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Human cytotoxic T-lymphocyte recognition of an HLA-A3 gene product expressed on murine L cells: The only human gene product required on the target cells for lysis is the class I heavy chain

(major histocompatibility antigen/class I molecule/DNA-mediated gene transfer)

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ABSTRACT **To dissect the molecular basis for T-cell recognition of class I major histocompatibility complex antigens, we have examined the ability of human cytotoxic T lymphocytes (CTL) to recognize murine L cells transformed with a human class I gene. Three transformed L-cell lines were generated that expressed the human HLA-A3 gene from donor E1 at levels comparable to those of the endogenous L-cell H-2K^k molecules. CTL were generated in secondary and tertiary mixed lymphocyte culture against the HLA-A3 subtype of donor E1 by culturing irradiated E1 peripheral blood lymphocytes with the peripheral blood lymphocytes of responder donor M3 (M3 shares all defined class I antigens with E1 but expresses a different HLA-A3 subtype). Each of the HLA-A3-transformed L cells was lysed by M3 anti-E1 CTL in a short-term ^{51}Cr release assay and this recognition was blocked by a monoclonal anti-HLA-A3 antibody. Antibodies specific for the human T8 and LFA-1 molecules on the CTL effectors (but absent from the transformed targets) also blocked lysis of each of the HLA-A3 transformed L-cell targets. Antibodies to other T-cell molecules failed to block lysis. The present results demonstrate that human CTL can recognize human class I molecules on targets that do not express any other human gene product and further suggest that effector T-cell molecules T8 and LFA-1 are functionally involved in this recognition process.**

Class I molecules are highly polymorphic, cell surface glycoproteins that consist of a major histocompatibility complex (MHC)-encoded heavy chain (45,000 M_r) noncovalently associated with the non-MHC-encoded β_2-microglobulin (β_2m) (12,000 M_r) (1). The specific recognition of foreign antigens and allogeneic cells by cytotoxic T lymphocytes (CTL) is determined by T-cell receptors that specifically interact with target cell determinants expressed on the class I molecule alone (alloreactive CTL) or in association with exogenous antigen (e.g., antiviral CTL) (2, 3). In addition, the antigen-specific receptor is noncovalently associated with the T3 polypeptide complex (4) that is thought to be required for surface expression of the receptor (5) and to be involved in transducing signals from the cell surface (6).

The interaction of the T-cell receptor–T3 complex with its molecular target is thought to be accompanied by the formation of a stable conjugate between the CTL and the target cell (7–9). Results of antibody-blocking studies indicate that conjugate formation can be inhibited by antibodies to molecules such as T8 (7, 8, 10), LFA-1 (11), and LFA-2 (11) in the human system. It has been suggested that these molecules act as cell adhesion molecules in human T-cell recognition by binding to molecules on the target cell, thereby facilitating CTL–target interaction (7–13). The target cell counterparts to these T-cell adhesion molecules have not yet been identified.

To assess the target cell molecules required for recognition and lysis by human CTL, several recent studies have examined the recognition of class I antigens in a variety of cells transformed by DNA-mediated gene transfer with human class I genes (14–17). These results demonstrated that human anti-A2- or -B7-specific CTL clones could recognize A2 or B7 molecules on human cell (14–17) or on monkey cell transformants (17) but not on any murine L-cell transformants (14–17). Cotransfection of the L cells with the human β_2m gene failed to facilitate recognition by human CTL (15–17). It was thus proposed that human molecules other than class I antigens are required for effective CTL–target interaction (14–17). These other human molecules would be the receptors for the putative T-cell adhesion structures such as T8, LFA-1, and LFA-2.

In this study, we have transformed murine L cells with a gene encoding the E1 subtype of HLA-A3 (18–20) and have utilized these transformants as target cells for human CTL specific for this HLA-A3 subtype. Contrary to the previously published results (14–17), in our system the class I heavy chain is the only human molecule required for target cell recognition and lysis by human CTL.

MATERIALS AND METHODS

Cells. E1B2 and M16B are Epstein–Barr virus-transformed B-cell lines of peripheral blood lymphocytes (PBL) from donors E1 and M16, respectively (20). DAP-3, a LMTK$^-$, BrdUrd-resistant, diaminopurine-resistant mouse L-cell fibroblast line (21) (provided by K. Ozato, National Institutes of Health), was maintained in monolayer culture in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum (Dutchland Laboratories, Denver, PA), 50 μg of gentamicin (GIBCO) per ml, and 6 mM glutamine (M. A. Bioproducts, Walkersville, MD).

DNA-Mediated Gene Transfer. The HLA-A3 gene from donor E1 encodes a molecule that represents an HLA-A3 subtype (18–20). A plasmid clone, p35-1 (unpublished data), was used to introduce the E1-A3 gene into the thymidine kinase (TK)-deficient L-cell line DAP-3 by cotransformation with the herpesvirus TK gene at a p35-1:TK ratio of 80:1 by using the calcium phosphate technique, as described (21). Transformed cells were maintained in DAP-3 medium containing 0.1 mM hypoxanthine, 0.01 mM aminopterin, 0.03 mM thymidine (HAT), and 10% fetal calf serum. In some

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Abbreviations: β_2m, β_2-microglobulin; CTL, cytotoxic T lymphocyte(s); HAT, hypoxanthine/aminopterin/thymidine; MLR, mixed lymphocyte culture; MHC, major histocompatibility complex; PBL, peripheral blood lymphocytes; IL, interleukin; LFA, lymphocyte function-associated antigen; TK, thymidine kinase; PHA, phytohemagglutinin.

experiments, cells were cultured overnight in HAT medium containing 10% heat-inactivated human serum in order to replace the bovine or mouse β_2m with human β_2m (22, 23).

Monoclonal Antibodies. The monoclonal antibodies used, and their respective specificities and sources, are listed in Table 1.

Indirect Immunofluorescence. L cells were removed from tissue culture flasks with Versene (GIBCO) and were analyzed for reactivity with specific monoclonal antibodies as described (32) on a FACS Analyzer (Becton Dickinson).

Generation and Assay of CTL. Bulk populations of CTL specific for the HLA-A3 subtype of donor E1 (18–20) were generated in secondary and tertiary mixed lymphocyte culture (MLR). Responder PBL (9×10^6) from donor M3 (HLA-A2,3, -B7,44, -Cw5,7, -DR1,2, -DPw2,4) were cultured with 3×10^6 2000-rad-irradiated PBL from donor E1 (HLA-A2,3', -B7,44, -Cw5,7, -DR2,5, -DPw4) for 9 days in 8 ml of culture medium [RPMI 1640 with glutamine (GIBCO) supplemented with 10 units of penicillin per ml and 10 μg of streptomycin per ml] with 5% heat-inactivated normal human plasma in upright 25-cm^2 flasks (Falcon 3013). Primed responder cells obtained from these cultures were restimulated in secondary MLR: 3×10^6 responders and 9×10^6 2000-rad-irradiated E1 PBL were cultured for 5 days in flasks (Falcon 3013) in 8 ml of culture medium with 20% plasma. These secondary responders were either tested directly for cytotoxicity or cryopreserved (33), thawed, and restimulated in tertiary MLR exactly as described for the generation of secondary MLR responders. CTL clone 8.9, specific for the class II HLA specificity DPw2, was generated and characterized as described (34). Four-hour ^{51}Cr release cytotoxicity assays were performed as described (34, 35). In antibody-blocking assays, effector cells were incubated for 30–60 min with antibodies prior to the addition of ^{51}Cr-labeled target cells.

RESULTS

Expression of the E1-A3 Gene Product on Murine L Cells Following DNA-Mediated Gene Transfer. A subtype of the HLA-A3 antigen detected by T-cell recognition (but not distinguishable by serological reagents) is expressed on the cells of donor E1 (18, 19). This subtype has been shown to differ from the prototype HLA-A3 molecule (36) at amino acid positions 152 and 156 (20). A recombinant plasmid, p35-1, was constructed by ligating a *Hin*dIII fragment containing the cloned E1 HLA-A3 gene into the plasmid vector pUC8 in the same orientation as the *lacZ* gene. The plasmid p35-1 was used to introduce the E1 HLA-A3 gene into the murine TK$^-$ L-cell line DAP-3 by cotransfection with the TK gene (21). Three colonies of HAT-resistant transfected cells (2-6, 2-7, and 2-8) were isolated and analyzed by flow cytofluorimetry for surface expression of the HLA-A3 antigen by using the HLA-A3-specific monoclonal antibody GAP.A3 (Fig. 1). Each of the three E1-A3 transformants expressed surface HLA-A3 at levels that were comparable to that of endogenous L-cell H-2K^k molecules (Fig. 1 *C–E*). Monoclonal antibodies specific for HLA-A2 or -B7 bound to E1B2 but did not bind to any of the E1-A3 transformants (Fig. 1 *B–E*). The nontransformed parent L cells, DAP-3, bound only the anti-H-2K^k reagent.

Recognition of E1-A3-Transformed L Cells by E1-A3-Specific CTL. CTL specific for the E1-A3 subtype were generated by coculture of irradiated E1 PBL with PBL from donor M3. [Although M3 and E1 are identical at all serologically defined class I loci, their A3 gene products can be functionally distinguished by CTL (18, 19).] As shown in Fig. 2, the specificity of these M3 anti-E1 CTL is such that only target cells from donor E1 are lysed efficiently, whereas unmatched targets, including two that express an alternate HLA-A3 subtype (FZ2 and M18), are not lysed.

We next assessed the ability of the M3 anti-E1 CTL to recognize the E1-A3 transformants (Fig 3*A*) and found that each of the transformants was efficiently lysed regardless of the serum used for target cell culture [indicating that the source of β_2m (22, 23) did not have any functional significance]. In contrast, the parental cell line DAP-3 was not lysed, nor was an H-2D^d L-cell transformant (data not shown). Antibody-blocking studies demonstrated that lysis of targets 2-7 and 2-8 could be inhibited by the HLA-A3-specific antibody GAP.A3 (Fig. 4*A*) but lysis was not inhibited by an anti-H-2K,D^k antibody (data not shown). An HLA-DPw2-specific CTL clone, 8.9, was also tested for its ability to lyse the E1-A3 transformants (Fig. 3*B*). Although

Table 1. Monoclonal antibodies used in this study

Antibody	Specificity	Form	Source	Ref.
GAP.A3	HLA-A3	Ascites fluid	A. Berger (Upjohn)	24
PA2.1	HLA-A2	IgG fraction	P. Parham (Stanford Univ. School of Medicine)	25
BB7.1	HLA-B7	Culture fluid	American Type Culture Collection	25
11-4.1	H-2K^k	IgG fraction	Becton Dickinson	26
OKT1	T1	IgG fraction	G. Goldstein (Ortho Pharmaceutical)	27
OKT3	T3	IgG fraction	G. Goldstein (Ortho Pharmaceutical)	28
OKT4	T4	IgG fraction	G. Goldstein (Ortho Pharmaceutical)	28
OKT8C	T8	IgG fraction	G. Goldstein (Ortho Pharmaceutical)	29
OKT8F	T8	IgG fraction	G. Goldstein (Ortho Pharmaceutical)	29
MHM23	LFA-1 β chain	Culture fluid	A. McMichael (Oxford Univ.)	13
MHM24	LFA-1 α chain	Culture fluid	A. McMichael (Oxford Univ.)	13
TS1/22	LFA-1 α chain	Ascites fluid	T. Springer (Dana–Farber Cancer Institute)	30
TS2/9	LFA-3	Ascites fluid	T. Springer (Dana–Farber Cancer Institute)	30
95-5-49	LFA-2	Ascites fluid	R. Quinones (National Institutes of Health)	*
Anti-Tac	IL-2 receptor	IgG fraction	T. Waldmann (National Institutes of Health)	31

We thank the suppliers of monoclonal antibodies for making their reagents available. IL, interleukin; LFA, lymphocyte function-associated antigen.

*R. Quinones and R. Gress, personal communication.

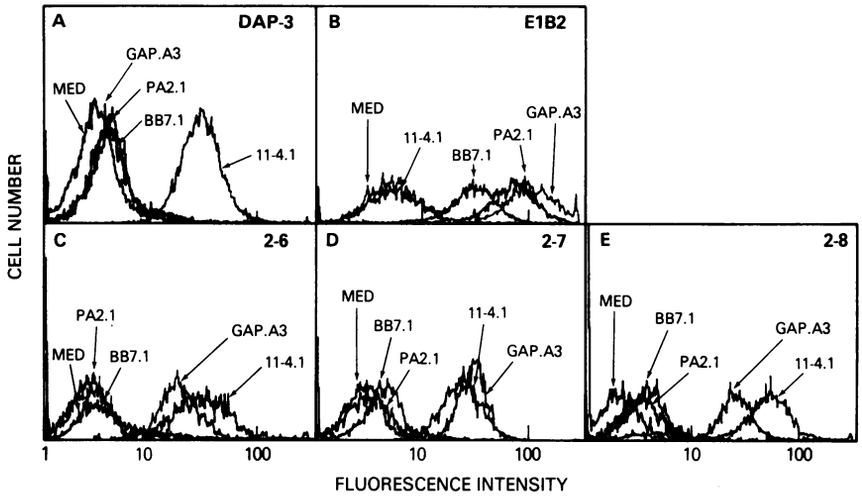

FIG. 1. Analysis of transformants for expression of class I molecules by indirect immunofluorescence. (*A*) DAP-3. (*B*) E1B2. (*C*) Transformant 2-6. (*D*) Transformant 2-7.(*E*) Transformant 2-8. Cells were allowed to react with culture medium (MED) or with monoclonal antibody GAP.A3 (specific for HLA-A3), PA2.1 (specific for HLA-A2), BB7.1 (specific for HLA-B7), or 11-4.1 (specific for H-2K^k).

8.9 lysed the DPw2-positive M16B target cell, it did not lyse any of the E1-A3 transformants. These data demonstrate that M3 anti-E1 CTL specifically recognize E1-A3 molecules on the murine L-cell transformants.

Blocking of CTL Recognition of Transformants by Antibodies to Human T-Cell Molecules. Several molecules have been implicated as cell adhesion molecules in T-cell recognition of targets. These molecules, which appear to aid in the formation of CTL–target cell conjugates (7, 8, 10, 11), include T8 (for class I-specific CTL) (7, 8, 10), LFA-1, LFA-2, and LFA-3 (11). To determine if any of these molecules are involved in the recognition of the E1-A3 L-cell transformants by M3 anti-E1 CTL, monoclonal antibodies to these molecules were used to block CTL-mediated target cell lysis. The results of two such studies are shown in Fig. 4. In one experiment (Fig. 4*A*), lysis of E1-A3 transformants 2-7 and 2-8 was blocked by the anti-T3 reagent OKT3, thus demonstrating that the lysis is mediated by T cells (4, 9). Two antibodies specific for T8 (OKT8C and F) almost completely blocked lysis and one antibody specific for the β chain of the LFA-1 molecule (MHM23) partially, but significantly, inhibited lysis of 2-7 and 2-8. Antibodies to LFA-2 (95-5-49), LFA-3 (TS2/9), T4 (OKT4), and T1 (OKT1) did not significantly inhibit lysis of the transformants.

In another experiment (Fig. 4*B*), we compared the ability of various antibodies to block lysis of E1 PHA blasts and the E1-A3 L-cell transformant 2-6. Antibodies that blocked the lysis of E1 PHA blasts and transformant 2-6 were identical to those that blocked the lysis of 2-7 and 2-8. In this experiment, two anti-LFA-1 antibodies that are specific for the LFA-1 α chain (MHM24 and TS1/22) each significantly inhibited lysis of both the transformant and the E1 PHA blasts. The lysis of E1 PHA blasts was also partially blocked by anti-LFA-2 and anti-LFA-3, whereas these antibodies had no apparent effect on the lysis of any of the transformants. The anti-IL-2 receptor antibody anti-Tac did not block lysis of either target.

The observation that anti-T8 and anti-LFA-1 antibodies block the M3 anti-E1 CTL-mediated lysis of E1 PHA blasts and the E1-A3 L-cell transformants suggests that the T8 and LFA-1 molecules are functionally involved in the human CTL recognition of the E1-A3 molecule expressed on murine L cells.

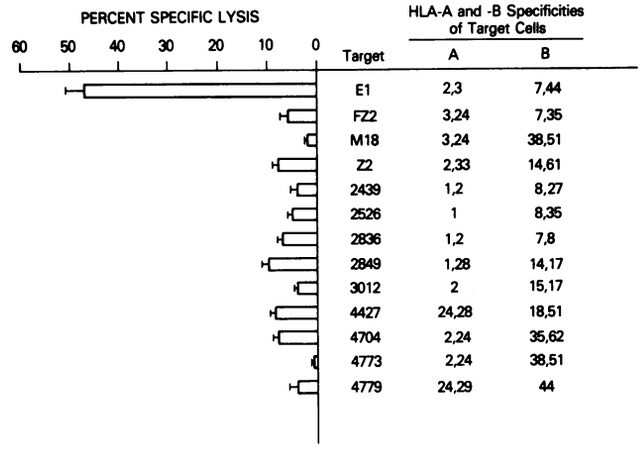

FIG. 2. Specificity of M3 anti-E1 CTL. M3 anti-E1 CTL generated in a secondary MLR were assayed on a panel of phytohemagglutinin (PHA) blast targets.

DISCUSSION

The experiments described here document the recognition of a human class I gene product on murine L cells by human CTL and thus demonstrate that the only human molecule required on target cells for human CTL recognition is the class I heavy chain. In contrast, previous investigators have observed that human CTL could recognize transfected human class I genes expressed on human (14–17) or monkey (17) cells but not on mouse cells. The failure of these investigators to achieve recognition of a human class I gene expressed on mouse L cells was attributed to the absence of species-specific target cell structures capable of acting as ligands for putative human CTL cell adhesion molecules, such as T8 and LFA-1. This species barrier would prevent effective adhesion of the CTL to the target cell and would thus preclude the effective interaction of the antigen-specific T-cell receptor with the class I target molecules. The results of the present study demonstrate that the recognition of human class I molecules on mouse L cells can be inhibited by antibodies to the T8 and LFA-1 molecules. Because no binding of anti-T8 or anti-human LFA-1 antibodies to the surface of the transformed targets was observed (data not shown), the inhibition of lysis must be due to effects of the antibodies on the CTL and not on the targets.

These results strongly suggest that the putative target structures for the T8 and LFA-1 molecules are present on these L-cell transformants. This being the case, we can conclude that these target structures are located either on the human class I molecule or on murine cell surface molecules that lack the species specificity postulated by other investigators (14–17). The T8 molecule has been postulated to bind to a nonpolymorphic region of human class I molecules (9, 37), which could be either the heavy chain, β_2m, or a conformational determinant formed by their association. The presence

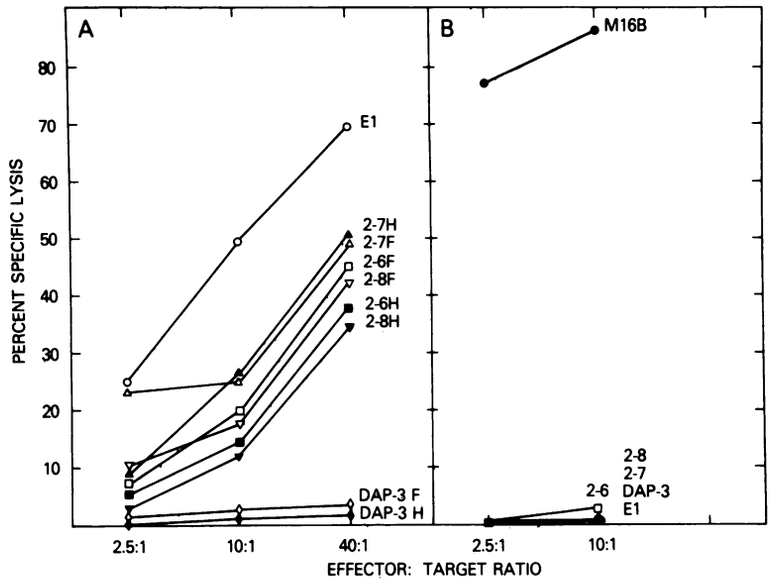

FIG. 3. Cytolytic activity of M3 anti-E1 CTL on L cells transformed with the E1-A3 gene. M3 anti-E1 CTL generated in a tertiary MLR (*A*) and the DPw2-specific clone 8.9 (*B*) were assayed for cytolytic activity on E1 PHA blasts (○), M16B (●), untransformed DAP-3 L cells (◇, ◆), and three different L-cell lines transformed with the E1-A3 gene: 2-6 (□, ■), 2-7 (△, ▲), and 2-8 (▽, ▼). L-cell targets either were grown in fetal calf serum (open symbols) or cultured overnight in human serum (solid symbols).

of human class I molecules on the surface of the transformants could thus provide the appropriate target structure for the binding of T8. It is also possible that endogenous L-cell class I molecules could serve as target structures for T8, since murine class I C2 domains are 75–80% homologous at the amino acid level to human class I α3 domains (1). In support of this, Swain *et al.* (37) have shown that lysis of mouse cell targets by human anti-mouse CTL specific for mouse class I antigens can be inhibited by the anti-T8 antibody Leu-2a. The target structure for the LFA-1 molecule is not known, although a recent study by Gromkowski *et al.* (38) suggests that it is not a class I molecule. If so, our data suggest that LFA-1 on human CTL can recognize target cell molecules of murine origin. This would not be surprising because human CTL can be readily generated against murine target cells (39).

The potential roles of LFA-2 and LFA-3 in CTL recognition of the E1-A3 transformants and E1-PHA blasts were also examined. Anti-LFA-2 and anti-LFA-3 antibodies partially blocked lysis of the E1 PHA blasts but did not significantly inhibit lysis of the transformants. These results suggest that LFA-2 and LFA-3 do not play a crucial role in the recognition of E1 cells by M3 anti-E1 CTL, and thus no firm conclusions can be made about the roles of LFA-2 and LFA-3 in CTL recognition of the transformants.

It is not clear why the results of previous studies differ from those reported here, although several possible explanations can be offered. (*i*) The L-cell lines used for transformation experiments may not be identical. The DAP-3 L cells used in the present experiments may possess an unusually high concentration of cell surface target structures that can be bound by putative human cell adhesion molecules or may possess molecules or determinants that are lacking in other

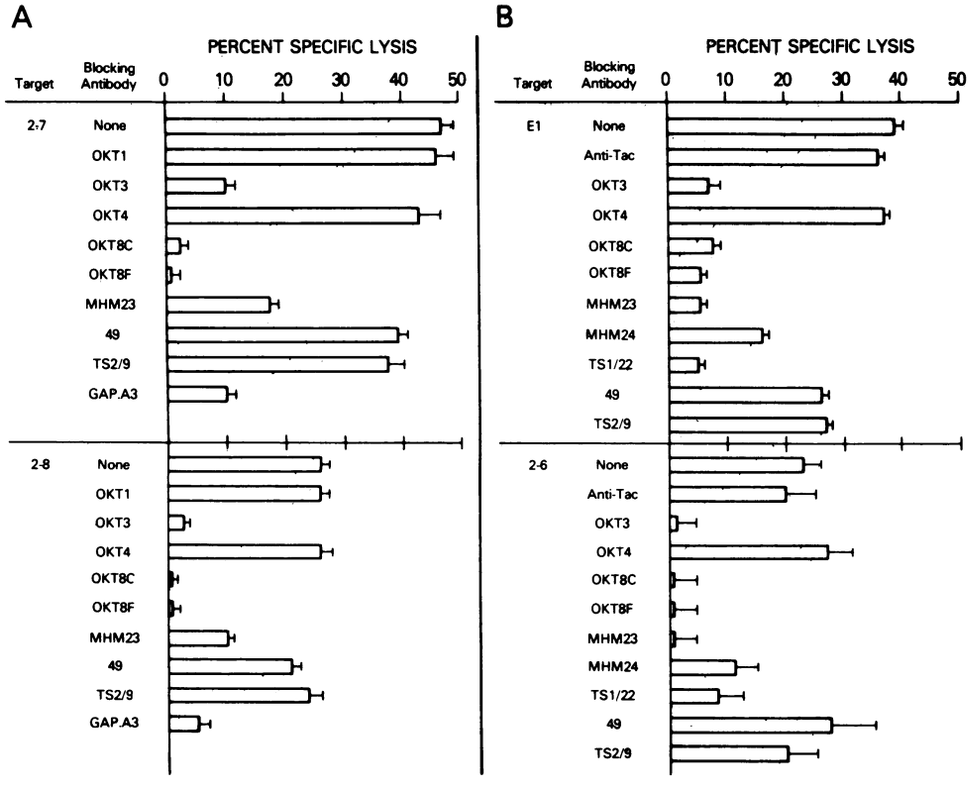

FIG. 4. Inhibition of the cytolytic activity of M3 anti-E1 CTL by monoclonal antibodies. Effector cells were assayed on 2-6, 2-7, and 2-8 targets at 40:1 effector:target ratio and on E1 PHA blasts at 10:1. The following antibodies were used at 25 μg/ml: OKT1, anti-Tac, OKT3, OKT4, OKT8C, OKT8F, and TS1/22. Antibodies 49, TS2/9, and GAP.A3 were used as a 1:50 dilution of ascites fluid. Antibodies MHM23 and MHM24 were used as a 1:2 dilution of culture fluid.

L-cell lines. However, the fact that the L-cell lines used by others are efficiently lysed by murine CTL (14) would seem to indicate that these other lines do not have an inherent molecular deficiency. (*ii*) The E1-A3 transformants express the HLA-A3 gene product on the cell surface at levels comparable to those of the endogenous H-2K^k molecules, based on indirect immunofluorescence. The level of expression of the transfected MHC gene products was not compared with that of the endogenous mouse MHC genes in other studies (14–17). It is therefore possible that the E1-A3 transformants are more readily recognized by CTL because they express an unusually high level of the transfected gene product when compared with the levels expressed by other transformants. (*iii*) The determinants recognized by CTL on certain class I molecules may vary depending upon the cell in which the gene is expressed. This could result from cell-specific differences in post-translational modifications, such as glycosylation, which could obscure CTL determinants or cause a conformational change that has the same effect. In this regard, it has been demonstrated recently that L cells glycosylate the product of a transfected murine class I gene in a different manner than cells naturally producing this molecule (40). Another possibility is that the molecular environment on the L-cell surface influences the conformation of class I molecules. The class I determinant recognized by M3 anti-E1 CTL may be particularly stable in this regard. (*iv*) A unique feature of our system is the specificity of the CTL we have employed. The M3 responder cells differ from the E1 stimulator cells in their class I loci only by their A3 subtype, and these subtypes differ by only two amino acids in the α2 domain, at positions 152 and 156 (ref. 20; unpublished data). Thus, M3 anti-E1 CTL are most probably directed against either a single determinant or a very limited number of determinants. This is in contrast to a population of classical allospecific CTL that presumably recognize a variety of determinants.

The results of this study demonstrate that allospecific human CTL require only the appropriate class I molecules on the target cell for effective recognition. By isolating a human class I molecule in a xenogeneic environment, the role of various T-cell accessory molecules and the mechanism by which they exercise their function can be examined more rigorously. In addition, the fine specificity of the CTL we have employed will allow better definition of those portions of the class I molecule that are functionally important.

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