EXPRESSION AND FUNCTION OF CD8 IN A MURINE T CELL HYBRIDOMA

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In addition to the TCR, a number of other cell surface molecules play a role in T cell activation and T cell effector function (see reference 1). Among the various cell surface antigens involved in T cell function are the human T cell–specific molecules CD8 (T8, Leu-2) and CD4 (T4, Leu-3). CD8 and its murine homologue, Lyt-2, are expressed on functionally similar T cell subpopulations (2). CD8 exists in homomultimeric configurations on the surface of human T cells, the most common being homodimers of 67 and 76 kD (3). These two species are thought to be conformational isomers varying only at inter- or intrachain disulfide bonds (3, 4). Comparison of the predicted amino acid sequence for human and murine CD8 yields an overall level of homology of 56% (5). CD8 may be placed in the Ig supergene family, implying the potential capacity for receptor function. At the amino acid level, it shares 30–35% homology with λ and Vκ region segments and 24% homology with TCR-α and -β chain V segments (6).

CD8 and Lyt-2 were initially believed to be phenotypic markers for T cells with cytotoxic or suppressor function while CD4 and its murine equivalent, L3T4, were markers for helper T cells (2, 7, 8). The demonstration of the existence of Lyt-2^+ helper T cells specific for products of the class I region of the MHC led to the alternate hypothesis that the Lyt-2^+ phenotype correlated with the class of MHC protein recognized by the T cell rather than with function (8, 9). This observation was extended by studies with human lymphocytes demonstrating $CD8^+$ helper cells (10, 11) and $CD4^+$ cytotoxic cells (12). The correlation between CD8 expression and class I restriction has led to the hypothesis that CD8 is a receptor for nonpolymorphic determinant on the class I MHC molecule (13), while CD4 is a receptor for nonpolymorphic determinants on the class II MHC molecule (14, 15).

It has been proposed that one function of the CD8 and Lyt-2 molecules is to increase the avidity with which T cells bind to target or stimulator cells. Avidity is the strength of interaction resulting from the summation of all receptor-ligand

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pairings. The studies that led to the hypothesis that these molecules play a supplementary or accessory role in the formation of conjugates examined the ability of mAbs directed at either CD8 or Lyt-2 to block the function of T cell lines (16, 17). T cells with a low-affinity TCR could be functionally inhibited by these mAbs, suggesting that lower affinity TCR bearing T cells would place greater reliance upon accessory molecules for cell–cell interactions.

Initially, it was assumed that inhibition of T cell function by anti-CD8 or anti-CD4 mAbs resulted from steric inhibition of the CD8 or CD4 receptor for its ligand. Recently, however, there has been evidence to suggest that anti-CD8 or anti-CD4 mAbs can inhibit T cell function in the absence of a stimulator cell expressing a putative ligand, the mAb possibly delivering an "off" or negative signal to the T cell. For example, anti-Lyt-2 mAb inhibits lectin-dependent cytolysis in the absence of target cells expressing MHC class I antigens (18). It has also been demonstrated that anti-CD8 mAb inhibits anti-CD3–triggered lysis of a class I MHC–negative target cell (19). Similarly, mAbs directed at CD4 may also generate an off signal. Anti-L3T4 mAbs have been shown to inhibit lectin-stimulated T cell activation in the absence of MHC class II–positive APCs (20, 21) and to inhibit lectin-activated IL-2 production by T cell hybridomas in a culture system devoid of Ia^+ accessory cells (22). mAbs to CD4 have also been shown to block anti-CD3 mAb-stimulated IL-2 production (23).

To address the role of CD8 in T cell activation and to define its ligand, a murine T cell hybridoma specific for human class II antigens was generated by immunizing C57BL/6 mice with the human EBV-transformed B cell line, JY. A cDNA clone encoding CD8 was efficiently and functionally expressed in this T cell hybridoma. $CD8^+$ hybridomas produced 10-fold greater IL-2 in response to JY stimulator cells than did the $CD8^-$ parent line. This enhancement was blocked by the addition of anti-CD8 mAb. The lack of enhancement seen when a class I–negative, class II–positive stimulator was used and the ability to block JY stimulation with anti–class I mAb implies that the ligand for CD8 is encoded on the class I MHC molecules.

Materials and Methods

Construction of the MNCT8 Producer Cell Line. Amphotropic helper cell lines producing the MNCT8 virus were generated by transfecting the xenotropic helper cell line, MXEN (Peterson, A., unpublished observations), using a modification of the DEAE-dextran procedure of Seed and Aruffo (24). 48 h after transfection the supernatants from the cells were passed through a 0.45-μm filter and were used to infect the amphotropic helper line, DAMP (Peterson, A., unpublished observations), in the presence of 10 μg/ml polybrene. 48 h following infection selection was initiated using 500 μg/ml G418. Pooled colonies of $G418^r$ DAMP cells were used as the MNCT8 producer line.

Expression of the CD8 Gene. BY155.16 (25) was infected with the recombinant retrovirus MNCT8 by coculture with the MNCT8 transfected producer line. $\sim 5 \times 10^5$ hybridoma cells were added to a half-confluent 10-cm monolayer plate of MNCT8 DAMP in the presence of 8 μg/ml polybrene. After 48 h, the cells were transferred to 24-well plates at 10^4 hybridoma cells per well in 1 ml of tissue culture medium containing the neomycin analog G418 at 2 mg/ml. Resistant cells were visible microscopically within 5 d of transfer. All lines were then passaged in the absence of G418 without alteration in the expression of membrane-associated CD8.

Indirect Immunofluorescent Staining. All staining was done in V-bottomed 96-well plates in a volume of 50 μl of antibody. The mouse mAb OKT8, which is specific for an epitope

of the CD8 molecule, was raised as an ascites and used at a concentration of 5 μg/ml. F23.1, a mouse mAb directed at a TCR allotype, and M17/5.2, a rat mAb specific for the murine lymphocyte function–associated antigen 1 (LFA-1)[1] molecule, were both used as undiluted culture supernatants. All groups were incubated with their first antibody (described above) on ice for 30 min. After two washes with cold PBS containing 2% FCS, 50 μl of a 1:25 dilution of fluorescein-conjugated $F(ab')_2$ anti–mouse or anti–rat Ig were added for 40 min on ice. All groups were then washed three times and fixed with 2% paraformaldehyde. Fluorescence was assessed by flow cytometry (Epics IV, Coulter Electronics, Hialeah, FL).

IL-2 Assay. 5×10^4 hybridoma cells were cultured with irradiated (5,000 rad) JY cells in a final volume of 1 ml of RPMI 1640 containing 2 mM glutamine, 10% heat-inactivated FCS, and 5×10^{-5} M 2-ME. Supernatants transferred from cells were frozen and thawed, and assayed for IL-2 content by their ability to support the incorporation of [^{3}H]TdR by the IL-2-dependent cell line, CTLL20 (26). The data are converted to units of IL-2 per milliliter by comparison to a rat Con A supernatant. Half-maximal ^{3}H incorporation with the supernatant is defined as 100 U of IL-2 per milliliter.

Antibodies. The fluorescein-conjugated $F(ab')_2$ antibodies goat anti–mouse IgG + IgM and goat anti–rat IgG + IgM were purchased from Tago, Inc., Burlingame, CA. Leu-3a was obtained from Becton Dickinson & Co., Mountain View, CA. OKT8C and F mAb were a generous gift of Dr. Gideon Goldstein, Ortho Pharmaceutical, Raritan, NJ. The OKT8-producing hybridoma was obtained through American Type Culture Collection (ATCC), Rockville, MD, and was initially described by Hoffman et al. (27). All HLA class I–specific hybridoma lines were purchased from ATCC. The HLA-B7-specific hybridomas ME1 and MB40.2 were initially described by Ellis et al. (28) and Parham (29), respectively. The HLA-A2-specific hybridomas, MA2.1 and PA2.1, were described by McMichael et al. (30) and Parham and Bodmer (31), respectively. The murine LFA-1-specific mAb M17/5.2 was described by Davignon et al. (32). The HLA-DR-specific mAb TS1/16 was generated by Sanchez-Madrid et al. (33). The mAb for Lyt-2.2 was obtained from ATCC and was initially described by Raulet et al. (34). The TCR-specific mAb F23.1 was generated by Staerz et al. (35).

Results

Expression of CD8 in a Murine T Cell Hybridoma. Murine T cell hybridomas were derived by the fusion of C57BL/6 spleen cells, from mice immunized with the human Epstein-Barr virus lymphoblastoid line JY (HLA-A2,2; -B7,7; -DR4,6), to the HAT-sensitive thymoma BW5147. The T cell hybridoma BY155.16, which produces IL-2 in response to the class II HLA-DR antigens on JY and Daudi cells, was isolated from this fusion.

The retroviral expression vector MNCT8 (Fig. 1) provided a technique to readily transfer the CD8 cDNA clone (Aruffo, A., and B. Seed, unpublished observations) into BY155.16. The promoter used to drive the CD8 cDNA clone is a fusion between the cytomegalovirus immediate early promoter and the *tat*-responsive element of the human immunodeficiency virus. It was hoped that this portion of the HIV genome would provide a means for upregulation of cDNAs driven by the resulting chimeric promoter upon transfection with the *tat* gene. Such an effect was not detected (Peterson, A., unpublished observation). MNCT8 confers resistance to G418 in a eukaryotic cell by virtue of the APH(3′) II gene *neo* from Tn5. This neo$^+$ element uses the left Moloney murine leukemia virus LTR as its site for initiation of transcription. A second vector containing the neo$^+$ element without CD8 was used to infect the parent line with the *neo* gene alone (25). Producer cell lines were constructed by transfecting the DAMP

[1] *Abbreviation used in this paper:* LFA-1, lymphocyte function–associated antigen 1.

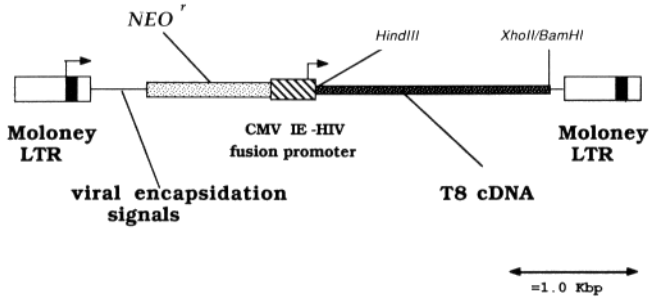

FIGURE 1. Structure of the retroviral vector MNCT8. MNCT8 has retroviral transcription and integration sequences in the long terminal repeats (LTR) derived from Moloney murine leukemia virus. The retroviral encapsidation signals are also derived from Moloney virus. The neo^r element is the APH (3′) II gene from Tn5 and confers resistance to G418 in eukaryotic cells. The messenger RNA for the *neo* gene and the viral genomic RNA are produced by transcription initiation in the left LTR and polyadenylation in the right LTR. The CD8 cDNA (isolated from the human T cell leukemia line HPB-ALL, Aruffo, A., and B. Seed, unpublished data) is expressed from a subgenomic RNA initiated at an internal promoter. The internal promoter in this case is a fusion between the cytomegalovirus immediate early promoter and the tat-responsive element of the HIV.

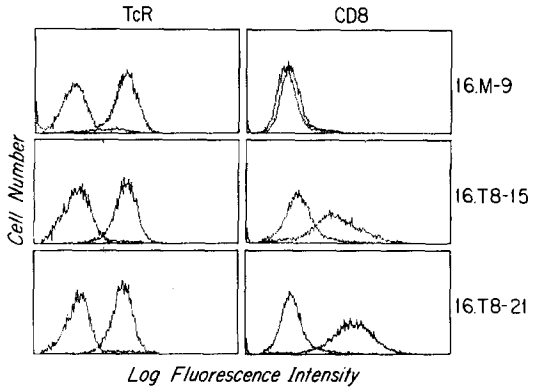

FIGURE 2. Flow cytometric analysis of murine TCR and CD8 expression. The neo$^+$, CD8$^-$ cell line 16.M-9 and CD8$^+$ cell lines 16.T8-15 and 16.T8-21 were assessed for TCR expression by indirect staining with the TCR mAb F23.1. CD8 expression was assessed by indirect immunofluorescence as described in Materials and Methods.

amphotropic packaging cell line (Peterson, A., unpublished observations) with these retroviral vectors.

Two infections of BY155.16 were carried out. Of the 48 G418-resistant cell lines derived from these infections, all were CD8$^+$ and ~50% retained the ability to secrete IL-2 in response to stimulation with JY cells. The flow cytometry profile of two representative CD8$^+$ hybridomas, 16.T8-15 and 16.T8-21, are presented in Fig. 2. Maximal density achieved using the MNCT8 expression system was equivalent to that found on IL-2-dependent human peripheral blood lymphocyte–derived T cell lines. To assess the effect of infection and selection on these T cell hybridomas, it was necessary to assess the expression of two other molecules involved in T cell function, LFA-1 (32, 33) and the antigen-specific TCR. Infection of cells by MNCT8 did not alter either the surface density of the antigen-specific TCR as assessed by the mAb F23.1 (Fig. 2) or LFA-1 as assessed by an anti-LFA-1 mAb (data not shown) when compared with the neo$^+$ infectant 16.M-9 or the parent cell line. All infectants derived from BY155.16 were negative for Lyt-2 expression as assessed by indirect immunofluorescence (data not shown).

CD8 Expression Enhances T Cell Hybridoma Function. CD8 function was assessed by examining the ability of the CD8$^+$ T cell hybridomas to respond to

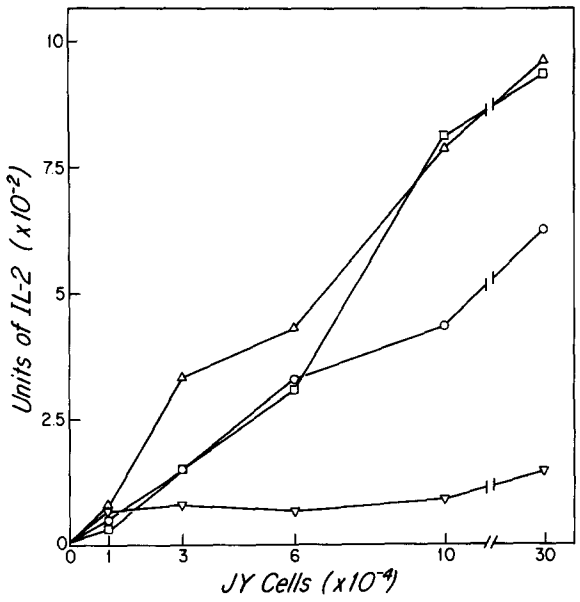

FIGURE 3. CD8 expression enhances T cell responsiveness. Antigen-specific stimulation of the neo$^+$, CD8$^-$ cell line 16.M-9 (▽) and CD8$^+$ cell lines 16.T8-15 (□), 16.T8-21 (△), and 16.T8-25 (○) with varying numbers of JY stimulator cells as indicated were assessed. Responder cells were cultured at 5×10^4 cells/ml with varying numbers of JY stimulators as indicated.

antigen in comparison to 16.M-9, the neo$^+$, CD8$^-$ infectant. On average, T cell hybridomas expressing CD8 produced 10-fold more IL-2 than the neo$^+$ cell line when cultured with 10^5 irradiated JY stimulator cells (Fig. 3). To determine the efficiency of responsiveness of these CD8$^+$ hybridomas, the IL-2 response to varying numbers of stimulator cells was assessed (Fig. 3). All three CD8$^+$ cell lines evaluated could be triggered to secrete IL-2 with 10-fold fewer stimulator cells than the CD8$^-$ control. Thus, the expression of CD8 allowed these T cell hybridomas to respond more efficiently to suboptimal levels of antigen.

mAbs to CD8 Inhibit the Response of CD8$^+$ Hybridomas. Stimulation of both 16.T8-25 and 16.M-9 by JY cells was completely inhibited by the addition to culture of anti-HLA-DR mAb (TS1/16), supporting the conclusion that the TCR of these hybridomas is specific for HLA-DR antigens on JY. mAbs to the CD8 molecule were used to further assess the contribution of CD8 to the response of a CD8$^+$ infectant, the 16.T8-25 cell line (Table I). OKT8 inhibited the response of 16.T8-25 to JY by 84%, suggesting that the CD8 molecule was functionally involved in T cell activation. This level of inhibition was detected with as little as 60 ng/ml mAb (data not shown). Concentrations of up to 5 μg/ml of OKT8 did not inhibit the neo$^+$, CD8$^-$ cell line, 16.M-9. In addition, a nonspecific mAb (Leu-3a) had no effect on either 16.M-9 or 16.T8-25. The OKT8C and OKT8F mAb, specific for epitopes on the CD8 molecule distinct from that defined by OKT8, were equally effective in inhibiting the response of 16.T8-25 (Table I).

mAb to Class I HLA Antigens Inhibits the Enhanced CD8 Response. The choice of BY155.16, a murine T cell line specific for the class II HLA-DR antigens, made it possible to assess whether HLA class I molecules bear the ligand for CD8. The response of a CD8$^+$ hybridoma to varying numbers of JY stimulator cells was analyzed in the presence or absence of anti–class I mAb specific for

TABLE I

CD8 Expressing Class II–specific T Cell Hybridomas Are Blocked by Anti-CD8 mAb

mAb added	Specificity	16.M-9		16.T8-25	
		IL-2	Inhibition	IL-2	Inhibition
		U/ml	*%*	*U/ml*	*%*
—		80	—	526	—
OKT8	Anti-CD8	98	0	86	84
OKT8C	Anti-CD8	98	0	80	85
OKT8F	Anti-CD8	74	7	80	85
Leu3a	Anti-CD4	78	2	534	0
TS1/16	Anti–class II	16	80	28	95

5×10^4 T cells were cultured with 3×10^5 irradiated JY stimulator cells in a final volume of 1 ml. All CD8-specific mAbs were used at a final dilution of 330 ng/ml. After 18–24 h, supernatants were assayed for IL-2 by the ability to support the growth of the IL-2-dependent T cell line, CTLL20.

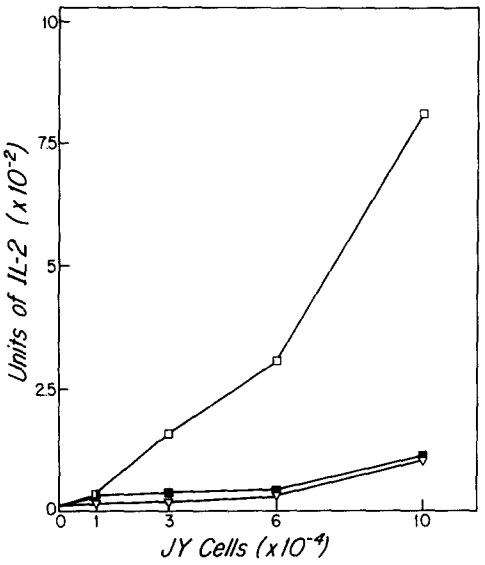

FIGURE 4. Inhibition of CD8-mediated enhancement by anti-HLA class I mAb. The anti-HLA-A2 mAbs PA2.1 and MA2.1 and the anti-HLA-B7 mAbs MB.1 and ME40.2 were added to cultures containing the $CD8^+$ cell line 16.T8-15 (■). Control cultures consisted of 16.T8-15 with no antibody (□) and the neo^+, $CD8^-$ cell line 16.M-9 without antibody (▽). Responders were cultured at 5×10^4/ml and stimulated with varying numbers of JY cells.

HLA-A2 and HLA-B7, the class I products expressed on the JY stimulator cell (Fig. 4). The addition of anti–class I mAb markedly inhibited the ability of 16.T8-15 to respond to JY. Specifically, the response of 16.T8-15 in the presence of the anti–class I mAb was reduced to that of the neo^+, $CD8^-$ cell line, 16.M-9. The anti-HLA class I mAb had no effect on 16.M-9 (data not shown). When mAbs to either HLA-A2 or -B7 alone were added to culture, there was only a partial inhibition of the response (data not shown), suggesting that the CD8 molecule can interact with either the HLA-A2 or HLA-B7 molecules. Thus, the addition to culture of mAbs directed to the class I antigens on the JY stimulator cells inhibited the enhanced T cell response observed with CD8 expression.

The requirement for expression of class I antigens on the stimulator for CD8 function was also demonstrated genetically. The human cell line Daudi is HLA-DR^+ but expresses no class I MHC antigens due to its inability to synthesize β2-

TABLE II
CD8-mediated Enhancement Requires Class I MHC Expression on the Stimulator

Cell line	IL-2 production stimulated by:	
	JY (class I^+,II^+)	Daudi (class I^-,II^+)
	U/ml	*U/ml*
16.M-9	400	105
16.T8-15 ($CD8^+$)	2,250	150
16.T8-21 ($CD8^+$)	2,500	250

5×10^4 T cells were cultured with 3×10^5 JY or Daudi stimulator cells in a final volume of 1 ml. After 18–24 h, the culture supernatants were assayed for IL-2 by the ability to support growth of the IL-2-dependent T cell line, CTLL20.

microglobulin (36). As can be seen in Table II, the $CD8^+$ infectants 16.T8-15 and 16.T8-21 responded at best twofold better than 16.M-9 to Daudi, but they responded up to 10-fold better than 16.M-9 to JY. These data suggest that the enhanced response observed with the $CD8^+$ hybridomas requires the expression of class I MHC antigens on the stimulator cell. Thus, both mAb blocking and stimulation with a class I–negative cell line suggest that class I HLA molecules encode the ligand for CD8.

Discussion

We have expressed a CD8 cDNA clone in a murine T cell hybridoma that is specific for human class II antigens. The function of CD8 was assessed by the demonstration of the increased efficiency with which these T cell hybridomas respond to suboptimal and optimal levels of antigen. Furthermore, mAbs to the CD8 molecule substantially decreased the response of these infectants. The ability of anti–class I–specific mAbs to inhibit the CD8-mediated enhancement supports the hypothesis that class I MHC proteins encode the physiological ligand for the CD8 molecule.

The ability to block T cell function with mAbs to epitopes on the CD8 molecule can result from several possible mechanisms. Inhibition may occur by the disruption of a receptor–ligand interaction that is essential to provide sufficient avidity for the T cell to interact with its stimulator or target cell. mAbs to CD8 might crosslink the molecule on the surface of a T cell, thereby generating an off signal that inhibits subsequent T cell activation. Either or both of these mechanisms of inhibition could be operative in the experiments reported. Our data, however, do not provide evidence for a potent off signal delivered by the OKT8 mAb. We were unable to totally suppress the response of the $CD8^+$ T cell hybridomas to the JY stimulator cell, which expresses class I and class II MHC antigens. The response in the presence of OKT8 was equivalent to that of the neo^+ control. In addition, the OKT8 mAb had no demonstrable inhibitory effect on the response of these $CD8^+$ T cells to the class I–negative cell line Daudi (data not shown). These results are consistent with OKT8 blocking a receptor–ligand interaction, not with induction of an off signal. In preliminary experiments, it has been

possible to significantly suppress the response to Daudi cells with the mAbs OKT8C and OKT8F (data not shown). These mAbs are to epitopes that are distinct from that of OKT8. These observations suggest that certain anti-CD8 mAbs might function to inhibit the CD8 receptor–ligand interaction between stimulator/target cells, but some of these mAbs might also function to transduce an off signal from the extracellular environment. Such an interpretation would be consistent with the studies of Hunig (18), who was able to inhibit the lectin-dependent lysis of H-2-negative target cells with mAbs to Lyt-2, the murine CD8 equivalent.

Noting that most murine class II–specific T cells are $L3T4^+$,$Lyt\text{-}2^-$ and most class I–specific T cells are $L3T4^-$, $Lyt\text{-}2^+$, Saizawa et al. (37) have hypothesized that a physical interaction occurs between the L3T4 molecule, the murine equivalent of CD4, and the class II–specific TCR. The model would also suggest a similar interaction occurs between Lyt-2 and a class I–specific TCR. The thesis states that there is an obligatory physical association between L3T4 and the TCR, inducing a conformational change in the TCR that results in its shift to a higher affinity state. mAbs to L3T4 inhibit by disrupting the TCR-L3T4 association, thus preventing the TCR affinity shift from occurring. Our results, demonstrating the ability of the CD8 molecule to augment activation of a T cell with a class II–specific TCR, would appear to be inconsistent with this hypothesis.

Shimonkevitz et al. (38) studied the sensitivity to inhibition by anti-Lyt-2 mAb in a series of K^d-specific murine cytolytic T cell clones. As a target cell, they used a cell line that expressed very low basal levels of the H-2 K^d antigen but could be induced to express greater levels by incubation with IFN-γ. T cell clones that could lyse uninduced targets were insensitive to anti-Lyt-2 mAb inhibition. Those that could only lyse IFN-γ-induced targets were susceptible to the effect of anti-Lyt-2 mAb. They concluded that high-avidity T cell clones, as defined by their ability to recognize a low density of MHC on target cells, functioned independently of Lyt-2. Conversely, the low-affinity clones, those that required the greater antigen density present on IFN-γ-induced targets, relied on Lyt-2 to increase their ability to adhere to target cells. Consistent with this conclusion, Dembic et al. (39) transfected a Lyt-2 cDNA clone into a mouse cytotoxic T cell, and thereby enhanced its ability to respond to antigen. Though we did not manipulate the antigen density per cell, by coculturing the hybridomas with suboptimal numbers of stimulator cells, we did manipulate the antigen available to these T cell hybridomas. The ability of the $CD8^+$ hybridomas to respond to 10-fold fewer stimulator cells would be in agreement with the interpretation of Shimonkevitz et al. (38).

Two conclusions may be drawn from our functional studies of the CD8 molecule. First, expression of CD8 in a murine T cell hybridoma confers the ability to respond to suboptimal amounts of antigen, and secondly, the increased responsiveness of these HLA class II–specific T cell hybridomas was dependent upon the expression of HLA class I products on the stimulator cells. The utility of this approach in understanding the role of T cell accessory molecules has been recently corroborated by expressing CD4 in the same T cell hybridoma used here (25). The data presented here further support the hypothesis that the ligand for CD8 is on the HLA class I molecule.

Summary

In general, the human CD8 molecule is expressed on T cells specific for HLA class I molecules. Studies designed to delineate the function and to define the ligand of the CD8 molecule have been complicated by the fact that the presumptive ligand for CD8 is on the HLA class I molecule, the same molecule encoding the ligand for the antigen-specific T cell receptor. The ability to express genes in cells other than their natural host has produced a new technology with which to approach CD8 functional studies. The insertion of a cDNA clone for CD8 in a defective retroviral vector has allowed the transfer of CD8 by infection with the resulting defective retrovirus. CD8 was then expressed in an HLA class II–specific T cell, thus separating the ligand requirements of the TCR and CD8. By this approach, the human CD8 molecule was expressed in a murine T cell hybridoma specific for human class II antigens. The resulting $CD8^+$ hybridomas demonstrated a 10-fold increase in IL-2 production over the parent cell line when stimulated with JY, a human B lymphoblastoid cell line expressing both class I and II HLA antigens, demonstrating that expression of CD8 increases T cell activation. mAbs directed against the CD8 molecule inhibited the response of $CD8^+$ hybridomas to JY, supporting the conclusion that the CD8 molecule was functional. The role of CD8 as a receptor for class I MHC antigens was addressed by stimulation with a cell line expressing HLA-DR antigens, but lacking the expression of HLA class I antigens (Daudi). Stimulation of the $CD8^+$ hybridomas by Daudi did not result in increased IL-2 production. The response to Daudi was unaltered by the addition of anti-CD8 mAb, in contrast to the ability of anti-CD8 mAb to block JY stimulation. Furthermore, mAbs directed against the class I antigens present on JY cells were able to block the enhanced response of the $CD8^+$ hybridomas to JY. These data support the hypothesis that HLA class I molecules are the ligands involved in the CD8-dependent enhancement of T cell activation.

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