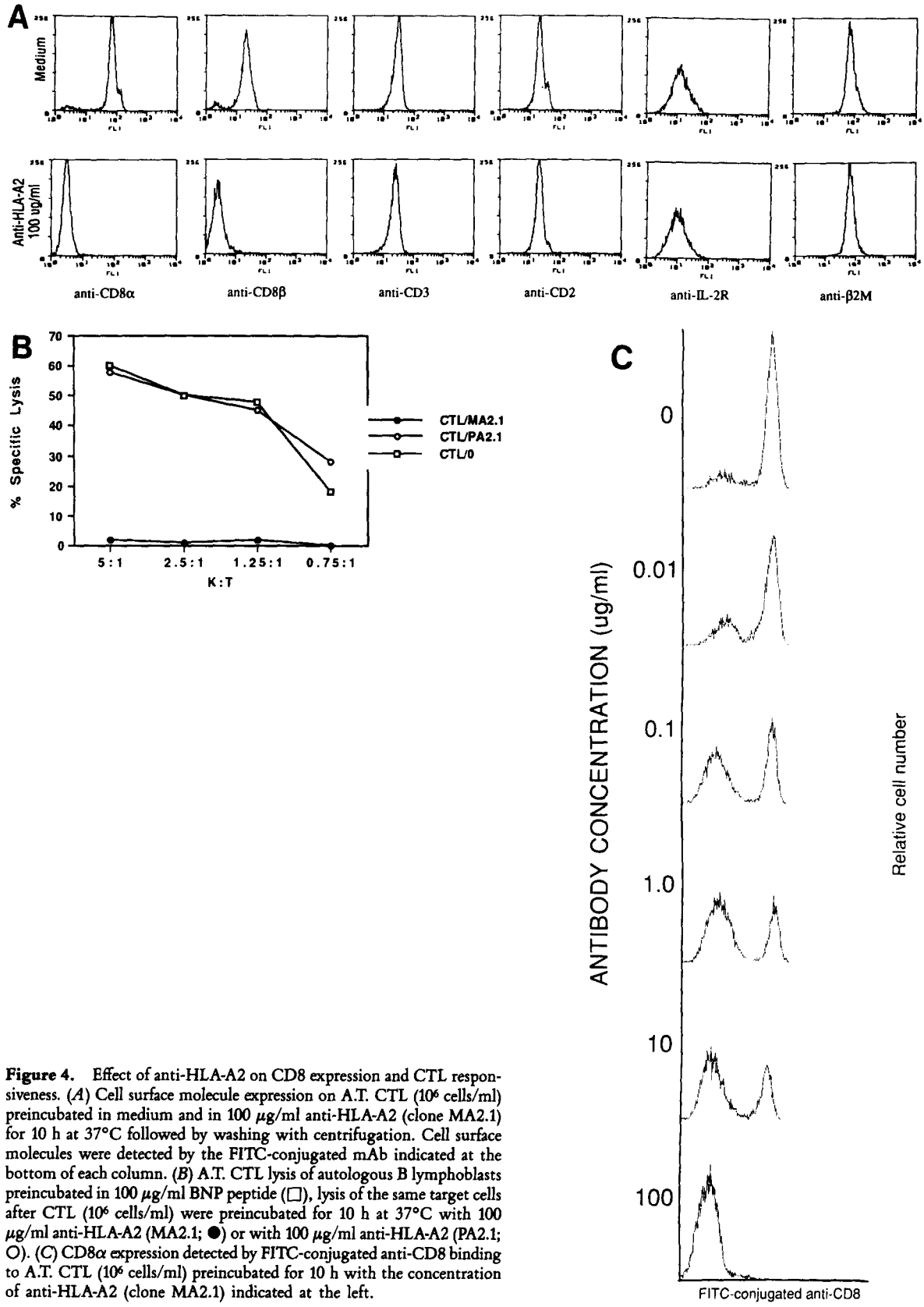

Figure 4. Effect of anti-HLA-A2 on CD8 expression and CTL responsiveness. (*A*) Cell surface molecule expression on A.T. CTL (10^6 cells/ml) preincubated in medium and in 100 μg/ml anti-HLA-A2 (clone MA2.1) for 10 h at 37°C followed by washing with centrifugation. Cell surface molecules were detected by the FITC-conjugated mAb indicated at the bottom of each column. (*B*) A.T. CTL lysis of autologous B lymphoblasts preincubated in 100 μg/ml BNP peptide (□), lysis of the same target cells after CTL (10^6 cells/ml) were preincubated for 10 h at 37°C with 100 μg/ml anti-HLA-A2 (MA2.1; ●) or with 100 μg/ml anti-HLA-A2 (PA2.1; ○). (*C*) CD8α expression detected by FITC-conjugated anti-CD8 binding to A.T. CTL (10^6 cells/ml) preincubated for 10 h with the concentration of anti-HLA-A2 (clone MA2.1) indicated at the left.

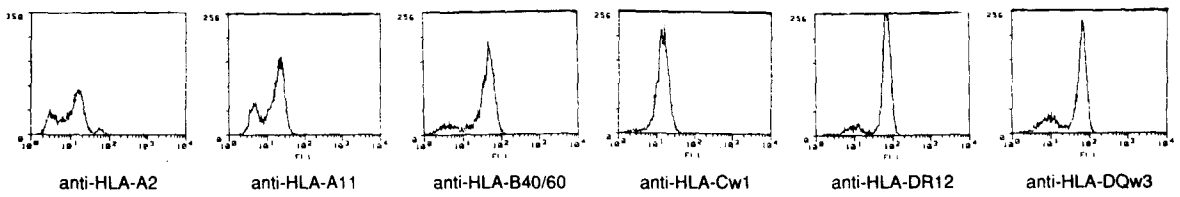

Figure 5. CD8 expression on A.T. CTL incubated with HLA alloantisera. FITC-conjugated anti-CD8 mAb binding to A.T. CTL incubated (10^6 cells/ml) for 10 h in 50% (vol/vol) of alloantisera containing the specificity indicated at the bottom of each picture.

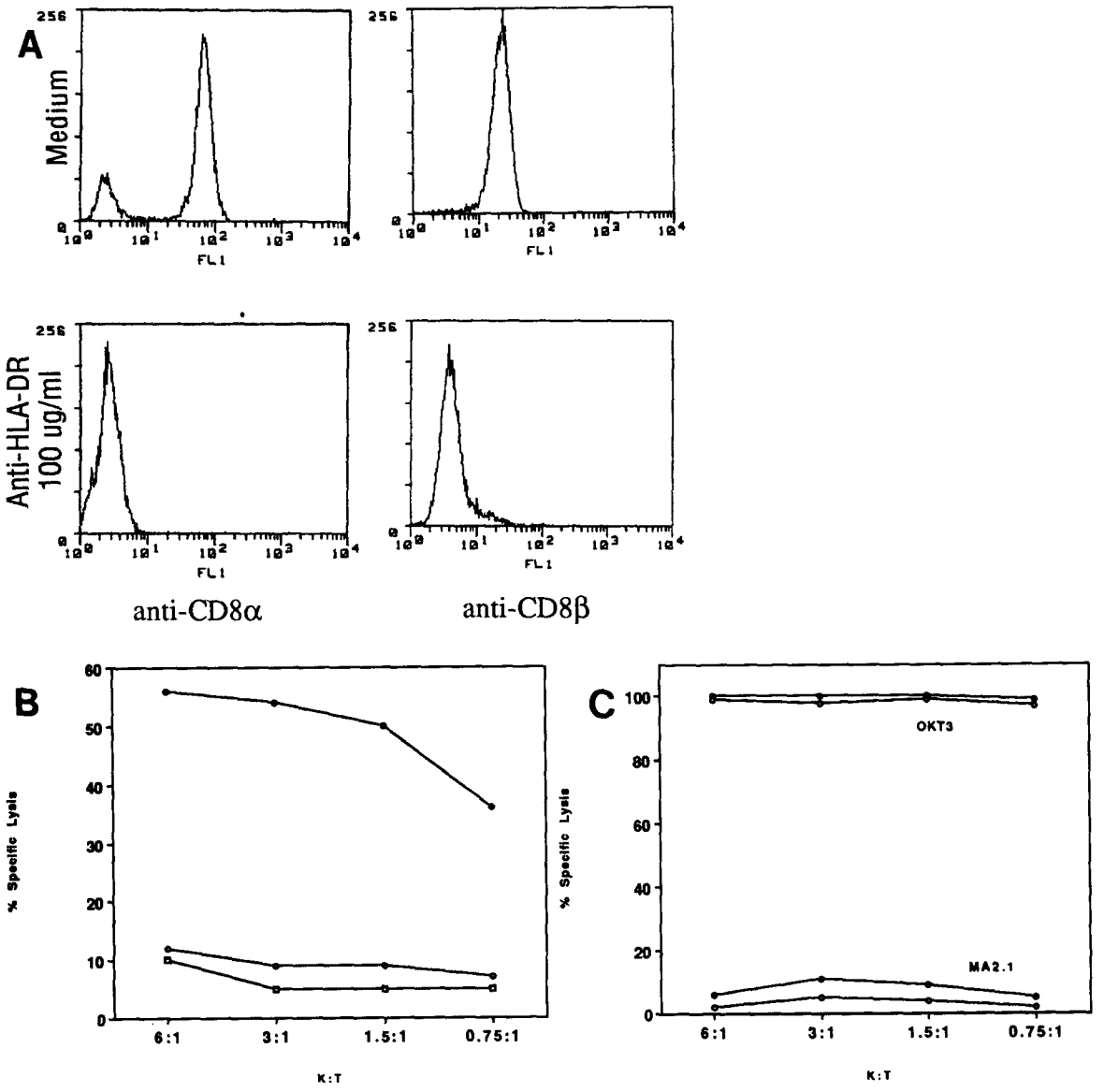

Figure 6. Effect of anti-HLA-DR mAb on CD8 expression and CTL responsiveness. (*A*) CD8 expression on A.T. CTL preincubated (10^6 cells/ml) in medium and in 100 μg/ml anti-HLA-DR (clone L243). (*B*) A.T. CTL lysis of autologous B lymphoblasts preincubated in 100 μg/ml BNP peptide (●), lysis of the same targets after CTL were preincubated for 10 h at 37°C in 100 μg/ml of anti-HLA-DR (clone L243; ○), and lysis by A.T. CTL of autologous B lymphoblasts without peptide (□). (*C*) A.T. CTL lysis of ^{51}Cr-labeled OKT3 hybridoma cells and MA2.1 hybridoma cells (○), and lysis of the same target cells after A.T. CTL were preincubated for 10 h with 100 μg/ml anti-HLA-DR (●).

(data not shown). An anti-HLA-DR mAb, L243, was found to downmodulate CD8α and CD8β expression after 10 h of incubation with A.T. CTL, too (Fig. 6). Changes in CD3, CD2, IL-2R, and β_2-microglobulin expression were equally undetectable, as in Fig. 4 (data not shown). This downmodulation was complete using a 100 μg/ml of anti-HLA-DR mAb but also depended on antibody concentration (data not shown; see Fig. 4 *B*). When these cells were tested in a CTL assay after 10 h of incubation with anti-HLA-DR, they were unresponsive to peptide on APC but not degranulated, because they could still lyse OKT3 hybridoma cells (Fig. 6). Thus, both anti-HLA class I and class II mAbs induced nonresponsiveness of CTL and downmodulated CD8 expression. The degree of these effects depended on antibody concentration, time of exposure, and the presence of antibody aggregates.

Aggregates of mAbs. When anti-HLA mAbs are crosslinked they are better at inhibiting mitogen-induced proliferation of PBL (35). In this study, the presence of antibody aggregates also correlated with an increased capacity of a mAb to downmodulate CD8. mAbs were purified from ascites by gel filtration and checked by SDS-PAGE for purity, as described (25). Nevertheless, different mAb aliquots showed variability in downmodulating CD8 expression. Adding to this anomaly was the observation that CD8 expression was only completely downmodulated using 100 μg/ml of antibody, a concentration way above saturation (see Fig. 4 *C*). FPLC examination of 17 samples of purified anti-HLA-DR and anti-HLA-A2 mAbs revealed that monomers, which eluted between fractions 45 and 50, under the FPLC conditions used, never had any activity on CD8 expression, at any concentration, and were the only protein peaks present immediately after purification of antibody from ascites (data not shown). In contrast, mAb aliquots that downmodulated CD8 had additional peaks of protein in fractions 31 and 41, as well as the monomer peak in fractions 45–50 (Fig. 7). These fractions of higher molecular weight stained HLA-A2-positive cells, as did the monomer peak, but all the functional activity of downmodulating CD8 was mainly in fraction 31 (active at 4 μg/ml), with partial activity in fraction 41. The activity in these peaks could be absorbed out by HLA-A2 transfectants (data not shown). These results suggest that the fractions of higher molecular weight most likely represent aggregated mAbs, which are biologically active at concentrations below that of saturation. The aggregation may facilitate crosslinking of HLA molecules.

Anti-HLA mAb Does Not Block Anti-CD8 Binding. Because CD8 molecules have been reported to coassociate with HLA-A2 molecules (36), the ability of anti-HLA-A2 to block anti-CD8 binding was examined. Incubation of A.T. CTL with 100 μ/ml anti-HLA-A2 for 15 min at 4°C (mAb activity shown in Fig. 4 *B*) completely blocked anti-HLA-A2 mAb binding, while anti-CD8 mAb binding was not affected at all (data not shown). As well, the anti-HLA-DR mAb did not block anti-CD8 mAb binding at 4°C under identical conditions (data not shown). Furthermore, when resting PBL were incubated for 10 h at 37°C with 100 μg/ml of anti-HLA-A2 mAb, no CD8 modulation was detected unless cells were first activated by mitogen (Fig. 8). Again, similar results were obtained with the anti-HLA-DR mAb. These results suggest the effect on CD8 expression by HLA-specific mAbs is not due to simple cell surface blocking of CD8 expression.

Discussion

In this study, CTL preincubated with their specific viral peptide in the absence of APC become nonresponsive. This decrease in responsiveness could be demonstrated without any detection of self-lysis. When the cell surface of CTL preincubated with viral peptide was examined, both CD8α and CD8β were downmodulated without changes in CD3, HLA-A2, HLA-DR, or β_2-microglobulin expression. This sug-

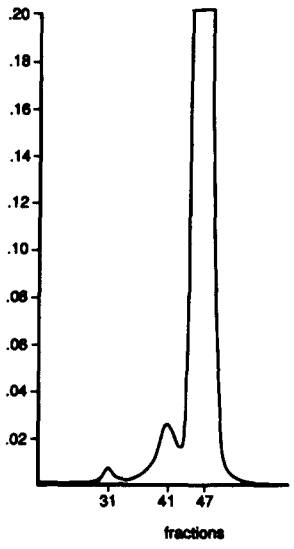

Figure 7. Aggregates of mAb downmodulate CD8. HPLC profile of a 100-μl aliquot of anti-HLA-A2 (clone MA2.1) loaded at 6 mg/ml onto a Superose 12 column on a FPLC system (Pharmacia Fine Chemicals) run at 0.25 ml/min collecting 250-μl fractions. The left axis indicates the UV monitor sensitivity meter, reading OD_{280}, set at 0.2 × 1. The bottom axis indicates relevant positions of the fractions collected.

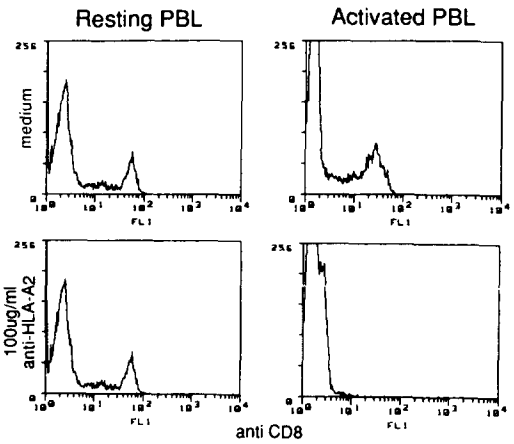

Figure 8. Anti-HLA-A2 does not block anti-CD8 binding. FITC-conjugated anti-CD8 mAb binding to A.T. PBL preincubated in medium or in 100 μg/ml anti-HLA-A2 (MA2.1) for 10 h at 37°C and to A.T. PBMC activated for 4 d with 5 μg/ml tuberculosis purified protein derivative followed by preincubation in medium or in 100 μg/ml anti-HLA-A2 (MA2.1) for 10 h at 37°C.

gests that downmodulation was specific to CD8 and not due to cocapping of several cell surface molecules. The degree of nonresponsiveness and CD8 downmodulation varied between experiments. For example, when CTL were incubated for 10 h with peptide and put in a 5-h CTL assay, there was no lysis of target cells (data not shown). Although, in two separate experiments shown in Fig. 2 and 3, CTL incubated for 10 h with the BNP peptide still expressed some CD8; it was never completely downmodulated after CTL were incubated in the BNP peptide. The difference between CTL responsiveness and CD8 expression may reflect the TCR affinities' presence within the CTL line. Partial CD8 downmodulation might be expected if caused by T cell recognition of the HLA-A2-peptide complex, as cells with downmodulated CD8 coreceptor would be impaired in their ability to recognize peptide-HLA-A2 complexes on remaining T cells. However, it is also possible that the difference between nonresponsiveness and CD8 expression might be accounted for by loss of other cell surface molecules not examined in this study. A change in TCR expression could have a profound effect on CTL responsiveness. Expression of CD3 molecules usually taken as an indication of TCR expression was not downmodulated by any of the procedures. However, the lack of TCR mAbs reacting with the TCRs on these CTL prohibited examining TCR expression directly. TCRs might be lost from the cell surface with similar or different kinetics from CD8 expression. However, because these CTL require the CD8 coreceptor in recognition of the BNP peptide (Fig. 1), impairment of CD8 expression would be enough to abolish responsiveness of these CTL.

Downmodulation of CD8 expression was specifically mediated by the BNP viral peptide. The influenza A virus matrix peptide, which binds HLA-A2 (29), had no effect on CD8 expression on AT CTL (Fig. 3). That both peptides bind HLA-A2 but only the BNP downmodulated CD8 expression implies downmodulation occurred from the BNP peptide binding directly to the TCR or from T cell recognition of the BNP peptide in HLA-A2 molecules on the CTL. In the process of sorting out these possibilities, an anti-HLA-A2 mAb was found to have an similar effect to the BNP peptide. It both downmodulated CD8α and CD8β expression and made the CTL nonresponsive. An anti-HLA-DR mAb also downmodulated CD8α and CD8β expression and made the CTL nonresponsive. In this latter case, when CTL were nonresponsive, they were still able to kill OKT3 (anti-CD3) hybridoma cells but not MA2.1 (anti-HLA-A2) hybridoma cells, suggesting that CTL treated with anti-HLA mAbs might be nonresponsive because of loss of CD8 expression and not from some nonspecific degranulation of the CTL. That incubation of the CTL with either the viral peptide or with anti-HLA mAb selectively altered CD8 expression without changing expression of other cell surface molecules (Figs. 2, 4, and 6) suggests CD8 downmodulation obtained with the viral peptide might be due to an interaction involving HLA-A2. That such an effect does not occur because of direct peptide binding to HLA is supported by the lack of an effect obtained with the matrix peptide in Fig. 3. Furthermore, that the effect with anti-HLA mAbs requires aggregated antibody suggests again that HLA molecules may not be simply bound by anti-HLA antibodies but crosslinked. Hence, the downmodulation of CD8 resulting from incubation of CTL with the BNP viral peptide may be due to CTL recognition of the BNP peptide in HLA-A2 molecules on neighboring CTL. This T cell recognition may crosslink HLA molecules.

How crosslinking HLA molecules actually brings about changes in CD8 cell surface expression remains to be determined. Certainly, it seems clear that interactions between distinct cell surface glycoproteins can be a means for altering cell function. But whether these interactions occur via the cell surface or through the cytoplasmic domain has yet to be determined in the cases where TCR crosslinking increases adhesiveness of LFA-1, where binding to TNF receptor downmodulates CSF receptors, or where binding to the IL-4R alters expression of IL-2R (37–39). Cell surface interactions between HLA molecules and CD8 molecules cannot be formally ruled out since HLA class I molecules have been shown to bind CD8 molecules (9) and associate intracellularly with CD8 molecules (36). However, HLA class II molecules have never been shown to bind CD8 molecules, and neither HLA class I nor class II molecules have been shown to bind CD8β chains A cell surface interaction between HLA and CD8 is unlikely therefore to explain the CD8 downmodulation.

Internalization of CD8 could be a possible mechanism to account for downmodulation of CD8. But it is controversial as to whether CD8 molecules are internalized after phosphorylation; two processes that are usually associated (40, 41). This stems from the observation that CD8 phosphorylation on thymocytes and peripheral lymphocytes is not accompanied by internalization (11). However, both CD8 phosphorylation and the disappearance of CD8 expression occur on CTL treated with PMA (20). On the other hand, CD8 molecules have been found in the serum of individuals with extensive viral infections and in the culture medium of PHA-activated lymphocytes (42). This might be taken as evidence for a mechanism of CD8 shedding. In the case of the mouse homing receptor, it is shed after proteolytic cleavage from the cell surface (43). Thus, such a mechanism might also operate for downmodulation of CD8 molecules.

A number of reports have shown that anti-HLA class I and class II mAbs inhibit mitogen-induced T cell proliferation (44–47). In these cases, the anti-HLA antibodies may be mimicking T cell recognition as they apparently do in our study. The type of T cell interaction whereby a CD8$^+$ T cell recognizes HLA class I–peptide complexes on neighboring T cells might account for one mechanism of immunoregulation. Certainly, the notion that CD8$^+$ T lymphocytes can immunoregulate peripheral T cell responses has existed for 20 yr since first suggested by Gershon and Kondo (48), but the mechanism for this type of immunoregulation has proved elusive.

In conclusion, downmodulation of cell surface molecules could be a mechanism for immunoregulation by inducing nonresponsiveness of immune cells. Downmodulation of IgM has been reported to induce tolerance to a self antigen in peripheral B cells (18), and in the case of peripheral T cell tolerance, reduced CD8 expression was found (17), possibly

resulting from CD8 downmodulation. The results reported here suggest that when cytotoxic T lymphocytes become nonresponsive, this is accompanied by downmodulation of CD8 expression, which occurs after crosslinking of HLA molecules by antibody or after T cell recognition of their peptide-HLA complex.

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References

1. Zinkernagel, R.M., and P.C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T cell restriction-specificity, function, and responsiveness. *Adv. Immunol.* 27:51.
2. McDonald, H.R., A.J. Glasebrook, G. Bron, A. Lelso, and J.C. Cerottini. 1982. Clonal heterogeneity in the functional requirement for Lyt 2/3 molecules on cytolytic T lymphocytes (CTL). *Immunol. Rev.* 68:89.
3. Goldstein, P., B. Goridis, A. Schill-Verhulst, N. Hazlot, A. Pierres, A. van Agthoven, Y. Kaufman, Z. Eshhar, and M. Pierres. 1982. Lymphoid cell surface interaction structure detected using cytolysis inhibiting monoclonal antibodies. *Immunol. Rev.* 68:89.
4. Boyse, E.A., M. Miyazawa, T. Aoki, and L.J. Old. 1968. Ly-A and Ly-B: two systems of lymphocyte isoantigens in the mouse. *Proc. R. Soc. Lond. Biol. Sci.* 170:175.
5. Cantor, H., and E.A. Boyce. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* 141:1376.
6. Swain, S.L., A. Badde, M. English, and R.W. Dutton. 1979. Ly phenotypes and MHC recognition: the allohelper that recognizes K or D is a mature Ly 123 cell. *J. Immunol.* 123:2716.
7. Swain, S.L. 1981. Significance of Lyt phenotypes: Lyt 2 antibodies block activities of T cells that recognize class I major histocompatibility complex antigens regardless of function. *Proc. Natl. Acad. Sci. USA.* 78:7101.
8. Doyle, C., and J.L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature (Lond.).* 341:619.
9. Norment, A.M., R.D. Salter, P. Parham, V.H. Englehard, and D.R. Littman. 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature (Lond.).* 336:79.
10. Potter, T., T.V. Rafan, R.F Dick, and J.A. Bluestone. 1989. Substitution at residue 227 of H-2 class I molecules abrogates recognition by CD8-dependent but not CD8-independent cytotoxic T lymphocytes. *Nature (Lond.).* 337:73.
11. Blue, M.L., D.A. Hafter, K.A. Craig, H. Levine, and S.F. Schlossman. 1987. Phosphorylation of CD4 and CD8 molecules following T cell triggering. *J. Immunol.* 139:3949.
12. Veilette, A., M.A. Bookman, E.M. Horak, and J.B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell.* 55:301.
13. Barber, G.K., J.D. Dasgupta, S.F. Schlossman, J.M. Trevillyan, and C.E. Rudd. 1989. The CD4 and CD3 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc. Natl. Acad. Sci. USA.* 86:3277.
14. Engleman, E.G., C.J. Benike, C. Metzler, P.A Gatenby, and R.L. Evans. 1983. Blocking of human T lymphocyte function by anti-Leu 2 and anti-Leu3 antibodies: differential inhibition of proliferation and suppression. *J. Immunol.* 130:2623.
15. Welte, K., E. Platzer, C.Y. Wang, E.A. Rinnooy Kan, M.A.S. Moore, and R. Mertelsman. 1983. OKT8 antibody inhibits OKT3-induced IL-2 production and proliferation in OKT8 cells. *J. Immunol.* 131:2356.
16. Dembic, A., W. Haas, R. Zamoyska, J. Parnes, M. Steinmetz, and H. von Boehmer. 1987. Transfection of the CD8 gene enhances T-cell recognition. *Nature (Lond.).* 326:510.
17. Kisielow, P., J. Bluthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. *Nature (Lond.).* 333:742.
18. Goodnow, C., J. Crosbie, H. Jorgensen, P.A. Brink, and A. Basten. 1989. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature (Lond.).* 342:385.
19. Blue, M.L., J.F. Daley, H. Levine, K.R. Branton, and S.F. Schlossman. 1989. Regulation of CD4 and CD8 surface expression on human thymocyte subpopulations by triggering through CD2 and the CD3-T cell receptor. *J. Immunol.* 142:374.
20. DiSanto, J.P., J.S. Klein, and N. Flomenberg. 1989. Phosphorylation and down-regulation of CD4 and CD8 in human CTLs and mouse L cells. *Immunogenetics.* 30:494.
21. Robbins, P.A., L.A. Lettice, P. Rota, J. Santos-Aquado, J. Roth-

bard, A.J. McMichael, and J.L. Strominger. 1989. Comparison between two peptide epitopes presented to cytotoxic T lymphocytes by HLA-A2. Evidence for discrete locations within HLA-A2. *J. Immunol.* 143:4098.
22. Lamb, J.R., B.J. Skidmore, J.M. Chitter, and M. Feldman. 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J. Exp. Med.* 157:1434.
23. Parham, P., C. Clayberger, S.L. Zorn, D.S. Ludwig, G.K. Schoolnik, and A. Krensky. 1987. Inhibition of alloreactive cytotoxic T lymphocytes by peptides from the α1 domain of HLA-A2. *Nature (Lond.).* 325:626.
24. Gotch, F., A.J. McMichael, G. Smith, and B. Moss. 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. *J. Exp. Med.* 165:408.
25. Robbins, P.A., E.L. Evans, A.H. Ding, N.L. Warner, and F.M. Brodsky. 1987. Monoclonal antibodies that distinguish between class II antigens (HLA-DP, DQ and DR) in 14 haplotypes. *Hum. Immunol.* 18:301.
26. Norment, A.M., and D.R. Littman. 1988. A second subunit of CD8 is expressed in human T cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3433.
27. Robbins, P.A., V.C. Maino, N.L. Warner, and F.M. Brodsky. 1988. Activated T cells and monocytes have characteristic patterns of class II antigen expression. *J. Immunol.* 141:1281.
28. Gotch, F., J. Rothbard, K. Howland, A. Townsend, and A. McMichael. 1987. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. *Nature (Lond.).* 326:881.
29. Kranz, D.M., and H.N. Eisen. 1987. Resistance of cytotoxic T lymphocytes to lysis by a clone of cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 84:3375.
30. Young, J.D.E., and C.C.Liu. 1988. How do cytotoxic T lymphocytes avoid self-lysis? *Immunol. Today.* 9:14.
31. Santos-Aquado, J., J.A. Barbosa, P.A. Biro, and J.L. Strominger. 1988. Molecular characterization of serologic recognition sites in the human HLA-A2 molecule. *J. Immunol.* 141:2811.
32. Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley. 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature (Lond.).* 329:512.
33. Salter, R.D., C. Clayberger, C.E. Lomen, A.M. Krensky, and P. Parham. 1987. In vitro mutagenesis at a single residue introduces B and T cell epitopes into class I molecule. *J. Exp. Med.* 166:283.
34. Huet, S., D.F. Nixon, J. Rothbard, A. Townsend, S.A. Ellis, and A.J. McMichael. 1989. Structural homology between two HLA-B27 restricted peptides suggest residues important for interacting with HLA-B27. *Int. Immunol.* 2:311.
35. Gilliland, K.L., N.A. Norris, L.S. Grosmaire, S. Ferrone, P. Gladstone, and J.A. Ledbetter. 1989. Signal transduction in lymphocyte activation through crosslinking of HLA class I molecules. *Hum. Immunol.* 25:269.
36. Blue, M.L., K.A. Craig, P. Anderson, K.R. Branton, and S.F. Schlossman. 1988. Evidence for specific association between class I major histocompatibility antigens and the CD8 molecules of human suppressor/cytotoxic cells. *Cell.* 54:413.
37. Dustin, M.L., and T.A. Springer. 1989. T-cell receptor cross-linking transiently stimulates adhesiveness thorough LFA-1. *Nature (Lond.).* 341:619.
38. Shieh, J.H., R.H.F. Peterson, D.J. Warren, and M.A.S. Moore. 1989. Modulation of colony-stimulating factor-1 receptors in macrophages by tumor necrosis factor. *J. Immunol.* 143:2534.
39. Longhanan, M.S., and G.J.V. Nossal. 1989. Interleukins 4 and 5 control expression of IL-2 receptor on murine B cells through independent induction of its two chains. *Nature (Lond.).* 340:76.
40. Cantrell, D.A., A.A. Davis, and M. Crumpton. 1985. Activators of protein kinase C downregulate and phosphorylate the T3/T cell antigen receptor complex of human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 82:8158.
41. Krangel, M.S. 1987. Endocytosis and recycling of the T3-T cell receptor complex. The role of T3 phosphorylation. *J. Exp. Med.* 165:1141.
42. Tomkinson, B.E., M.C. Brown, H.S. Ip, S. Carrabis, and J.L. Sullivan. 1989. Soluble CD8 during T cell activation. *J. Immunol.* 142:2230.
43. Jung, T.LM., and M.O. Dailey. 1990. Rapid modulation of homing receptors (gp90MEL-14) induced by activators of protein kinase C. *J. Immunol.* 144:3130.
44. Akiyama, Y., R. Zicht, S. Ferrone, G.D. Bommard, and R.B. Herberman. 1985. Effect of monoclonal antibodies (MoAb) to class I and class II HLA antigens on Lectin- and MoAb OKT3-induced lymphocyte proliferation. *Cell. Immunol.* 91:477.
45. Turco, M.C., M. DeFelice, L. Corbo, G. Morrone, R. Mertelsmann, S. Ferrone, and S. Venuta. 1985. Regulatory role of a monomorphic determinant of HLA class I antigens in T cell proliferation. *J. Immunol.* 135:2268.
46. Taylor, D.S., P.C. Nowell, and J. Kornbluth. 1986. Functional role of HLA class I cell-surface molecules in human T-lymphocyte activation and proliferation. *Proc. Natl. Acad. Sci. USA.* 83:4446.
47. Dasgupta, J.D., K. Cemach, D.P. Dubey, E.J. Yunis, and D.B. Amos. 1987. The role of class I histocompatibility antigens in the regulation of T cell activation. *Proc. Natl. Acad. Sci. USA.* 84:1094.
48. Gershon, R.K., and Kondo, K. 1971. Infectious immunological tolerance. *Immunology.* 21:903.