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Major histocompatibility complex class I-specific and -restricted killing of β_2-microglobulin-deficient cells by CD8$^+$ cytotoxic T lymphocytes

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ABSTRACT **Cytotoxic T lymphocytes (CTLs) recognize major histocompatibility complex (MHC) class I molecules, normally composed of a heavy chain, a β_2-microglobulin (β_2m), and peptide antigens. β_2m is considered essential for the assembly and intracellular transport of MHC class I molecules as well as their peptide presentation to CTLs. Contrary to this dogma, we now report the generation of allospecific and restricted CD8$^+$ and TCR$\alpha\beta^+$ CTLs (where TCR is T-cell receptor) capable of killing β_2m-deficient cells. Such CTLs were obtained by priming mice with live allogeneic β_2m$^-$ spleen cells or mutant lymphoma cells producing MHC class I protein but no detectable β_2m. Although both β_2m$^-$ and β_2m-expressing lymphoma cells were rejected in allogeneic mice, only the former were efficient inducers of CTLs recognizing β_2m$^-$ cells. These CTLs were MHC class I (H-2K^b or D^b)-specific and CD8-dependent and did not require serum as a source of external β_2m in the culture. They could be induced across major and minor histocompatibility barriers. The H-2-restricted CTLs generated in the latter case failed to kill the antigen-processing-deficient target RMA-S cells. The results show that MHC class I heavy chains in β_2m$^-$ cells can be transported to the cell surface and act as antigens or antigen-presenting molecules to allospecific and MHC-restricted CTLs.**

Cells deficient in β_2-microglobulin (β_2m) do not export major histocompatibility complex (MHC) class I molecules efficiently and fail to express them in a form that can be recognized by conventional cytotoxic T lymphocytes (CTLs) or antibodies specific for the peptide-binding $\alpha_1\alpha_2$ domain (1–6). This is relevant in at least two situations. (*i*) Loss of β_2m is one cause of MHC class I deficiency in tumors and has been suggested as an escape mechanism from T cells (2). (*ii*) More recently, it has become possible to generate β_2m-deficient cells in normal tissues by the use of gene knockout technology (5, 7); this has led to speculations on approaches to avoid graft rejection in transplantation. It has thus been generally assumed that MHC class I heavy chains cannot be transported to the cell surface in a form that can be recognized as antigen or antigen-presenting molecules in the absence of β_2m. However, support for this theory has come mainly from studies of CTL responses induced by β_2m-expressing cells (1, 2). We have addressed this issue critically by using a protocol based on *in vivo* priming of allogeneic major or minor histocompatibility antigen-mismatched animals with live β_2m-deficient cells of either normal or malignant origin.

MATERIALS AND METHODS

Mice. All the mice were bred and maintained at the Department of Tumor Biology, Karolinska Institute (Stockholm). The following mice were used: A/Sn (H-2^a), A.BY (H-2^b on strain A background), C57BL/6 (B6, H-2^b), C57BL/10 (B10, H-2^b), B10.D2 (H-2^d), and 129 (H-2)^b. The generation of the (B6 × 129)F$_2$ β_2m-deficient mice is described elsewhere (7).

Cell Lines. C4.4-25$^-$ is a β_2m-deficient variant of the EL-4 lymphoma (H-2^b), and E50.16$^+$ is a β_2m^b transfectant of C4.4-25$^-$ (8). C4.4-25$^-$ was originally derived from a panel of EL-4 mutant lines generated by K. Sturmhöfel and G. Hämmerling (9). RMA and the antigen-processing-defective mutant RMA-S are sublines of the RBL-5 lymphoma (4, 10–13). T2-K^b, -D^b, -L^d, -K^k, and -D^p are MHC class I transfectants of the antigen-processing-defective T2 line (0.174 × CEM^R) (14–17), all of which were a kind gift from P. Cresswell (Yale University School of Medicine, New Haven, CT). P815-K^b is a K^b transfectant of the P815 P1HTR subline (mastocytoma of DBA/2, H-2^d origin). P815-KKA and P815-AAK are P1HTR cells transfected with exon-shuffled chimeric genes between HLA-A2 and H-2K^b. The KKA gene product has the $\alpha_1\alpha_2$ domains from H-2K^b and the α_3 domain from A2. The AAK gene product has the $\alpha_1\alpha_2$ domains from A2 and the α_3 domain from K^b (18). P815, P815-K^b, P815-KKA, and P815-AAK cell lines were kind gifts from H. Strauss (University College London). All cell lines were grown in RPMI 1640 medium supplemented with 5% (vol/vol) fetal calf serum (FCS). AIM-V (GIBCO) was used for serum-free cultures.

Antibodies and Complement. Monoclonal antibodies (mAbs) of the following specificities were used: CD8 (YTS 169.4) and CD4 (RM-4-4) (Kemila, Stockholm); T-cell receptor (TCR)$\alpha\beta$ (H57-597) and TCR$\gamma\delta$ (GL-3) (PharMingen, San Diego; Scan Biotech, Falkenberg, Sweden). Rabbit complement was purchased from Pel-Freez Biologicals.

Generation of Con A-Activated Blasts. Freshly isolated spleen cells were erythrocyte-depleted by hypoosmotic shock and cultured at 5 × 10^6 cells per ml for 48 h in RPMI 1640 medium supplemented with 10% FCS and Con A at 5 μg/ml. In some experiments we used serum-free AIM-V medium either without serum supplement or with 1% serum from β_2m−/− mice.

Generation of Effector Cells. A/Sn or A.BY mice were inoculated with 10^5–10^7 live tumor or spleen cells either subcutaneously (s.c.) or intraperitoneally (i.p.). After 2–12 weeks, their spleen cells were cultured for 5 days *in vitro* in a mixed lymphocyte tumor culture (MLTC) (60–100 × 10^6 responders with 4–8 × 10^6 tumor cells or 20–30 × 10^6 spleen cells). Cells from these cultures were used as effectors in a standard ^{51}Cr-release assay.

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Abbreviations: β_2m, β_2-microglobulin; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLTC, mixed lymphocyte tumor culture; TCR, T-cell receptor.

Unlabeled Target Competition Assay. Effector cells and unlabeled target cells were mixed and preincubated for 60 min at 37°C, before addition of ^{51}Cr-labeled target cells. The normal lymphocytes used as unlabeled targets were freshly isolated spleen cells irradiated with 2000 rad (1 rad = 0.01 Gy) before use in the assay.

Effector-Cell Depletion and Antibody Blocking Assay. The effector cells were first incubated with antibody for 30 min on ice and then washed once. After addition of rabbit complement (diluted 1:8, 1 ml per 10^7 cells), the cells were incubated for 60 min at 37°C, washed twice in phosphate-buffered saline (PBS), and used as effector cells in ^{51}Cr-release assays. For effector-cell blocking, the effector cells were preincubated in microwells with mAb for 20 min at room temperature in 100 μl prior to addition of target cells.

Limiting Dilution Analysis of Cytotoxic Cell Lines. Cytotoxic cell lines from A/Sn anti-C4.4-25$^-$ effectors were serially diluted in microwells in 1:3 dilution steps in RPMI 1640 medium supplemented with 10% FCS, 30 μM 2-mercaptoethanol, and 8 international units of recombinant interleukin 2. Approximately 5×10^3 irradiated (10,000 rad) C4.4-25$^-$ stimulator cells and 4×10^6 irradiated (2000 rad) syngeneic A/Sn splenocytes were present in each well as feeder cells. The cell lines were established and expanded from wells originally seeded with three effector cells per well. At this dilution, <63% of the wells contained cytotoxic cells, which is the expected value from randomly seeding an average of one dividing cell per well.

RESULTS

Allogeneic mice were primed by s.c. inoculation with the following live tumor cells: (*i*) EL-4. (*ii*) C4.4-25$^-$, the EL-4-derived β_2m-deficient lymphoma, was shown (8) to be cell-surface-negative for MHC class I by immunofluorescence on intact cells, by using mAbs for the $\alpha_1\alpha_2$ domain and α_3 domain. The C4.4-25$^-$ is also devoid of intracellular β_2m protein, as assessed by immunoprecipitation of lysates from metabolically labeled cells with a panel of eight polyclonal rabbit antisera reacting with MHC class I heavy chains complexed with β_2m or β_2m alone (ref. 8; R.G. and H.-G.L., unpublished data). (*iii*) E50.16$^+$, a β_2m transfectant of C4.4-25$^-$, expresses the MHC class I and β_2m protein intracellularly and on the cell surface (8). All inocula grew to large tumors within a few weeks. To our surprise, the C4.4-25$^-$

Table 1. Target-cell specificity of effector cells

Exp.	Mouse	Effector cell	Target cell				
			EL-4	C4.4-25$^-$	E50.16$^+$	EL-4*	C4.4-25$^-$*
1	A/Sn	EL-4	30, 15	1, 0	26, 15	ND	ND
		C4.4-25$^-$	65, 55	49, 36	64, 49	77, 63	60, 36
		E50.16$^+$	59, 52	2, 1	52, 43	68, 64	7, 0
			EL-4	C4.4-25$^-$	E50.16$^+$	RMA	
2	A/Sn	EL-4	41, 19	10, 6	45, 23	53, 32	
		C4.4-25$^-$	42, 20	34, 14	48, 28	59, 39	
		E50.16$^+$	50, 32	9, 4	55, 43	56, 37	
			EL-4*	C4.4-25$^-$*	E50.16$^+$*	RMA-S	
	A/Sn	EL-4	57, 29	17, 6	52, 29	31, 11	
		C4.4-25$^-$	67, 35	57, 38	58, 27	70, 47	
		E50.16$^+$	72, 48	25, 14	63, 39	47, 27	
			P815	P815-K^b	P815-AAK	P815-KKA	
	A/Sn	EL-4	12, 7	47, 23	7, 9	50, 23	
		C4.4-25$^-$	9, 6	38, 15	5, 1	51, 34	
		E50.16$^+$	14, 6	50, 38	12, 5	55, 34	
			C4.4-25$^-$	T2	T2-K^b	T2-D^b	T2-K^k
3	A/Sn	C4.4-25$^-$	57, 47	7, 5	66, 63	48, 32	4, 6
		E50.16$^+$	20, 18	4, 4	63, 67	32, 16	3, 5
			β_2m−/−	β_2m+/−	A/Sn	B6	DBA
4	A/Sn	EL-4	10, 2	33, 14	0, 0	17, 14	3, 4
		C4.4-25$^-$	69, 58	74, 48	0, 0	37, 32	13, 2
		E50.16$^+$	44, 23	76, 64	0, 0	32, 28	4, 0
	A.BY	EL-4	18, 10	47, 26	0, 0	17, 9	0, 1
		C4.4-25$^-$	36, 12	43, 19	0, 0	22, 18	3, 0
		E50.16$^+$	3, 0	33, 17	0, 5	15, 15	0, 0
			β_2m−/−*	β_2m+/−*	RMA	RMA-S	
5 and 6	A/Sn	EL-4	0, 5	33, 31	ND	ND	
		C4.4-25$^-$	40, 20	55, 58	38, 40	51, 41	
		E50.16$^+$	ND	ND	49, 43	38, 22	
	A.BY	C4.4-25$^-$	ND	ND	57, 41	2, 2	
		E50.16$^+$	ND	ND	44, 36	2, 1	

Effector cells were generated in secondary MLTCs. Effector/target cell ratios used were as follows. Experiments: 1, 40:1 and 8:1; 2, 8:1 and 1.6:1; 3, 3.2:1 and 0.64:1 for C4.4-25$^-$ targets and 80:1 and 16:1 for T2 targets; 4, 8:1 and 1.6:1; 5, 50:1 and 10:1; 6, 40:1 and 8:1. Asterisks indicate cytotoxicity experiments performed in, and with target cells cultured in, serum-free medium. A/Sn is a responder mouse strain. EL-4 are stimulator cells used for priming *in vivo* (live) as well as for restimulation in MLTCs *in vitro* (after irradiation with 10,000 R). Data are percent specific lysis for the target cells indicated (mean of triplicates); representative results from repeated analyses are shown. ND, not done. All targets in experiment 4 were Con A-activated blasts, generated from the mouse strains indicated. The β_2m−/− and β_2m+/− targets tested in experiment 5 are Con A-activated blasts. RMA and RMA-S were tested in experiment 6.

Table 2. Effect of priming mice with β_2m^- tumor cells or β_2m^- lymphocytes

Exp.	Mouse	Effector cell: *In vivo* priming	Effector cell: *In vitro* MLTC	Target cell: Con A-activated blast		Target cell: Tumor cell	
1				$\beta_2m+/-$	$\beta_2m-/-$	E50.16^{+}*	C4.4-25^{-}*
	A/Sn	None	$\beta_2m+/-$	54, 41	67, 51	44, 17	0, 0
		None	$\beta_2m-/-$	20, 10	42, 21	15, 0	0, 0
		$\beta_2m+/-$	$\beta_2m+/-$	54, 42	61, 49	67, 64	15, 1
		$\beta_2m-/-$	$\beta_2m-/-$	72, 62	93, 73	69, 75	48, 38
2				B6	$\beta_2m-/-$	E50.16^{+}	C4.4-25^{-}
	A.BY	None	B6	7, 3	9, 7	6, 2	0, 0
		None	$\beta_2m-/-$	5, 8	8, 9	2, 1	0, 0
		$\beta_2m+/-$	B6	41, 42	29, 31	47, 46	0, 1
		$\beta_2m-/-$	$\beta_2m-/-$	40, 40	66, 69	45, 26	2, 6
3						E50.16^{+}	C4.4-25^{-}
	A/Sn	None	E50.16^{+}			52, 37	3, 1
		None	C4.4-25^{-}			22, 16	5, 3
		E50.16^{+}	E50.16^{+}			37, 40	2, 0
		C4.4-25^{-}	C4.4-25^{-}			55, 53	36, 38
4				$\beta_2m+/-$*	$\beta_2m-/-$*	A/Sn	A.BY	YAC-1
	A/Sn	B6	B6	96, 93	76, 61	11, 8	85, 87	7, 2
		$\beta_2m-/-$	$\beta_2m-/-$	77, 49	76, 60	14, 10	77, 47	15, 4
	A.BY	B6	B6	80, 54	43, 34	6, 5	20, 11	7, 3
		$\beta_2m-/-$	$\beta_2m-/-$	65, 35	64, 40	11, 5	22, 17	8, 2

Effector cells were from primary and secondary MLTCs. Effector/target cell ratios used were as follows; experiment 1, 10:1 and 2:1 for Con A-activated blasts and 50:1 and 10:1 for tumor cells; experiments 2 and 3, 40:1 and 8:1; experiment 4, 1.6:1 and 0.32:1. Live lymphocytes from $\beta_2m-/-$ or $\beta_2+/-$ mice (experiments 1, 2, and 4), or alternatively, E50.16^{+} or C4.4-25^{-} tumor cells (experiment 3), were used for *in vivo* priming. Irradiated cells were used for restimulation *in vitro* in MLTCs. A/Sn is a responder mouse strain. Asterisks indicate experiments performed with cells cultured and cytotoxicity tests performed in the absence of FCS (see Table 1). Data are percent specific lysis (mean of triplicates). In experiment 3, the experiment using target cells from $\beta_2m+/+$ and $\beta_2m-/-$ mice was not done.

grafts were then rejected as efficiently as the EL-4 parental and β_2m transfectant grafts (19). Restimulation of spleen cells from the mice that rejected EL-4 or E50.16^{+} grafts with the corresponding tumor cells *in vitro* yielded a strong secondary CTL response directed against H-2^{b}-expressing cells (Table 1). As expected (1–8), these CTLs failed to recognize the β_2m-deficient mutant C4.4-25^{-} (Table 1, experiments 1–3). In contrast, CTLs derived from mice primed and restimulated with the C4.4-25^{-} were able to kill the C4.4-25^{-} target cells and the β_2m-expressing control lines EL-4 and E50.16^{+} (ref. 19 and Table 1, experiments 1 and 2).

The CTLs induced in this way were MHC class I (H-2 D^{b} or K^{b})-specific. They did not kill the standard natural killer target YAC-1 (Table 2) and they were able to kill human T2 and murine P815 (H-2^{d}) target cells only after transfection with H-2K^{b} or H-2D^{b} genes (Table 1, experiments 2 and 3), but not after transfection of D^{d}, D^{p}, K^{k}, or L^{d} genes (data not shown). The H-2K^{b} killing was dependent on the K$^{b}\alpha_1\alpha_2$ domains, as shown by P815 cells transfected with exon-shuffled genes chimeric for H-2K^{b} and HLA-A2 (Table 1, experiment 2). CTLs primed and restimulated with β_2m-deficient cells were also able to kill C4.4-25^{-} cells cultured in serum-free medium for 6 weeks. The effector cells from the MLTCs were washed extensively in PBS and the cytotoxicity test itself was also performed in serum-free medium (Table 1, experiments 1 and 2).

The exact status of the MHC class I and β_2m genes in the C4.4-25^{-} is not known, and low remaining levels of β_2m protein escaping antibody mediated precipitation cannot be excluded. It was therefore important to confirm the CTL recognition by using target cells with a known β_2m defect that are also otherwise known to be normal with respect to the MHC class I genes. We found that CTL killing of Con A-activated blasts from $\beta_2m-/-$ (H-2^{b}) mice were even more efficient than CTL killing of C4.4-25^{-} (Table 1, experiment 4). Again, this killing was seen even if the $\beta_2m-/-$ splenocytes were kept in medium without serum or with serum from $\beta_2m-/-$ mice for the whole Con A stimulation period and cytotoxic assay (Table 1, experiment 5).

H-2 K^{b}/D^{b}-specific CTLs recognizing β_2m^- targets could also be induced by priming and restimulation with $\beta_2m-/-$ spleen cells (Table 2). Use of such cells even allowed induction of CTLs in primary mixed lymphocyte culture, while the expansion of CTLs in response to C4.4-25^{-} cells required priming *in vivo*. Note also that Con A-activated blasts from $\beta_2m-/-$ mice often were killed even by CTLs induced by $\beta_2m+/-$ cells (Table 2).

CTL lines obtained by limiting dilution of bulk cultures stimulated with β_2m-deficient cells killed either T2-K^{b} or T2-D^{b} and always killed C4.4-25^{-} (Table 3). Moreover, A/Sn anti-C4.4-25^{-} bulk CTLs specific for H-2K^{b} (in this case, defined by their ability to kill T2-K^{b} but not T2 targets) could be inhibited using either unlabeled C4.4-25^{-} cells or unlabeled β_2m^- Con A-activated blasts (Fig. 1). Altogether this proved that the β_2m-deficient cells were recognized by MHC class I-specific clones in the effector-cell population. CTLs raised against β_2m-deficient cells could also recognize β_2m-positive cells in a class I-specific manner, as shown in direct cytotoxicity (Table 1) and unlabeled target competition assays (Fig. 1*b*). Note that unlabeled target inhibition was seen also with normal spleen cells, provided they expressed H-2^{b} (Fig. 1).

The killing of $\beta_2m-/-$ Con A-activated blasts and C4.4-25^{-} tumor cells was abrogated by mAb plus complement-mediated depletion of CD8^{+} cells (Fig. 2 *b* and *d*) and by blocking of the effector cells with anti-CD8 mAb during the assay (Fig. 2 *f* and *h*). There was no inhibitory effect of CD4 mAb (Fig. 2 *b*, *d*, and *f*). The killing could also be abrogated by treatment with antibody against the TCR$\alpha\beta$ plus comple-

Table 3. Specificity of CTL lines recognizing β_2m-deficient target cells

CTL	Target cell					E/T ratio
	C4.4-25$^-$	E50.16$^+$	T2	T2-K^b	T2-D^b	
14	42	56	0	4	34	0.8:1
	23	34	0	2	13	0.16:1
19	31	46	0	29	5	4:1
	14	31	0	11	0	0.8:1

E, effector cell; T, target cell. Data are percent specific lysis (mean of triplicates).

ment, while an anti-TCR $\gamma\delta$ mAb plus complement had no effect (Fig. 2 *a* and *b*).

Similar CTLs could be induced by priming H-2-matched but minor-histocompatibility-antigen-disparate A.BY mice with β_2m−/− spleen (Table 2) or C4.4-25$^-$ lymphoma cells (Table 1, experiments 4–6). Again, the priming protocol with s.c. inoculation of A.BY mice with live C4.4-25$^-$ lymphoma cells resulted in regression after initial outgrowth to manifest tumors (data not shown). The effector cells generated after restimulation *in vitro* specifically killed β_2m−/− H-2^b Con A-activated blasts (Table 1, experiment 4) that are expected to share at least some genes encoding minor histocompatibility antigens with the C4.4-25$^-$ cells used for priming. External β_2m from serum was not required for killing (Table 2).

The B6 minor-histocompatibility antigen-specific CTLs generated against β_2m-deficient tumor cells killed the B6-derived lymphoma line RMA but consistently failed to kill the TAP-2-defective RMA-S mutant (4, 10–13) derived from it (Table 1, experiment 6). In contrast, the CTLs generated with the same priming cells (i.e., C4.4-25$^-$ or β_2m$^-$ spleen cells) over an MHC barrier consistently killed RMA-S better than the control RMA line (Table 1, experiments 2 and 6).

DISCUSSION

Although β_2m-independent CTLs have previously not been observed, there are several findings in the literature that are relevant for the interpretation of the present data. (*i*) Free heavy chains can interact with short high-affinity peptides in the absence of β_2m *in vitro* (20). (*ii*) Free heavy chains (H-2D^b and K^b) can exist on the surface of cells, as assessed by their ability to bind exogenous β_2m (6). They are considered to originate from regular class I complexes by dissociation from β_2m. (*iii*) H-2D^b heavy chains can reach the cell surface in β_2m$^-$ cells; however, completely folded class I molecules have not been demonstrated by antibody binding assays on the cell surface of β_2m$^-$ cells (1–3, 5–8, 21) (including the C4.4-25$^-$ cells and the β_2m−/− Con A-activated blasts used in this study; ref. 8 and unpublished observations). (*iv*) β_2m$^-$ cells cannot be recognized by CTLs primed by MHC class I-positive cells; certain β_2m$^-$ cells can, however, be killed by CTLs when the medium is supplemented with FCS containing bovine β_2m and/or specific synthetic peptide (5, 21), presumably allowing stabilization or refolding of heavy chains reaching the cell surface.

The present results are thus consistent with the following molecular interpretation: a fraction of MHC class I heavy chains can be transported to the cell surface without β_2m, either alone or in association with short minor histocompatibility antigen peptides. These heavy chains fall below the threshold for a clear signal in antibody binding assays and usually also below the threshold for CTLs induced by MHC class I-positive stimulator cells. However, they are sufficient to trigger certain T-cell clones in the afferent phase of the

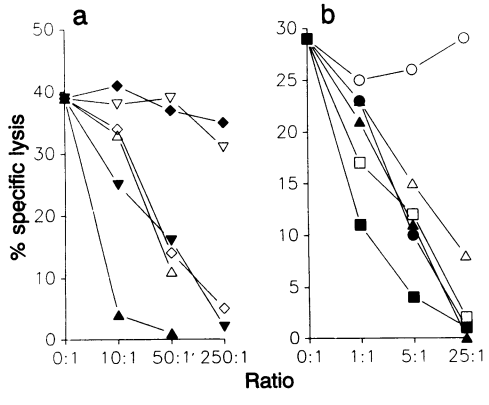

FIG. 1. Unlabeled target inhibition with ^{51}Cr-labeled C4.4-25$^-$ (*a*) or T2-K^b (*b*) target cells tested by using A/Sn anti-C4.4-25$^-$ effectors. Unlabeled target inhibitor cells were as follows. (*a*) △, C4.4-25$^-$; ▲, E50.16$^+$; ▽, lymphocytes from A/Sn (H-2K^{k}D^d); ▼, A.BY (H-2K^{b}D^b); ◇, B10 (H-2K^{b}D^b); ◆, B10.D2 (H-2K^{d}D^d). (*b*) ○, T2; ●, T2-K^b; △, C4.4-25$^-$; ▲, E50.16$^+$; □, Con A-activated blasts from β_2m−/− mice; ■, Con A-activated blasts from β_2m+/− mice from H-2^b background. The unlabeled/labeled target cell ratio is shown. The same effectors yielded 0% lysis against T2 targets in the same experiment. When the ^{51}Cr-labeled target cells were incubated with the unlabeled target cells alone, no cytotoxicity was seen. Effector/target ratios were 8:1 in *a* and 2.5:1 in *b*.

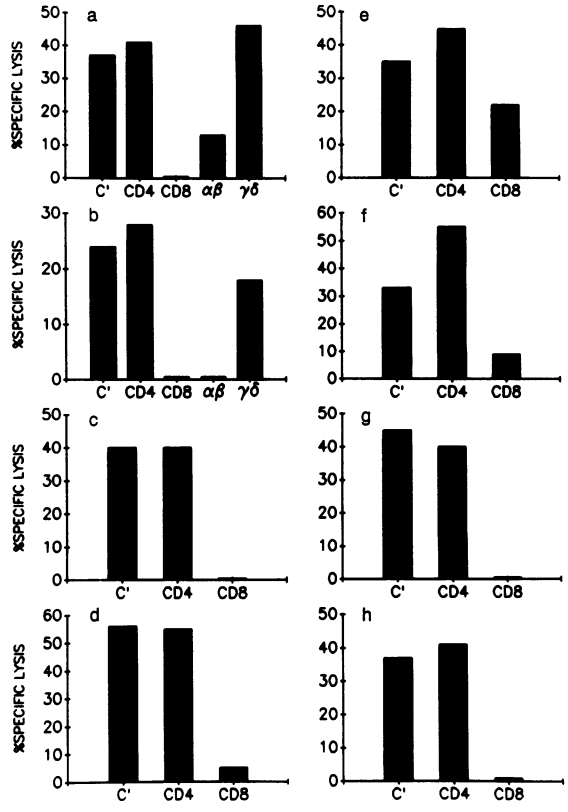

FIG. 2. Effector-cell depletion and blocking by mAb against CD4/CD8 or TCR$\alpha\beta$/TCR$\gamma\delta$. A/Sn anti-EL-4 (*a* and *e*), A/Sn anti-C4.4-25$^-$ (*b* and *f*), A/Sn anti-B6 (*c* and *g*), or A/Sn anti-β_2m−/− (*d* and *h*) effectors were depleted of cellular subsets with mAb and complement (*a–d*) or preincubated and tested in the presence of mAb (*e–h*). Cytotoxicity was tested against EL-4 (*a* and *e*), C4.4-25$^-$ (*b* and *f*), B6 Con A-activated blasts (*c* and *g*), or β_2m−/− Con A-activated blasts (*d* and *h*). (*a* and *c*) Experiments were performed in RPMI 1640 medium containing 5% FCS. (*b–d* and *f–h*) Experiments were performed under serum-free conditions (i.e., with target cells cultured and assay performed in serum-free medium). In *e* and *f*, the antibody concentration was 2 μg/ml and 10 μg/ml for the anti-CD8 (YTS 169.4) and anti-CD4 (RM4-4) mAbs, respectively. In *g* and *h*, a mixture of two anti-CD8 (169.4.2.1 and 156.7.7) or anti-CD4 (191.1.2 and 3.1.2) mAbs was used at a total concentration of 100 μg/ml.

response, at least *in vivo*. These clones expand and are able to recognize heavy chains (with or without peptide) in β_2m^- cells also in the effector phase. This interpretation is in line with the notion that T cells can detect as few as 200 MHC–antigen ligands per cell (22) and may thus be more sensitive than antibodies in flow cytometry analysis for detection of functional MHC molecules. The results further indicate that β_2m-deficient Con A-activated blasts are superior to the lymphoma mutant cells with respect to induction of and susceptibility to CTLs. This may be due to higher expression of heavy chains on the former.

The results with peptide loading of defective RMA-S targets indicate that minor-histocompatibility-specific CTLs induced by β_2m^- stimulator cells are peptide-dependent, like conventional H-2-restricted (4, 11, 23) and many allo-H-2-specific (24, 25) CTLs. The efficient killing of the RMA-S (and also of T2-K^b and T2-D^b) by CTLs generated against β_2m^- cells over an MHC barrier is intriguing but, due to leakiness of the peptide-loading defect in these mutants (26–29), it remains open whether these CTLs are independent of peptide and of β_2m.

A role for serum β_2m in the induction of the CTLs described here cannot be entirely excluded. However, the results from attempts to prime with β_2m-transfected lymphoma cells suggest that the activation of β_2m-independent CTLs is reduced or even prevented by the presence of β_2m. At the effector cell stage, the role of serum-derived β_2m could be excluded by the use of serum-free medium for culture of target cells and cytotoxic assays. There is still a possibility of β_2m being provided by secretion from effector cells, and it therefore remains open whether MHC class I-dependent recognition can take place in the total absence of β_2m. However, the major implication of this study is that enough free heavy chains can be transported to the cell surface without β_2m to trigger CTL precursors and effectors. For practical considerations this is a most important point, since serum β_2m would be present anyhow in individuals with β_2m-deficient tumors or grafts. Note that the recognition of β_2m^- cells does not depend upon the particular properties of the incompletely folded ($\alpha_1^-\alpha_2^-\alpha_3^+$) H-2$D^b$ molecules that have been reported to reach the cell surface in several β_2m^- cells (3, 5). (*i*) Some of the β_2m-independent CTLs were specific for H-2K^b rather than for H-2D^b (Table 1, experiment 3, and Table 3). (*ii*) R1E-D^b, with readily detectable levels of ($\alpha_1^-\alpha_2^-\alpha_3^+$) H-2$D^b$ molecules at the cell surface (3), was in general not sensitive to the CTLs studied here (data not shown), whereas C4.4-25$^-$, on which such molecules have not been detected (data not shown), was reproducibly killed.

We conclude that MHC class I heavy chains in β_2m-deficient cells can present major and minor histocompatibility antigen epitopes for CD8$^+$ TCR$\alpha\beta^+$ CTLs and their precursors. At least in the final effector–target interaction, triggering of such CTLs occurs in the absence of serum β_2m. These results challenge the current dogma and are of potential importance in relation to transplantation and tumor immunology. We predict that engraftment of β_2m-deficient cells or organs will require suppression of the type of CTLs described here, and conversely, that such CTLs may be possible to induce against autologous β_2m-deficient tumor clones observed in many cancers (30).

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1. Hyman, R. & Throwbridge, I. (1975) *Cold Spring Harbor Symp. Quant. Biol.* **40**, 407–415.
2. Arce-Gomez, B., Jones, E. A., Barnstable, C. J., Solomon, E. & Bodmer, W. F. (1978) *Tissue Antigens* **11**, 96–112.
3. Allen, H., Fraser, J., Flyer, D., Calvin, S. & Flavell, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7447–7451.
4. Townsend, A., Öhlèn, C., Bastin, J., Ljunggren, H.-G., Foster, L. & Kärre, K. (1989) *Nature (London)* **340**, 443–448.
5. Zijlstra, M., Bix, M., Simister, N. E., Lauring, J. M., Raulet, D. H. & Jeanisch, R. (1990) *Nature (London)* **344**, 742–746.
6. Rock, K. L., Gamble, S., Rothstein, L., Gramm, C. & Benacerraf, B. (1991) *Cell* **65**, 611–620.
7. Koller, B. H., Marrack, P., Kappler, J. & Smithies, O. (1990) *Science* **248**, 1227–1230.
8. Glas, R., Sturmhöfel, K., Hämmerling, G. J., Kärre, K. & Ljunggren, H.-G. (1992) *J. Exp. Med.* **175**, 843–846.
9. Sturmhöfel, K. (1990) Ph.D. thesis (Deutsches Kiebsforschungs Zentrum, Heidelberg).
10. Kärre, K., Ljunggren, H.-G., Piontek, G. & Kiessling, R. (1986) *Nature (London)* **319**, 675–678.
11. Ljunggren, H.-G., Stam, N., Öhlén, C., Neefjes, J., Höglund, P., Heemels, M. T., Bastin, J., Schumacher, T., Townsend, A., Kärre, K. & Ploegh, H. (1990) *Nature (London)* **346**, 476–480.
12. Powis, S. J., Townsend, A. R. M., Deverson, E. V., Bastin, J., Butcher, G. W. & Howard, J. C. (1991) *Nature (London)* **354**, 528–531.
13. Attaya, M., Jameson, S., Martinez, C. K., Hermel, E., Aldrich, C., Forman, J., Fischer-Lindahl, K., Bevan, M. J. & Monaco, J. J. (1992) *Nature (London)* **355**, 647–649.
14. Alexander, J., Payne, J. A., Murry, R., Frelinger, J. & Cresswell, P. (1989) *Immunogenetics* **29**, 380–388.
15. Cerundolo, V., Alexander, J., Anderson, K., Lamb, C., Cresswell, P., McMichael, A., Gotch, F. & Townsend, A. (1990) *Nature (London)* **345**, 449–452.
16. Hosken, N. & Bevan, M. (1990) *Science* **248**, 367–370.
17. Alexander, J., Payne, J. A., Shigekawa, B., Frelinger, J. & Cresswell, P. (1990) *Immunogenetics* **31**, 169–178.
18. Samberg, N. L., Scarlett, E. C. & Stauss, H. J. (1989) *Eur. J. Immunol.* **19**, 2349–2354.
19. Glas, R., Franksson, L., Öhlèn, C., Höglund, P., Koller, B., Ljunggren, H.-G. & Kärre, K. (1992) *J. Cell. Biochem.* Suppl. 16D, 12 (abstr.).
20. Elliot, T., Cerundolo, V., Elvin, J. & Townsend, A. (1991) *Nature (London)* **351**, 402–406.
21. Vitiello, A., Potter, T. A. & Sherman, L. A. (1990) *Science* **250**, 1423–1426.
22. Rosemary-Christinck, E., Luscher, M. A., Barber, B. & Williams, D. B. (1991) *Nature (London)* **352**, 67–70.
23. Townsend, A. & Bodmer, H. (1989) *Annu. Rev. Immunol.* **7**, 601–624.
24. Aosai, F., Öhlén, C., Ljunggren, H.-G., Höglund, P., Franksson, L., Ploegh, H., Townsend, A., Kärre, K. & Stauss, H. J. (1991) *Eur. J. Immunol.* **21**, 2767–2774.
25. Rötzschke, O., Falk, K., Faath, S. & Rammensee, H. G. (1991) *J. Exp. Med.* **174**, 1059–1072.
26. Esquivel, F., Yewdell, J. & Bennink, J. (1992) *J. Exp. Med.* **175**, 163–168.
27. Hosken, N. & Bevan, M. (1992) *J. Exp. Med.* **175**, 719–729.
28. Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D. F. & Engelhard, V. H. (1992) *Science* **255**, 1264–1266.
29. Wei, M. L. & Cresswell, P. (1992) *Nature (London)* **356**, 443–446.
30. Smith, M. E. F. (1991) *Semin. Cancer Biol.* **2**, 17–21.