REACTIVITY OF Vβ17a⁺ CD8⁺ T CELL HYBRIDS

Analysis Using a New CD8⁺ T Cell Fusion Partner

By HANS-GERHARD BURGERT, JANICE WHITE,
HANS-ULRICH WELTZIEN,¶ PHILIPPA MARRACK,*‡§‖
AND JOHN W. KAPPLER*§

From the *Howard Hughes Medical Institute, Department of Medicine, National Jewish
Center for Immunology and Respiratory Medicine, Denver, Colorado 80206;
the ‡Departments of Biochemistry, Biophysics, and Genetics, §Microbiology
and Immunology, and ‖Medicine, University of Colorado Health Sciences Center,
Denver, Colorado 80262; and the ¶Max-Planck Institute for Immunobiology,
Freiburg, Federal Republic of Germany

Progenitor T cells undergo a complex differentiation process in the thymus, in which the T cell repertoire in the periphery is created. After the initial rearrangement of TCR genes, at least two selection steps are thought to influence the further development. Both involve interactions of the TCR and MHC antigens (1–3). During the first process T cells bearing certain receptors are positively selected for further development upon "proper" interaction with thymic MHC antigens. A number of recent reports using chimeric mice (4), in vivo mAb treatment (5), or transgenic mice methodology (6, 7) support this view.

A reduction of the TCR repertoire occurs in a second process termed negative selection or tolerance (8, 9). Here, T cells recognizing self antigens together with self MHC are functionally inactivated. The first evidence for a possible mechanism came from studies on the Vβ17a TCR (8). We previously showed that TCRs containing the Vβ17a element are eliminated during thymic development in IE⁺ mice, at or after the CD4⁺ CD8⁺ double-positive stage. More recently, similar observations have been made in other self antigen/TCR systems, such as Mls-1ᵃ with Vβ6 and Vβ8.1 (10, 11), and Mls-2/3ᵃ with Vβ3 (12).

In mature T cells a close association exists between the expression of either the CD4 or CD8 surface molecules and the ability to recognize antigen associated with either class II or class I MHC molecules, respectively (13, 14). Therefore, it was surprising that in these tolerance experiments, despite the fact that the antigen in each case was associated with class II MHC, deletion of CD8⁺ T cells was often nearly as complete as that of CD4⁺ T cells.

There are two possible explanations for this phenomenon; either, tolerance to these antigens occurs when thymocytes are immature and express CD4 and CD8 simultaneously; or, alternatively, CD8⁺ mature T cells are able to recognize these class II antigens and are subject to deletion. In the latter case, it should be possible to

detect such a reactivity in vitro using hybridoma technology. However, the BW5147 (BW)[1] fusion partner for T cells does not support expression of CD8 after fusion to CD8⁺ T cells, and hybrids made from these cells generally do not react with the particular antigen under study (15). The recent cloning of the mouse CD8 molecule (16, 17, and for a review, 18) makes this study now feasible.

To test these ideas for Vβ17a⁺ T cells, we generated CD8⁺ Vβ17a⁺ T cell hybrids in two ways: first, previously established Vβ17a⁺ T cell hybrids, either CD4⁺ or CD4⁻, were transfected with a CD8 cDNA construct. Second, a new CD8⁺ fusion cell line was established and used to generate a large number of Vβ17a⁺ CD8⁺ T cell hybrids. These hybrids were tested for their ability to recognize IE molecules. In no case was any CD8-dependent IE reactivity found. Furthermore, the occasional IE reactivity of Vβ17a⁺ CD8⁺ hybrids was in nearly every case correlated with derepressed CD4 expression. These results indicate that the propensity of T cells bearing Vβ17a to interact with IE is independent of the MHC class selecting the cell, but that this interaction is highly dependent on the CD4 molecule. These findings strongly support the view that tolerance to this antigen among CD8⁺ T cells occurs at an early stage in the thymus when these cells bear CD4 as well as CD8.

In addition, we have demonstrated that a transfected CD8 molecule can function in class I–restricted T cell hybrids, generated with a new CD8⁺ fusion partner. The CD8⁺ cell line should prove useful for studying antigen processing requirements of class I MHC-restricted T cells.

Materials and Methods

Mice. Mice were either bred in our own facility or purchased from The Jackson Laboratory, Bar Harbor, ME. The production of the Vβ17a⁺ B10 mouse strains indicated in Fig. 1 was described recently (19).

mAbs and Flow Cytometry. The mAbs used in this study are listed in Table I. For direct immunofluorescence staining of cells, mAbs KJ23a, 53-6.7, GK1.5, 145-2C11, and RG7/9.1 were labeled with FITC or biotin-*n*-hydroxysuccinimide. To detect biotinylated antibodies, phycoerythrin-streptavidin (PE-Av; Tago Inc., Burlington, CA) was used as a secondary reagent. Cells were stained and analyzed using an Epics C flow cytometer (Coulter Electronics Inc., Hialeah, FL) as previously described (20).

Cells. BWα⁻ and BWα⁻β⁻ are x-ray-induced derivatives of the BW5147 AKR thymoma (33) that have lost the TCR-β or the α and β genes, respectively (34). The chicken OVA (cOVA)-specific T cell hybrid 2QO23-36.19.21 has been generated by immunizing SWR mice with cOVA in the base of the tail. 1 wk later cell suspensions from inguinal, mesenteric, and cervical lymph nodes (LNs) were prepared and T cells isolated by passage over a nylon wool column (35). These cells were stimulated in vitro for 4 d with antigen and SWR spleen cells. For the generation of Vβ17a⁺ T cell hybrids, T cells were incubated on plates that had been coated with mAb KJ23a (100 μg/ml purified antibody in PBS) overnight and washed several times with MEM. After 1 h at 37°C, nonadherent cells were removed, the culture dish was washed three times with medium, and adherent cells were incubated for 4 d in the presence of IL-2 (200 U/ml). Subsequently, they were shaken off the plate, incubated another 2 d in IL-2-containing medium, and fused to BWα⁻ (36.19.21) or BWα⁻β⁻ (3QO23-24.4). The fusion was done as described (30). Both T cell hybrids recognize cOVA in conjunction with IA�q, as well as IE molecules of the k, s, and d haplotypes. The other IE-reactive hybrid, 2Q23-34.7.10, is a CD4⁻ derivative of 2Q23-34.7.9 that has been described previously (36).

Transfections.	The vector pSFSVn-Ly2α, used for expression of the CD8 molecule, was kindly provided by Dr. J. Parnes (Stanford University, Stanford, CA). It consisted of the Eco RI–Pvu II fragment of pBR322 containing the ampicillin resistance (Amp^R) gene, the SV40 promoter/enhancer region driving the selectable marker gene, neophosphotransferase (*neo*), followed by SV40 sequences with splice acceptor sites and poly(A) signals (37). Linked to this portion of the plasmid is an Eco RI–Bam H1 fragment containing the Ly2α cDNA flanked at the 5' end by the spleen focus-forming virus (SFFV) long terminal repeat (LTR) and at the 3' end by SFFV poly(A) signals. Transcription of the Ly2α DNA occurs in opposite direction to the *neo* gene. The vector is essentially identical to one previously used for expression of the CD4 molecule, except that the CD4 cDNA was replaced by a CD8α cDNA (38).

Before transfection, the pSFSVn-Ly2α plasmid was cut with the restriction enzyme Nde I. A corresponding restriction site is located close to the pBR322 origin of replication. DNA was sterilized by ethanol precipitation and solubilized in H_2O at a concentration of 3 μg/ml. 5×10^6 T cells were washed once with HBSS and then with transfection buffer (TB) that consisted of the following ingredients: 0.28 M glucose, 1 mM $KH_2 PO_4/K_2HPO_4$, pH 7.1, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, and 50 μg/ml freshly added BSA. Finally, cells were resuspended in 95 μl TB, 5 μl DNA (15 μg) was added, and the mixture was incubated on ice for 10 min. Transfection was performed using an electroporator (2000; Baekon, Inc., Saratoga, CA). The settings were as follows: 9 KV amplitude, 256 pulses, each 160 μs long. The burst time was 12.8 s and the distance between electrode and the surface of the transfection buffer was 3 mm. Seven cycles were carried out. Under these conditions, 50–80% of the cells died during transfection or within 24 h. After 10 min on ice, the cells were resuspended in 120 ml of medium and plated in five 24-well plates (Becton Dickinson & Co., Mountain View, CA). 40 h later the selective drug G-418 (Gibco Laboratories, Grand Island, NY) was added to a final concentration of 800–1,500 μg/ml depending on the T cell line transfected. The optimal concentration for each hybrid was determined before transfection. To control the efficiency of the drug, cells were mock transfected (without DNA) and cultured in parallel. The medium was changed every 4–5 days. 2 wk after transfection, cells of individual wells, mostly clonal, were analyzed for cell surface expression of the CD8 molecule. In our hands, successful transfection of T cell hybrids required the particular TB used in this study. Other buffers like PBS or HBSS did not result in any colonies.

Preparation of Responder and Stimulator Cells for MLR.	Cells of mesenteric, inguinal, brachial, renal, and axillary LNs were pooled and enriched for T cells through a nylon wool column (35). This cell preparation (12×10^7 cells) was incubated with 5 ml culture supernatant of mAb GK1.5 for 45 min at room temperature, washed three times, and then transferred to two plastic dishes (75 cm^2) precoated with goat anti-rat Ig antiserum (100 μg/ml). Incubation was at 4°C for 45 min. This adsorption step was repeated twice. 3.4×10^7 nonadherent CD8-enriched T cells were recovered, washed, and cocultured (2×10^6/ml) with splenocytes of DBA/2 mice (4×10^6/ml) that had been pretreated with Gey's solution (39) to remove erythrocytes, and irradiated with 2,000 rad. No IL-2 was added in order to prevent growth of preactivated, nonspecific T cells (40). After 4 d T cell blasts were isolated by centrifugation on a LSM cushion (Organon Teknika Corp., Durham, NC) and incubated for another 2 d in medium supplemented with IL-2. Before fusion, the cells were tested for CD8, CD4, and Vβ17a expression.

Fusion of CD8⁺ T Cells with BWLyt2-4.	The fusion was carried out essentially as described previously (30), with the exception that 24 h later, HAT and G-418 (400 μg/ml) were added to select for CD8⁺ hybrids. The concentration of G-418 was chosen to be low compared with the drug concentration used for selection of the BWLyt2-4 fusion partner (700 μg/ml) in order to avoid additional stress to the growing hybrids. Sometimes this low concentration of G-418 allowed the growth of CD8⁻ cells (probably due to chromosome loss), which could often be eliminated by increasing the G-418 concentration to 600–800 μg/ml.

IL-2 Assay.	IL-2 production of T cell hybridomas was determined using the IL-2-dependent cell line HT-2 as described previously (30). Briefly, 10^5 T cell hybrids were incubated with 10^6 splenocytes or 10^5 P815 cells or B lymphoma cells in the presence or absence of different mAbs. In antibody blocking experiments, mAbs were added either as 50 μl undiluted culture supernatant or as 1:100 diluted ascites fluid. The final volume of the cultures was always

TABLE I

mAbs Used in This Study

mAb	Relevant specificity	Reference
KJ23a	Vβ17a	20
H57-597.2	TCR	21
145-2C11	T3ϵ	22
53-6.7	CD8	23
GK1.5	CD4	24
RG7/9.1	rat κ	25
20-8-4	K^d, K^b, Qa	26, 27
30-5-7	L^d	28
34-5-8	D^d	28
142-23	K^k, D^k, K^b	29
34-1-2	K^d, D^d, s, Qa	27, 28
MK-D6	A^d	30
M5/114	A^d, E^d	31
Y17	E^k, E^s	32

250 μl. Stimulation of T cell hybrids with mAbs against the TCR was achieved by coating microtiter wells overnight with a TCR-specific antibody (20). 24 h later, 80 μl of supernatant was transferred to a new microtiter plate, where serial dilutions were performed and HT-2 cells added. The amount of IL-2 was assayed visually (30) and with the MTT-ELISA (41).

Results

Deletion of CD8⁺ and CD4⁺ Vβ17a⁺ T Cells in IE⁺ Mice. Vβ17a⁺ T cells are strongly reactive to IE molecules. This has been demonstrated by deletion of mature Vβ17a⁺ thymocytes in IE⁺ mouse strains, as well as by the overwhelming IE reactivity of Vβ17a⁺ CD4⁺ T cell hybrids (8, 18).

Fig. 1 illustrates the point that in IE⁺ mice, both CD4⁺ and CD8⁺ T cells bearing Vβ17a were deleted. In these experiments, good expression of Vβ17a⁺ T cells was seen on both CD4⁺ or CD8⁺ T cells in mouse strains lacking an IE molecule. On the other hand, most Vβ17a⁺ T cells, including both CD4⁺ and CD8⁺ cells, were deleted from IE⁺ strains. For example, the percentage of Vβ17a⁺ CD4⁺ T cells in B10.HTTβL (IE⁺) dropped by ~90% compared with B10.S(7R)βL (IE⁻). A similar reduction of ~70% was observed in the CD8⁺ Vβ17a⁺ T cell subset of B10.HTTβL (Fig. 1).

We performed a number of experiments to test the relative efficiencies of CD4 and CD8 to act as accessory molecules for IE recognition by Vβ17a⁺ T cells in order to distinguish whether tolerance to IE involves deletion of CD8⁺ T cells at a CD4⁺ CD8⁺ or a CD4⁻ CD8⁺ stage.

Effects of Murine CD8α Transfected into Vβ17a⁺ T Cell Hybrids. So far, the double-positive thymocyte population is not directly accessible to functional tests. Therefore, we decided to investigate the reactivity of Vβ17a⁺ CD8⁺ T cells in vitro. Up to now, generation of such cells using hybridoma technology was hampered by the fact that expression of the incoming CD8 molecule was specifically shut down in hybrids derived from the BW5147 fusion partner (15).

We hoped to avoid this problem by transfecting into T cell hybrids a cDNA construct of CD8α in which the potential 5′ regulatory element was replaced with a

Vβ⁸ Strain	H-2				T cells Expressing Vβ17a		
	K	A	E	D	% of CD4⁺		% of CD8⁺
SWR	q	q	-	q			
SJL	s	s	-	s			
B10.S(7R)βL	s	s	-	d			
B10.βJ	b	b	-	b			
B10.A(5R)βL	b	b	b	d			
B10.HTTβL	s	s	s	d			
B10.BRₐₐₐ	k	k	k	k			

FIGURE 1.   Elimination of Vβ17a⁺ CD4⁺ as well as Vβ17a⁺ CD8⁺ T cells in the periphery of IE⁺ mice. The production of the mouse strains indicated in the left panel has been described previously (19). Nylon wool–purified LN T cells were stained with either FITC-anti-CD4 or FITC-anti-CD8 and biotinylated anti-Vβ17a plus PE-Av.

strong viral promoter (see Materials and Methods for more detailed information). The purpose of this experiment was twofold, first, to test whether the CD8 cDNA can be generally expressed in these cells and, second, whether CD8 can be used as an alternative accessory molecule in order to recognize IE antigens. Two Vβ17a⁺ T cell hybrids were chosen as transfection recipients: 2Q23-34.7.10, a CD4⁻ variant of a Vβ17a⁺ hybrid (36), which retained a weak reactivity to IE after loss of the CD4 molecule; and 2QO23-36.19.21, a CD4⁺ Vβ17a⁺ hybrid, originally selected for reactivity to cOVA/IAq, but also capable of CD4-dependent IE recognition. For simplicity, we refer to these cell lines in the text as 34.7.10 and 36.19.21. Both cell lines

FIGURE 2.   Cell surface expression of CD8 molecules upon transfection of Vβ17a⁺ T cell hybrids. Cells were stained with indirect immunofluorescence using mAbs FITC-RG7/9.1 (BGD), KJ23a (Vβ17a), GK1.5 (CD4), and 53-6.7 (CD8). KJ23a binding was detected by FITC-labeled goat anti-mouse IgG serum and the rat antibodies against CD4 and CD8 by FITC-RG7/9.1. FACS histograms are shown with the green fluorescence (x-axis) as a 3 decadic log scale. The numbers are the linearized mean value of fluorescence. The number of cells is plotted on the y-axis. The recipient cell lines for transfection, 34.7.10, 36.19.21, and one representative clone derived from each transfection, are shown. The histograms are from the analysis of 5,000 (34.7.10 cell lines) and 10,000 (36.19.21 cell lines) cells.

were transfected by electroporation with the pSFSVn-Ly2α plasmid containing the *neo* gene as selectable marker in addition to the Ly2α cDNA. Fig. 2 shows the results of a FACS analysis from the parental cell lines and one of each transfectant. The recipient cell lines 34.7.10 and 36.19.21 did not express CD8, whereas the two transfectants 36.19.21Ly2-6 and 34.7.10Ly2-2 expressed high levels of this molecule. The amount of CD4 and TCR on the plasma membrane of transfectants was comparable with the untransfected cell lines. Expression of the CD8 molecule was stable as indicated by its unaltered expression after continuous culture of transfectants for 3 mo without G-418 (data not shown). Thus, the cDNA construct gave rise to high and stable expression of the CD8 molecule in T cell hybridomas.

We then tested whether or not the transfected CD8 molecule contributed to the recognition of IE molecules by these hybrids. Hybrids were cocultured with B10.BR spleen cells (IE^k) in the presence and absence of mAbs against CD4, CD8, IE^k, and K^{k}D^k molecules. As a readout system, IL-2 production was measured using the IL-2-dependent cell line HT-2 (30). The response (U/ml IL-2) of the CD4$^-$ CD8$^+$ 34.7.10 transfectants was basically the same as that of the untransfected cell line (Table II). Neither CD8- nor CD4-specific mAbs inhibited IL-2 production. However, inhibition by the mAb directed to IE indicated that the amount of IL-2 produced depended upon stimulation by IE molecules and not by class I MHC molecules. This was confirmed by the lack of significant IL-2 production when the hybrids were cultured together with IE$^-$ B10A.(4R) spleen cells (data not shown).

Similar results were obtained comparing IL-2 production of CD4$^+$ CD8$^+$ 36.19.21 transfectants with the recipient cell line. In this case, however, the mAb against CD4 completely inhibited the response, indicating that the CD8 molecule expressed in the transfectants could not substitute for CD4 in this recognition. Also, the response of 36.19.21 transfectants towards OVA/IA^q was unchanged compared with that of

TABLE II

CD8 Does Not Replace the Accessory Function of CD4 in Transfected IE-reactive Vβ17a⁺ T Cell Hybrids

	Cell surface expression		IL-2 produced upon stimulation by:				
				B10.BR spleen cells			
	CD4	CD8	Medium	αCD4	αCD8	αIE^k	αK^{k}D^k
			U/ml				
2Q23-34.7.10	–	–	160	120	160	20	160
2Q23-34.7.10Ly2-1	–	+	120	120	120	25	80
2Q23-34.7.10Ly2-2	–	+	160	160	160	10	160
2QO23-36.19.21	+	–	520	<10	200	25	480
2QO23-36.19.21Ly2-6	+	+	· 400	<10	200	20	120
2QO23-36.19.21Ly2-10	+	+	200	<10	110	10	80

Recipient cell lines 2Q23-34.7.10 or 2QO23-36.19.21 and their CD8⁺ derivatives were cocultured with B10.BR spleen cells in the presence or absence of the following mAbs: anti-CD4, GK1.5; anti-CD8, 53-6.7; anti-IE^k, Y17; and anti-K^{k}D^k, 142-23. IL-2-containing supernatants were serially diluted on HT-2 cells and U IL-2 was determined as described (30). In this assay, <10 was the smallest amount of IL-2 to be detected. B10.BR spleen cells alone produced <10 U/ml of IL-2. Very similar results have been obtained from 12 more CD8⁺ clones generated by the two transfections.

the untransfected cell line. Furthermore, none of the hybrids reacted with class I antigens either expressed on P815 cells or L cells (data not shown). We concluded that the CD8 molecule does not influence the reactivity of these hybrids to their respective antigens. These data suggested that CD8 is not a functional accessory molecule for these particular TCRs in recognition of the IE molecule or of the cOVA/IA^q.

Generation of a New Fusion Cell Line Expressing CD8. However, one could argue that, first, the two TCRs examined with this transfection technique were preselected in the thymus together with CD4 and therefore might not be functional with CD8. Second, only a small number of TCRs (two) were tested; and third, we have not presented evidence that the transfected CD8 molecule is functional at all in the BW5147 background.

As CD8$^+$ T cells are restricted to class I MHC antigens, we tested the function of the introduced CD8 molecule by generating class I–restricted T cell hybrids. This was done in two ways. First of all, the CD8α cDNA construct was transfected into the BW$\alpha^-\beta^-$ fusion cell line (34). We hoped to use such a cell line as a fusion partner for class I–restricted T cells. Transfection of BW$\alpha^-\beta^-$ with the CD8α cDNA vector led to expression of CD8 at the plasma membrane, confirming that BW was unable to downregulate the CD8 antigen when expressed from this construct (data not shown). One of the CD8$^+$ BW clones, BWLyt2-4, which expressed a high level of CD8 (Lyt2α) on the cell surface, was chosen for further studies.

Generation of Class I–restricted, Alloreactive T Cell Hybrids Using the CD8$^+$ Fusion Cell Line. Class I–restricted T cells were generated by a primary MLR using C57L cells as responders and DBA/2 cells as stimulators. C57L mice were chosen because they express Vβ17a$^+$ TCRs and because the C57 background seems to give good alloresponses in primary MLR (40). Nylon wool–purified LN T cells were enriched for CD8$^+$ cells by removal of CD4$^+$ cells with panning. This procedure resulted in a T cell population highly enriched for CD8 expression (>95%). This T cell preparation was incubated for 4 d with irradiated DBA/2 spleen cells. The isolated T cell blasts were expanded for 2 more d in the presence of IL-2. Before fusion, T cell blasts were examined using FACS analysis. 98.4% were CD8$^+$, only 1.4% expressed the CD4 marker, and 5.9% of T cells were Vβ17a$^+$. These T cells were fused to BWLyt2-4. Hybrids were expanded and functionally analyzed measuring IL-2 production. Table III summarizes the reactivity pattern obtained. Of the 29 hybrids tested, four did not produce IL-2 upon stimulation with crosslinked anti-CD3 mAb, six failed to react with either DBA/2 spleen cells or P815 cells, and for five, a weak IL-2 production was observed but a specificity could not be assigned. The reactivity of 14 hybrids could be identified. Three seemed to recognize class II molecules and were not further studied; 11, however, reacted with class I molecules of H-2^d cells. To identify the particular class I molecule recognized, each hybrid was incubated with P815 cells and DBA/2 spleen cells in the presence and/or absence of mAbs against individual H-2^d gene products. In addition, the susceptibility of the T cell hybrids to inhibition by CD4- and CD8-specific mAbs was tested. The analysis of three representative hybrids is shown in Fig. 3. BD8-5 recognized H-2K^d as indicated by the specific inhibition of IL-2 production by mAb 20-8-4 directed against K^d (26). This result was confirmed by stimulation of this hybrid with D2.GD but not with B10A.(5R) spleen cells, expressing K^d, A^d, D^b, and K^b, A^b, E$^{k/b}$, L^d, and D^d MHC

TABLE III

Reactivity Pattern of T Cell Hybrids Generated from Alloreactive T Cells and the BWLyt2-4 Fusion Partner

Reactivity pattern	T cell hybrids
No IL-2 production with anti-CD3	4
No reactivity to DBA/2 or P815	6
Reactivity not identified	5
Reactivity to class II MHC	3
Reactivity to class I MHC	11
Total	29

T cell hybrids were produced with the BWLyt2-4 cell line as fusion partner as described in Materials and Methods. To examine the general capability of T cell hybrids to produce IL-2 via their TCR, cells were incubated on wells coated with anti-CD3 mAb (22). The IL-2 response was measured visually (30) and with the MTT-ELISA (41). Reactivities were identified by antibody blocking studies as illustrated in Fig. 3. In general, the response of hybrids whose reactivity could not be assigned for was <20 U/ml IL-2 when stimulated by DBA/2 splenocytes.
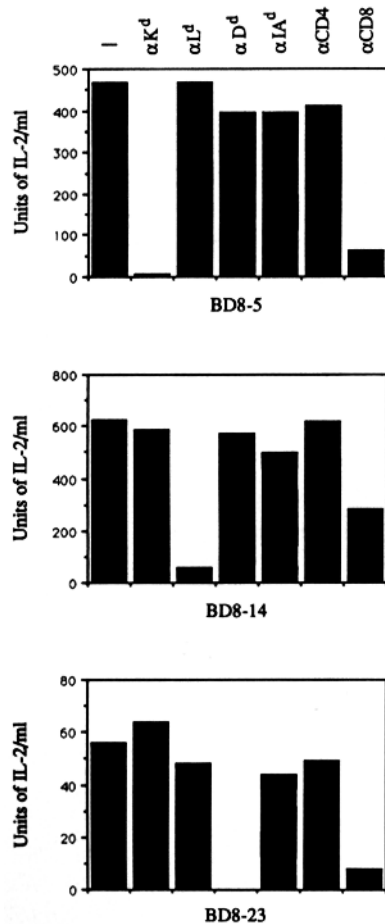
FIGURE 3. The reactivity of CD8⁺ T cell hybrids can be specifically blocked by class I MHC- and CD8-specific antibodies. Three different T cell hybrids derived from a fusion of alloreactive CD8⁺ T cells (primary MLR; C57L → DBA/2) with BWLyt2-4 were incubated with DBA/2 spleen cells only (-) or together with 50 µl of cell culture supernatants of the following mAbs: αK^d (20-8-4), αL^d (30-5-7), αD^d (34-5-8), αIA^d (MK-D6), $\alpha CD4$ (GK1.5), and $\alpha CD8$ (53-6.7). IL-2 production was monitored by titrating supernatants on the IL-2-dependent cell line HT-2. The assays were read visually and with an MTT-dependent ELISA (41).

molecules, respectively (data not shown). In similar fashion, the reactivity of the other hybrids was identified (Fig. 3 and Table IV).

More important was the fact that the response of the vast majority of class I–restricted T cell hybrids was inhibited by CD8-specific antibodies. A summary of the data is shown in Table IV. Three class I–reactive clones (BD8-4, BD8-12, and BD8-A5) did not express appreciable amounts of CD8 on the cell surface. Accordingly, their IL-2 response could not be inhibited by anti-CD8 mAb. All of the CD8$^+$ class I–restricted hybrids (8/8) were inhibited by mAb 53-6.7 directed to murine CD8, indicating very strongly that CD8 serves as a functional accessory molecule for these T cell hybrids. These experiments also demonstrated the general usefulness of the BWLyt2-4 cell line for generating class I–restricted antigen-responsive T cell hybrids.

In addition, a CD8$^-$ T cell hybrid derived from a TNP-specific, class I–restricted T cell clone was transfected with CD8 and its function reconstituted (H.-G. Burgert, unpublished results).

Generation and Reactivity towards IE Molecules of Vβ17a$^+$ CD8$^+$ T Cell Hybrids. We

TABLE IV

The Introduced CD8 Molecule Is Functional in Class I–restricted,
Alloreactive T Cell Hybrids

BD8-T cell hybrid	FACS analysis		Reactivity average of IL-2		Inhibition by anti-CD8
	TCR	CD8			
BD8-3	32	86	K^d	210	+ + +
BD8-4	19	–	L^d	570	–
BD8-5	45	140	K^d	590	+ + +
BD8-8	22	98	K^d	10	+
BD8-9	16	71	K^d	17	+ + +
BD8-10	34	64	IA^d	7*	+
BD8-12	44	–	K^d	290*	–
BD8-14	19	112	L^d	1,470*	+ +
BD8-15	ND	ND	D^d	85‡	+ + +
BD8-17	28	119	IA^d	900*	–
BD8-21	12	66	IA^d	23*	+
BD8-23	26	112	D^d	80*	+ +
BD8-A3	37	160	K^d	1,230*	+ +
BD8-A5	18	–	L^d	700	–

Alloreactive T cell hybrids have been generated by a primary MLR (C57L → DBA/2) and subsequent fusion with the BWLyt2-4 cell line. Only those hybrids are listed whose reactivity could be identified by antibody blocking studies. The FACS data are given as linearized mean values of fluorescence, after staining cells with mAb H57-597.2, directed to a TCR framework determinant (21), and with the anti-CD8 mAb, 53-6.7. The reactivity given was identified as described in the legend to Fig. 3. The average amount of IL-2 produced by a particular T cell hybrid is expressed as U/ml and is a mean value from three to five independent experiments, taking into account the data from both P815 cells and DBA/2 spleen cells. The degree of inhibition by anti-CD8 mAb is shown as: + + +, >80%; + +, 55-80%; +, 30-55%; –, 0-30% inhibition. The latter is considered unspecific since CD8$^-$ T cell hybrids fall in this category.

* IL-2 (U/ml) after stimulation with DBA/2 cells only. These hybrids produced much more IL-2 (4-20 times more) in response to DBA/2 splenocytes than to P815 cells.

‡ Data from one experiment.

concluded that the introduced CD8 molecule was functional in the BWLyt2-4 cell line and, therefore, one should be able to generate functional CD8⁺ Vβ17a⁺ T cell hybrids. To this end, CD8⁺ Vβ17a⁺ T cells from SWR or B10.βJ(19) mice were stimulated to grow by culturing them on anti-Vβ17a-coated plates. The resulting T cell blasts were 98% positive for the CD8 marker and 99% positive for KJ23 (data not shown). After 2 more d in IL-2, T cell blasts were fused to BWLyt2-4. Large numbers of T cell hybrids were obtained from both fusions (396 from SWR, 210 from B10.βJ). Of those, 115 and 48, respectively, were tested for expression of CD8 and Vβ17a by two-color flow cytometry. The vast majority of clones expressed both antigens at least to some extent.

Only those hybridomas that were >90% positive for Vβ17a and CD8 were examined functionally. 11 T cell hybrids derived from C57BL/10.βJ, and 27 derived from SWR, referring to B and Q, respectively, were tested for their response to B10.A (IEᵏ), B10.HTT (IEˢ), and B10 (IE⁻) spleen cells. In addition, their general capability to produce IL-2 via their TCR was examined by incubation on KJ23-coated wells. The amount of IL-2 produced is indicated in Table V.

Two T cell hybrids from each strain (B7, B24, Q35, and Q114) did not produce IL-2 upon stimulation with the mAb against Vβ17a and were excluded from further analysis. Surprisingly, only one of nine B10.BJ-derived T cell hybrids (B40) and 7 of the 25 SWR-derived hybrids seemed to react with IEᵏ and/or IEˢ.

Contribution of CD4 and CD8 to IE Reactivity of Vβ17a⁺ T Cell Hybrids. We further investigated whether or not CD8 was involved in this recognition process by antibody inhibition studies (Table VI). In general, the anti-CD4 and anti-CD8 antibodies showed some nonspecific inhibitory activity that decreased the IL-2 production of CD4⁻ (eg., B40, Q120) or CD8⁻ (3QO23-24.4) T cell hybrids when stimulated by one or both types of spleen cells. For the CD4 antibody, we calculated this to be 32%, for the CD8-specific antibody, 41%. Taking these nonspecific effects into account, none of the IE-reactive hybrids was significantly inhibited by the anti-CD8-specific antibody, indicating that the CD8 molecule did not act as accessory molecule for IE recognition. Surprisingly, however, four of the six SWR-derived hybrids produced reduced levels of IL-2 in the presence of the anti-CD4 antibody. We therefore analyzed whether or not these hybrids expressed CD4. All four clones inhibited by the CD4-specific antibody contained a variable proportion of cells (17-90%) positive for the CD4 molecule (data not shown). Moreover, double staining experiments showed that CD4 and CD8 were expressed on the same cell. Fig. 4 shows representative data of the FACS analysis. The control cell lines, BWLyt2-4 and 3QO23-24.4, were CD8⁺ and CD4⁺, respectively, indicated by the signal distribution pattern. Q2, an IE-nonreactive T cell hybrid, and Q120, an IE-reactive one, did not express CD4. However, the CD4-inhibitable T cell hybrids Q10, and especially Q25, contained a proportion of cells (22 and 68% in this experiment) that coexpressed CD4 and CD8. Similar results were obtained for the other CD4-inhibitable T cell hybrids (Q15, 22%; Q129, 3%). Apparently, CD4 had become derepressed in these particular hybrids, an observation made previously by Carbone et al. (15) studying hybrids produced by fusion of CD8⁺ T cells to BW5147.

In summary, five of seven IE-reactive T cell hybrids (71%) expressed CD4 to various degrees. As these expression data correlated with the inhibition experiments, we attributed the IE reactivity to the expression of CD4. A similar high percentage

TABLE V

*Only a Small Percentage of Vβ17a⁺ CD8⁺ T Cell Hybrids
Responds to IE Molecules*

T cell hybrid	IL-2 produced upon stimulation by:			
	B10.A	B10.HTT	B10	KJ23a
	U/ml			
BWLyt2-4	10	12	11	2
3QO23-24.4	1,210	2,570	23	2,630
B1	7	15	3	1,330
B7	2	2	1	3
B13	4	5	5	144
B16	5	5	7	1,740
B23	5	6	4	170
B24	4	6	3	3
B27	6	9	6	260
B32	7	3	3	80
B38	7	5	3	130
B40	390	26	4	3,460
B51	11	8	3	1,630
Q2	8	9	3	1,720
Q3	9	12	ND	70
Q5	8	32	3	960
Q6	6	8	5	2,420
Q10	40	16	3	750
Q14	6	5	4	270
Q15	20	70	6	1,830
Q17	8	6	5	340
Q22	4	7	4	450
Q25	110	230	4	1,290
Q30	6	4	4	80
Q33	2	2	1	1,620
Q35	3	2	3	5
Q50	4	2	2	180
Q56	2	3	3	190
Q62	5	7	ND	770
Q68	2	1	2	160
Q73	3	1	2	63
Q85	5	3	2	53
Q108	6	10	3	970
Q114	2	6	3	3
Q120	9	40	2	1,870
Q125	2	1	2	960
Q129	13	150	2	270
Q131	3	3	5	1,080
Q134	3	5	3	1,320
Q135	4	9	1	180
No hybrid	5	6	4	ND

T cell hybrids derived from the fusion of BWLyt2-4 of either B10.βJ CD8⁺ Vβ17a⁺ T cells (B1-B51) or CD8⁺ Vβ17a⁺ SWR T cells (Q2-Q135) were stimulated by spleen cells or the mAb against Vβ17a (KJ23a). IL-2 response was measured as before (see Table III). In this assay the background IL-2 production of B10.A, B10.HTT, and B10 spleen cells was 5, 6, and 4 U/ml, respectively. T cell hybrids that had IL-2 responses <10 were considered nonresponsive. B1, B51, and Q3 were subsequently shown to be nonresponsive. The CD4⁺ Vβ17a⁺ T cell hybrid 3QO23-24.4 was used as a positive control; its production is described in Material and Methods.

TABLE VI

Frequent Inhibition of IE Reactivity by Anti-CD4-specific Antibodies

T cell hybrid	IL-2 produced upon stimulation with:									
	B10.A					B10.HTT				
	–	αCD4	αCD8	αIE	αK^{k}D^k	–	αCD4	αCD8	αIE	αH-2^s
3QO23-24.4	1,080	4	480	60	610	2,570	70	1,880	430	2,840
B40	260	260	250	30	140	30	8	19	0	21
Q5	3	2	1	0	0	34	17	23	0	27
Q10	26	4	26	2	24	16	0	12	1	15
Q15	14	0	5	0	3	50	0	34	0	31
Q25	70	1	60	11	19	260	21	140	40	250
Q120	4	1	3	0	4	43	29	18	0	20
Q129	8	2	13	ND	ND	240	110	135	ND	ND

The indicated T cell hybrids were cocultured with B10.A and B10.HTT spleen cells with or without soluble mAbs against CD4 (GK1.5), CD8 (53-6.7), IE (Y17), K^{k}D^k (142-23), and H-2^s (34-1-2). The anti-CD4- (33%) and anti-CD8- (41%) specific mAbs affected the IL-2 response nonspecifically given the data from cells that did not express CD4 (eg., B40, Q120) or CD8 (3QO23-24.4). mAb 142-23 recognizes only one MHC class I antigen on B10.A splenocytes. Basically, the same results were obtained when, in addition, anti-L^d and anti-D^d mAbs were used. The amount of IL-2 was determined as described in Table III. Background IL-2 production of B10.A and B10.HTT splenocytes was subtracted.

(60–90%, depending on the mouse strain) of IE reactivity was found previously among Vβ17a⁺ CD4⁺ T cell hybrids (20). In contrast, we found only one hybrid from each fusion (B40 and Q120) that was IE reactive but not inhibited by either CD4- or CD8-specific antibodies. These two hybrids were considered to have high affinity TCRs for IE molecules that were not dependent on the help of accessory molecules. Thus, overall, we did not find any hybrid whose IE reactivity was inhibited by anti-CD8,
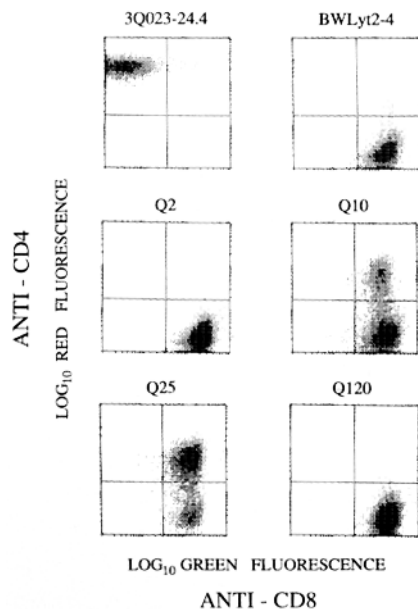

FIGURE 4. Many IE-reactive T cell hybrids express both the CD4 and the CD8 molecule at the cell surface. Two-color fluorescence histograms of control cell lines 3QO23-24.4 (Vβ17a⁺, CD4⁺ CD8⁻), BWLyt2-4 (CD4⁻ CD8⁺) and different Vβ17a⁺ CD8⁺ T cell hybrids are shown. The cells were incubated with biotinylated GK1.5 mAb and then with PE-Av and FITC-labeled 53-6-7 antibody. The gates were set after analysis of the controls. The T cell hybrids Q2, Q10, Q25, and Q120 were generated by fusing BWLyt2-4 with CD8⁺ Vβ17a⁺ T cells of SWR mice. Q2 does not recognize IE molecules, whereas the three latter ones do. The percentages of CD4⁺ CD8⁺ cells in the other IE-reactive clones not discussed in the text were: 4% (Q5), 22% (Q15), and 2.4% (Q129). These values had considerably decreased since the first FACS experiment was done. At that time the proportion of CD4⁺ CD8⁺ cells was as follows: Q5, 17%; Q10, 35%; Q15, 58%; Q25, 97%; Q120, 0%; Q129, 19%; B40, 0%).

and we therefore conclude that CD8 does not contribute to the recognition of IE molecules in vitro by these T cells.

Discussion

Vβ17a$^+$-containing T cells exhibit a strong affinity towards IE molecules and an unknown, probably B cell–specific, self antigen (20, 36). In mice expressing this ligand, Vβ17a$^+$ cells are clonally deleted during development in the thymus (8). In the periphery of IE$^+$ mice, Vβ17a expression is reduced in both the CD4$^+$ and CD8$^+$ T cell subsets (19, and Fig. 1). This result prompted us to investigate directly the reactivity of CD8$^+$ Vβ17a$^+$ T cells. To obtain such cells we transfected either established CD4$^-$ and CD4$^+$, Vβ17a$^+$ T cell hybrids, or an $\alpha^-\beta^-$ derivative of the BW5147 T cell fusion partner with an expressible CD8α (Lyt-2α) gene. CD8 was thus expressed on the surfaces of the preexisting T cell hybrids (Fig. 2), and on hybridomas produced by fusion of Vβ17a$^+$ CD8$^+$ T cells to the CD8$^+$ BW derivative (BWLyt2-4).

Interestingly, expression of CD8 in the preestablished Vβ17a$^+$ hybridomas did not alter their reactivity to IE molecules (Table II). Moreover, the Vβ17a$^+$ CD8$^+$ hybrids derived from the fusion with BWLyt2-4 were mostly IE nonreactive (Table V), and for those that did have some IE reactivity, this response was not inhibited by anti-CD8 antibodies (Table VI). Thus, CD8 seems not to contribute to IE reactivity.

It is well established that CD4 and CD8 can bind directly to class II and class I MHC proteins, respectively (42, 43). Since the target cells used in our assays expressed both IE and class I molecules, we expected that additional expression of the CD8 molecule on the effector T cells would increase the overall avidity of the T cell/target cell interaction, and thereby lead to increased IL-2 production. We did not, however, detect an increased response, nor did we observe any additional reactivities on the part of the T cell hybrids to, for example, class I antigens.

It is possible that one or the other of the interacting molecules was not expressed at high enough levels in this system. We think this is unlikely, since genuinely class I–reactive T cell hybridomas produced by fusion of alloreactive T cells to the CD8$^+$ BW derivative did use the CD8 as an accessory molecule. Alternatively, the affinity for IE of the α/β receptors on the previously established Vβ17a$^+$ hybridomas, 34.7.10 and 36.19.21, might have been high enough that the contribution from CD8 remained undetected. Rather, the opposite is probably true for the majority of hybrids that were produced by fusion to CD8$^+$ Vβ17a$^+$ T cells, cells that had presumably been selected in the thymus for class I reactivity. Another possibility was that in spite of the fact that these cells bore Vβ17a, the rest of the components of their TCRs precluded IE reactivity. We think this is an unlikely explanation because any of these hybrids that were or became CD4$^+$ did manifest CD4-dependent IE reactivity. For the two preexisting hybridomas that were transfected, one could argue that since these hybrids were originally CD4$^+$, their receptors had been selected in the thymus to operate with CD4, and could not properly interact with CD8. We feel that the fact that our findings apply both to these hybrids, and to hybridomas made by fusion to CD8$^+$ cells, argues against this possibility.

It is more likely that these data reflect the idea that CD4 and CD8 are closely associated with the TCR in some way, and are able to contribute to the reactivity of T cells for their targets more efficiently if the target molecule, class I or class II, for the TCR is the same as that for the operative accessory molecule, CD8 or CD4.

If this idea is correct, it follows that the TCR and CD8 can interact with the same class I molecule, a view that is supported by the fact that CD8 binds to a region of class I molecules, the $\alpha3$ domain, with which TCRs are not thought to react (44, 45).

This idea has been suggested by much work in the past (13, 14, 46–49). Fazekas de St. Groth et al. (46), for example, isolated two class II–specific T cell clones bearing both CD4 and CD8, and showed that only CD4 contributed to antigen recognition by these cells. Our results with the $CD4^+$ $CD8^+$ $V\beta17a^+$ cells corroborate these findings. Also, the idea has been given momentum from antibody-induced comodulation and crosslinking data that provide evidence for an association between CD4/CD8 and TCRs (47–49). However, there are other experiments that have shown that the inappropriate accessory molecule can also play a role in reactivity (50–52). For example, we have previously shown that CD4/class II interactions can stimulate the reactivity for class I of the mouse T cell hybridoma, 3DT-52.5 (50, 52), and Ratnofsky et al. (51) have similar findings expressing human CD8 in mouse T cell hybrids specific for human HLA-DR antigens. Whether these represent special cases, or some lapse in the system, in which nonhomologous interactions can contribute under certain circumstances (e.g., suboptimal stimulation) to T cell/target binding, remains to be seen.

Overall, these findings suggest that $CD4^-$ $CD8^+$ $V\beta17a^+$ T cells recognize IE rarely. Unless induction of tolerance by clonal deletion is much more sensitive to low concentrations of antigen than is T cell stimulation, we can only account for the deletion of $CD8^+$ and $CD4^+$ cells at the $CD4^+CD8^+$ thymocyte stage. Other experiments have shown that deletion at this stage is likely. Fowlkes et al. (53), for example, showed that they could block the elimination of $V\beta17a^+$ $CD8^+$ T cells in IE^+ mice by treatment in vivo with an anti-CD4 antibody. Similar conclusions have been reached from the study of TCR transgenic mice or of thymocyte development in normal mice expressing superantigens (9, 54).

In the experiments described in this paper, we have expressed by DNA-mediated gene transfer, the $CD8\alpha$ (Lyt-2α) gene, but thymocytes and peripheral T cells express also $CD8\beta$ (Lyt-3). One could argue that additional expression of $CD8\beta$ might add to the IE reactivity of the hybrids. Transfection of $CD8\alpha$ was sufficient, however, to allow CD8-dependent reactivity of the class I–specific hybridomas (Table IV, Fig. 3), and, moreover, restored reactivity to a preexisting $CD8^-$, class I–restricted T cell hybridoma (data not shown). Other investigators (55, 56) have also found that transfection with $CD8\alpha$ was sufficient to restore CD8-dependent class I reactivity. In fact, subsequent examination of the BWLyt2-4-derived T cell hybrids revealed that, similar to the physiologic situation, the great majority actually expressed the $CD8\beta$ molecule. This implies that the incoming $CD8\beta$ is regulated differently than the incoming $CD8\alpha$ molecule, expression of which is suppressed by BW (15). It will be interesting to determine the basis for this differential regulation.

Finally, in this paper we have described the production and properties of a $CD8^+$ fusion partner that can be used in the production of CD8-dependent T cell hybridomas. CD8 expression by the BW derivative and hybridomas produced by fusion to it is stable, providing levels of G-418 are kept sufficiently high during the critical phase of hybridoma establishment. Hybridomas produced by fusion of the line to class I–specific, alloreactive T cells were functional and dependent upon the activity of the CD8 molecules they bore. The specificity of these hybrids could be assayed

with ease using the IL-2 assay. Since this readout system does not depend upon living cells, all the experiments done with CD4$^+$ T cell hybrids and fixed target cells (57) or isolated MHC class I molecules (58) should now be possible with class I–restricted hybrids too. We hope, therefore, that the BWLyt2-4 cell line will help us to gain further insight into the antigen processing and presentation requirements of class I–restricted T cells.

Summary

Tolerance to IE molecules leads to deletion of Vβ17a-bearing T cells. Both, the CD4$^+$ as well as the CD8$^+$ T cell subsets are affected. A large percentage of CD4$^+$ Vβ17a$^+$ T cell hybrids recognize IE molecules. We now have investigated the reactivity for IE antigens of CD8$^+$ Vβ17a$^+$ T cell hybrids. Using a transfection approach, we have introduced the murine CD8 molecule into different Vβ17a$^+$ T cell hybrids. Furthermore, the CD8 cDNA was transfected into the BW5147$\alpha^-\beta^-$ fusion partner. This allowed us to generate a large number of Vβ17a$^+$ T cell hybrids by fusion with the appropriate T cells. Only 6% of T cell hybrids were stimulated to produce IL-2 upon incubation with IE$^+$ cells. However, in those, the CD8 molecule seemed not to contribute to the IE reactivity of the hybrid, since mAbs against the CD8 molecule failed to inhibit their reactivity. This low percentage of Vβ17a$^+$ CD8$^+$ IE-reactive T cell hybrids contrasts with the strong reduction of CD8$^+$ Vβ17a$^+$ T cells in IE$^+$ mice, strongly suggesting that elimination of such cells in the thymus occurs when they are coexpressing CD4 and CD8. This view was confirmed by the occasional expression of CD4 in some hybrids in which case IE reactivity was detected. Furthermore, we demonstrated the functional integrity of the introduced CD8 molecule by: (*a*) reconstitution of the IL-2 response in a class I–restricted TNP-specific T cell hybrid; and (*b*) by generation of alloreactive class I–restricted T cell hybrids using the new CD8$^+$ fusion cell line. This CD8$^+$ fusion partner, BWLyt2-4, should prove useful to study antigen processing and antigen presentation requirements of class I–restricted T cells.

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