USE OF DNA-MEDIATED GENE TRANSFER TO ANALYZE THE ROLE OF *H-2L^d* IN CONTROLLING THE SPECIFICITY OF ANTI-VESICULAR STOMATITIS VIRUS CYTOTOXIC T CELLS*

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The major histocompatibility complex (MHC)[1] is a cluster of closely linked genes that control cellular interactions in the immune system. Class I MHC genes encode for 40–45,000-dalton cell membrane glycoproteins that noncovalently associate with beta-2 microglobulin (1). While the function of many of these class I molecules is not understood, some of them play a role in determining the specificity of cytotoxic T lymphocytes (CTL) (2, 3).

The number of class I MHC genes in the mouse genome, as estimated by gene cloning, is ~40 (4). Although some of these hybridizing DNA fragments could be pseudogenes (5, 6), these data suggest that several other genes besides *H-2K*, *D*, and *L* play a role in *H-2* restriction of T cell specificity. To identify the function of individual *H-2* genes, it is necessary to isolate the gene and study the function of its product. Recently, such technology has allowed for the isolation and transfer of such genes into mouse C3H L cells (7, 8).

We have recently demonstrated that inoculation of vesicular stomatitis virus (VSV) into BALB/c animals results in the generation of CTL that are restricted by the *H-2L^d* gene (9). This evidence was obtained using two approaches; analysis of the response in H-2 recombinant and H-2D-region mutant strains, and blocking of lytic activity by precoating target cells with H-2-specific antisera and monoclones.

More recently, the *H-2D* region of *H-2^d* has been shown to control additional antigenic molecules, namely, L2^d and L2q, besides H-2D, L, M, and R (10, 11). This raises the possibility that these or other presently unknown H-2 products could be responsible for restriction of anti-VSV CTL activity. In this respect, serological reagents may not define the antigens since they could react with other molecules that share allodeterminants. Therefore, to definitively establish the role of *H-2L^d* as the restricting element for anti-VSV CTL, we used the method of DNA-mediated gene transfer (7). This technique permitted the transfer of a single BALB/c gene, *H-2L^d*, into L (H-2^k) cells. While VSV-infected normal L cells are not lysed by BALB/c anti-VSV CTL, L cells transformed with the *H-2L^d* gene express its product and are lysed by anti-VSV CTL. Therefore, by using this technology we are able to unequivocally

* Supported by grants AI13111 and AI11851 from the National Institutes of Health.

[1] *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; FCS, fetal calf serum; HAT, hypoxanthine, aminopterin, thymidine; MHC, major histocompatibility complex; PFU, plaque-forming units; VSV, vesicular stomatitus virus; tk, thymidine kinase.

demonstrate that a single *H-2* gene, *H-2L^d*, functions as a restricting element for anti-VSV CTL.

Materials and Methods

Mice. Mice were obtained from our colony at the University of Texas Health Science Center.

Virus. VSV of the Indiana serotype was prepared by passage of the virus stock in the rat cell line R(B77). The source and culture conditions of R(B77) have been previously described (12). VSV was titered by a plaque assay using R(B77) monolayers.

Cells. P815 tumor cells (a DBA/2 mastocytoma) were maintained by twice-weekly passage in vitro in RPMI medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Alloreactive cytotoxic cells were induced following previously published procedures (9). Spleen cells obtained from mice immunized 6 d previously with 10^8 plaque-forming units (PFU) of VSV were restimulated in vitro with VSV-infected stimulator cells to generate secondary anti-VSV CTL. The procedure has been described in detail elsewhere (13). Secondary anti-VSV CTL were used in all experiments described in this report.

Cells were transfected with the *H-2L^d* gene as previously described (7). Briefly, two cell lines derived from transformation of C3H mouse L cells were used. Ltk$^+$ cells (designated tk$^+$) were obtained by transfection of thymidine kinase (tk)-negative (Ltk$^-$) cells with the cloned herpes viral *tk* gene, and 8-5 cells were derived by the co-transformation of Ltk$^-$ cells with the herpes viral *tk* gene and DNA from the genomic clone 27.5 containing the BALB/c *H-2L^d* gene (7, 14). Transformants were selected and cloned in alpha-minimal essential medium containing 10% FCS and hypoxanthine, aminopterin, and thymidine (HAT medium) as described (7). Both 8-5 and tk$^+$ cells were maintained in HAT medium as adherent cell lines in 75-cm^2 tissue culture flasks. Before use as targets, cells were removed from the plastic by incubation with 0.02% trypsin at 37°C for 3–5 min. Trypsin activity was terminated by the addition of medium containing 10% FCS.

Assay for CTL Activity. CTL activity was measured using a standard chromium release assay (9). Various numbers of normal or immune lymphocytes were added to a constant number (10^4) of target cells in microtiter plates. After 4 h of incubation at 37°C, the cells were pelleted by centrifugation and the supernatant was measured for radioactivity. Cold target competition analysis was performed following previously published procedures (9) using 2.5×10^5 effector cells and 2.5×10^3 target cells together with various numbers of inhibitor cells.

When hybridoma products were tested for their ability to inhibit CTL activity, labeled targets were incubated with the indicated quantities of antibodies for 45 min at 37°C. The effector cells were then added and the mixtures incubated and processed as described for the CTL assay.

Net release of isotope was calculated by substracting the percent ^{51}Cr release from target cells cultured in media alone from the percent ^{51}Cr release from target cells cultured in the presence of effector cells. The standard error from three replicates in each group was <5%. Spontaneous release ranged from 8 to 34%.

Maximum release of isotope (water lysis) from P815 cells was generally 85%, whereas from L cell targets, the release was usually 10–15% lower. The calculation of percent isotope release does not take this factor into account.

Monoclonal Antibodies. Monoclonal antibodies were the generous gift of Dr. Ted Hansen, Merck Institute for Therapeutic Research, Rahway, NJ, and have been described previously (9, 15). Briefly, 30-5-7 is directed against H-2L^d (2.65) and 34-2-12 is specific for H-2D^d but not H-2L^d or H-2R^d (H-2.4). Both are IgG_{2a} antibodies. UPC10 myeloma (IgG_{2a}) protein (Litton Bionetics, Kensington, MD) at a concentration of 1 mg/ml was used as a control.

Fluorescence-activated Cell Sorter (FACS) Analysis. Cells were analyzed for fluorescence and light scatter on a FACS III (B-D FACS Systems Becton, Dickinson & Co., Sunnyvale, CA) instrument at a fluorescent gain of 4. Cells were prepared for analysis by incubation with monoclonal antibodies or myeloma protein (UPC10) followed by washing and exposure to fluoresceinated (F)- F(ab')$_2$ anti-mouse Ig (N. L. Cappel Laboratories, Cochranville, PA) in the presence of medium containing heat-inactivated normal rabbit serum to prevent nonspecific binding to Fc receptors. 10,000 cells were analyzed.

Results

Recognition of H-2L^d in Transformed H-2L Cells. tk$^-$ C3H (*H-2^k*)-derived L cells (Ltk$^-$) were transformed with either the herpes simplex virus (HSV) *tk* gene or the *tk* gene together with the BALB/c DNA genomic clone 27.5, which encodes for *H-2L^d*. L cells referred to as 8-5 represent a cloned tk$^+$ 27.5 transformant selected in medium containing HAT that expresses H-2L^d (7). Moreover, two-dimensional gel analysis of

TABLE I

Sensitivity of 8-5 Cells to Lysis by Alloreactive CTL

Effector cells	Experiment	Target cells*								
		P815			8-5			tk$^+$		
		100‡	50	10	100	50	10	100	50	10
BALB/c anti-B10.BR	1	6§	5	0	59	58	32	28	26	15
	2	7	6	3	63	64	59	44	38	26
B10.BR anti-BALB/c	1	62	59	25	31	24	9	4	5	1
	2	69	71	58	51	45	24	4	4	2

* Target cells were uninfected in experiment 1 and infected with VSV in experiment 2.
‡ Effector/target ratio.
§ Net release.

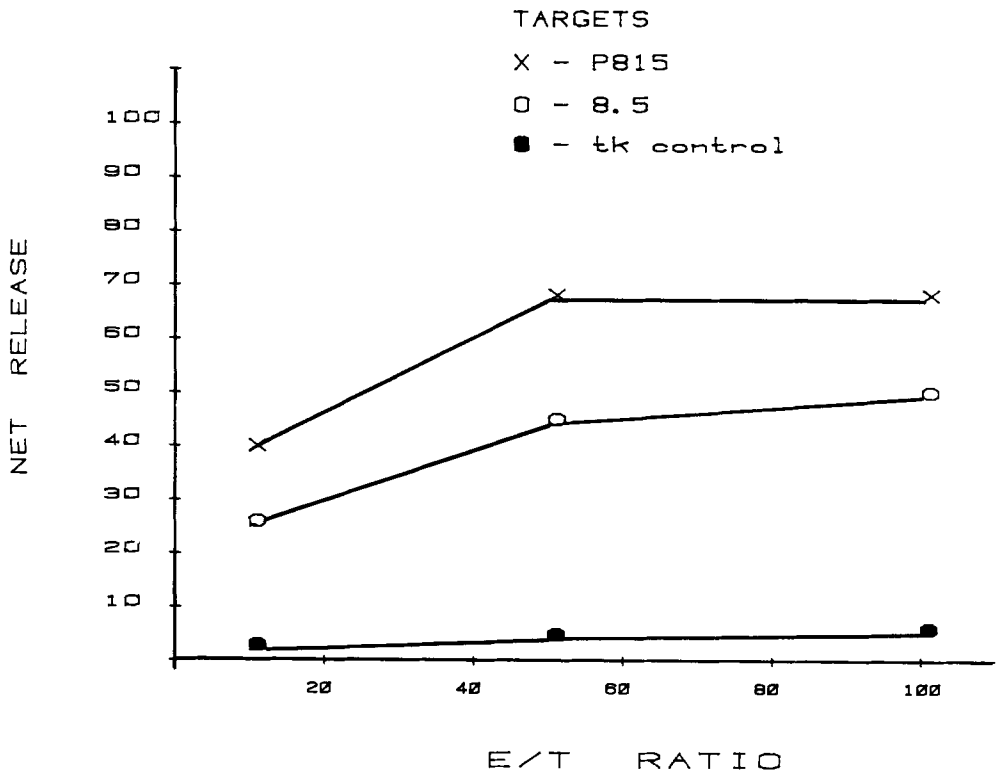

FIG. 1. Cytotoxic activity of anti-H-2L^d CTL. Net release of isotope from P815 (X), 8-5 (O), and tk$^+$ (●) target cells in the presence of dm2 anti-BALB/c effector cells.

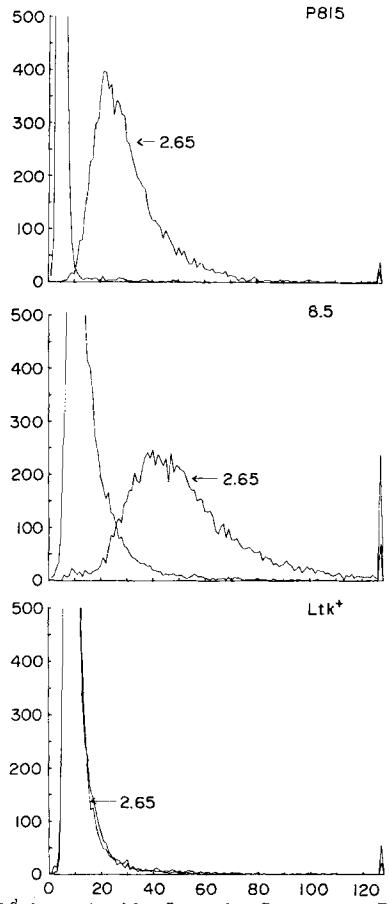

FIG. 2. Expression of H-2L^d determined by flow microfluorometry. P815 (top panel), 8-5 (middle panel), and tk$^+$ (bottom panel) cells were exposed to monoclonal antibody (30-5-7) that detects specificity 2.65 (H-2L^d) followed by F-F(ab')$_2$ anti-Ig. 10,000 cells were then analyzed. For control, an aliquot of cells was incubated with UPC10 myeloma protein rather than 30-5-7. Dead cells were excluded from analysis. Relative increasing intensity of fluorescence on the X axis is plotted vs. the number of cells/channel on the Y axis.

the product expressed by this cell line shows no differences with H-2L^d obtained from BALB/c splenocytes (7). To determine if CTL recognize the H-2L^d antigen on the transfected cells, we generated anti-H-2^d and anti-H-2L^d specific CTL and tested them against 8-5 target cells.

The data presented in Table I demonstrated that B10.BR (*H-2^k*) anti-BALB/c (*H-2^d*) effector cells display a lytic effect against P815 (*H-2K^d*, *H-2D^d*, *H-2L^d*) as well as 8-5 (*H-2^k*, *H-2L^d*) target cells, whereas cells transfected with the *tk* gene only (tk$^+$ cells) are not killed. Further, the same target cells infected with VSV before exposure to anti-H-2^d CTL show the same pattern of lysis. BALB/c anti-B10.BR (anti-H-2^k) CTL display lysis against 8-5 and tk$^+$ but not P815 cells, as expected, although the extent of lysis of the tk$^+$ cells was usually less than that observed against the 8.5 targets.

The above effector cells are potentially directed against several H-2^d encoded

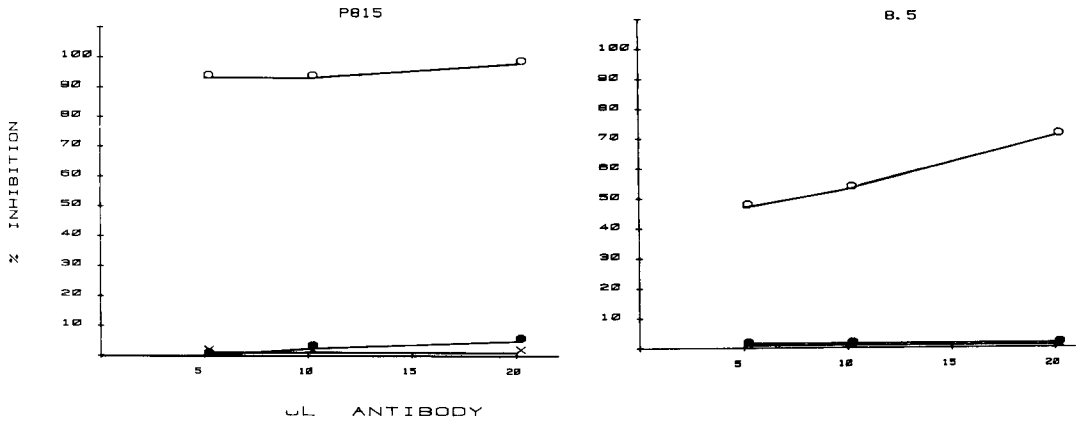

FIG. 3. Ability of monoclonal antibodies to inhibit lysis by anti-H-2L^d CTL. P815 (left panel) or 8-5 (right panel) target cells were exposed to dm2 anti-BALB/c effector cells in the presence of myeloma protein (control) (×), anti-H-2L^d (monoclone 30-5-7) (○), or anti-H-2D^d (monoclone 34-2-12) (●). Net release of isotope in the absence of antibody against P815 target cells was 41% and against 8.5 cells was 17%. Percent inhibition = [1 − (percent net release in the presence of antibody)/(percent net release in the absence of antibody)] × 100.

alloantigens. Therefore, to determine whether anti-H-2L^d CTL could also recognize target antigens on the 8-5 transfectants, we generated BALB/c-H-2^{dm2} (*H-2^{dm2}*, abbreviated dm2) anti-BALB/c CTL. We and others (9, 16) have previously shown that these effector cells are directed against H-2L^d but not H-2D^d. The data in Fig. 1 demonstrate that these effector cells are able to lyse P815 and 8-5 target cells but not the tk$^+$ targets. The reason for the higher degree of isotope release from the P815 targets relative to 8-5 cells in the presence of anti-H-2L CTL is not known. However, consistent with this finding is the observation that the extent of isotope release due to osmotic shock is greater from P815 than 8-5 cells (see Materials and Methods for data on H_2O lysis). In any event, it is not due to the density of H-2L^d, since 8-5 cells express a greater amount of this antigen than P815 cells, as determined by flow cytometry (Fig. 2).

To further establish that the alloantigen recognized is expressed on the H-2L^d molecule, monoclones directed against either H-2D^d or H-2L^d were tested for their ability to block lysis by effector cells. In agreement with our previous study (9), addition of anti-H-2L^d to both 8-5 and P815 target cells inhibited lysis by the dm2 anti-BALB/c effector cells, whereas anti-H-2D^d had no inhibitory effect (Fig. 3, left panel). Similar results were noted when 8-5 targets were used (Fig. 3, right panel). Therefore, these data demonstrate that H-2L^d, as defined by alloreactive CTL and monoclonal antibodies, is expressed on 8-5 target cells, and confirm the previous data of Örn et al. (17).

Ability of H-2L^d-transformed L Cells to Restrict Anti-VSV CTL. We previously demonstrated (9) that the *H-2L^d* gene restricted anti-VSV CTL in BALB/c animals. These data were obtained using H-2-recombinant and mutant mouse strains as well as monoclonal antibodies. However, since these monoclones could potentially react with unknown molecules controlled by a gene(s) in the *D region* of *H-2^d*, we used 8-5 target cells to unequivocally evaluate the role of *H-2L*.

BALB/c animals were infected with VSV, and 6 d later their spleen cells were

placed in culture with VSV-infected stimulator cells. After a 5-d culture period, the effector cells were tested against VSV infected or uninfected target cells for their sensitivity to lysis. VSV-infected P815 cells were lysed by the CTL, whereas uninfected P815 targets were not (Fig. 4). 8-5 target cells infected with VSV were also sensitive to lysis although to a somewhat lesser extent than P815 targets. Uninfected 8-5 cells as well as infected and uninfected tk$^+$ cells were not killed. Similar results were noted using DBA/2 (*H-2^d*) effector cells (Fig. 5).

Ability of Monoclonal Antibodies to Block Target Cell Lysis. To establish that the target antigen on 8-5 cells was not an H-2^k-controlled gene product, we used two anti-H-2^d monoclones to block lytic activity. Monoclones 30-5-7 and 34-2-12 react with H-2L^d and H-2D^d, respectively (9, 15). The dat presented in Fig. 6 demonstrate that BALB/c anti-VSV CTL are completely inhibited from killing VSV-infected 8-5 target cells in the presence of the anti-H-2L^d monoclone. As expected, the anti-H-2D^d monoclone had no blocking effect. Therefore, the fact that an anti-H-2L^d specific antibody blocks lysis indicates that H-2^k antigens do not account for anti-VSV CTL restriction.

Role of MHC Genes Other Than H-2L^d in Restricting Anti-VSV CTL in BALB/c Animals. The above data demonstrate that anti-VSV CTL generated in H-2^d animals recognize VSV in the context of *H-2L^d*. However, they do not exclude the possibility that other gene products in addition to H-2L^d also restrict these effector cells. If this

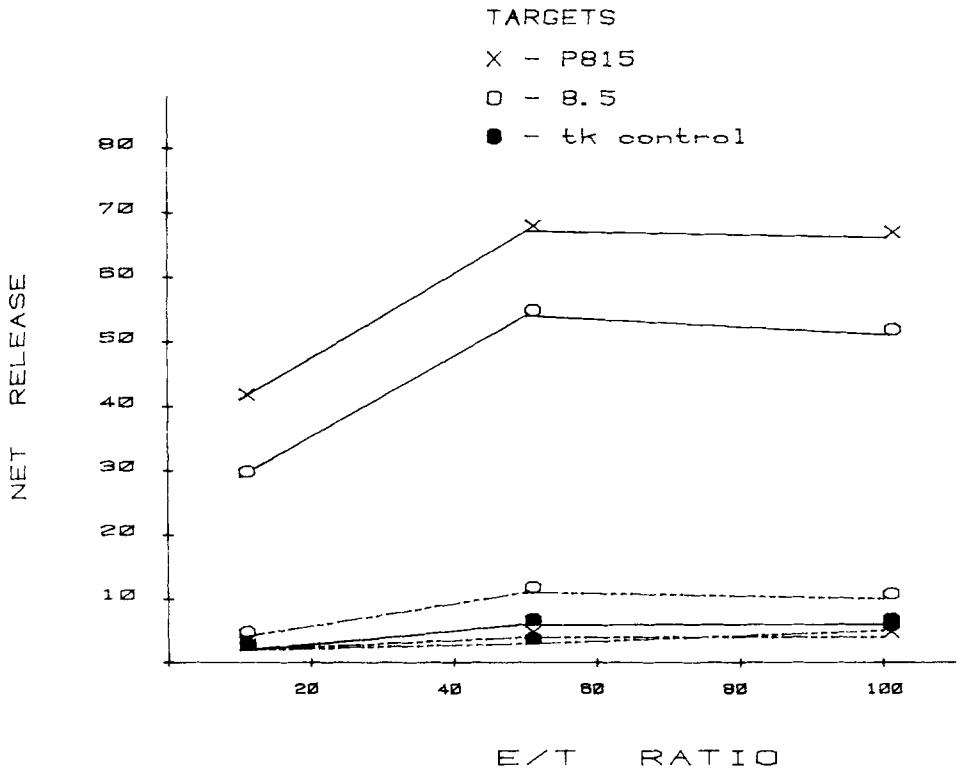

FIG. 4. Ability of anti-VSV CTL to lyse VSV-infected target cells. Net release of isotope from uninfected (-----) or infected (——) P815 (X), 8-5 (O), or tk$^+$ (●) target cells in the presence of BALB/c anti-VSV effector cells.

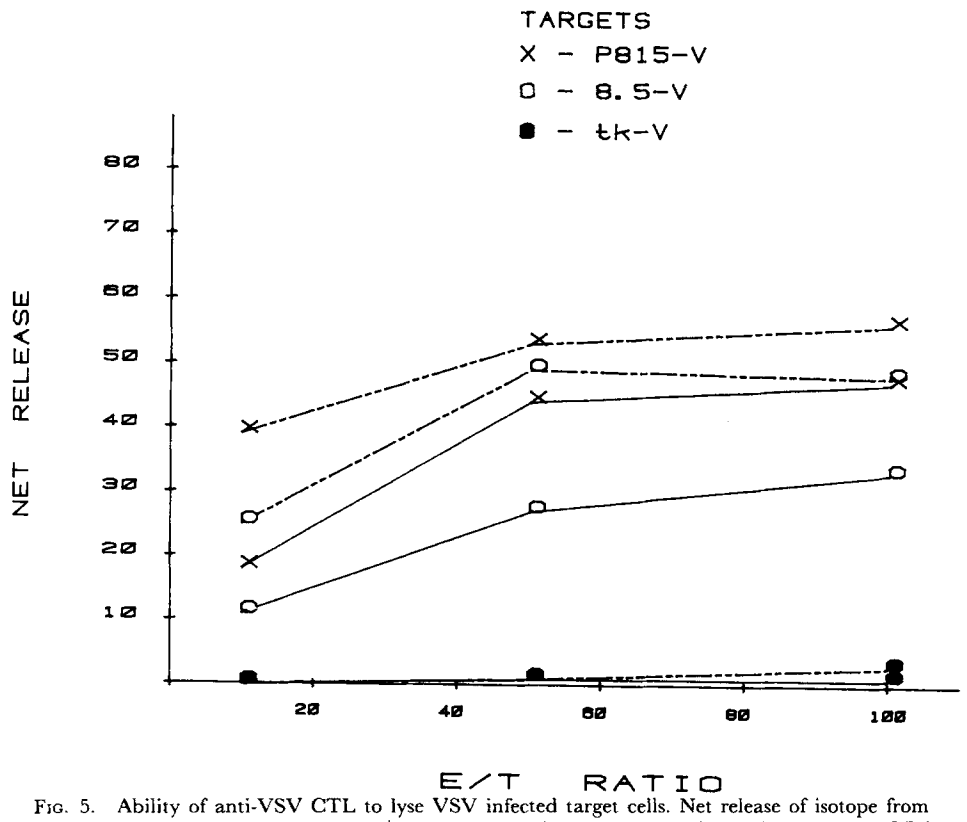

FIG. 5. Ability of anti-VSV CTL to lyse VSV infected target cells. Net release of isotope from VSV infected P815, (×), 8-5 (○), or tk^+ (●) target cells in the presence of DBA/2 (-----) or BALB/c (——) effector cells.

were the case, then anti-VSV CTL generated in BALB/c animals and tested against P815-VSV target cells in the presence of 8-5 unlabeled inhibitors should be only partially blocked in their lytic activity. The results of three such experiments (Fig. 7) show that neither VSV-infected tk^+ nor uninfected P815, 8.5, or tk^+ cells block CTL activity. On the other hand, most of the anti-VSV activity directed against infected P815 target cells is blocked by unlabeled P815-VSV inhibitors. Similar results were observed using 8-5-VSV cells as inhibitors, although the level of blocking was somewhat less than that of P815 cells in two of the three experiments. However, since 8-5 cells are less sensitive to lysis by anti-H-$2L^d$ CTL than P815 cells, this result is not unexpected (18). Taken together, these data demonstrate that the *H-2L*^{d} gene, as defined by the DNA sequence of clone 27.5 (14), is the major if not exclusive component controlling recognition by anti-VSV CTL in H-2^d animals.

Discussion

Recent estimates of the number of class I genes in the mouse MHC number ~40 (4). The genes that have been delineated by gene cloning include *H-2K*, *D*, *L*, *Qa-2*, and *TL* (19). Some of the genes in this complex could be pseudogenes (5, 6). All of the class I genes from BALB/c mice have been identifed and used to transform L cells, which permits the identification of all class I-restricting elements. The results of our

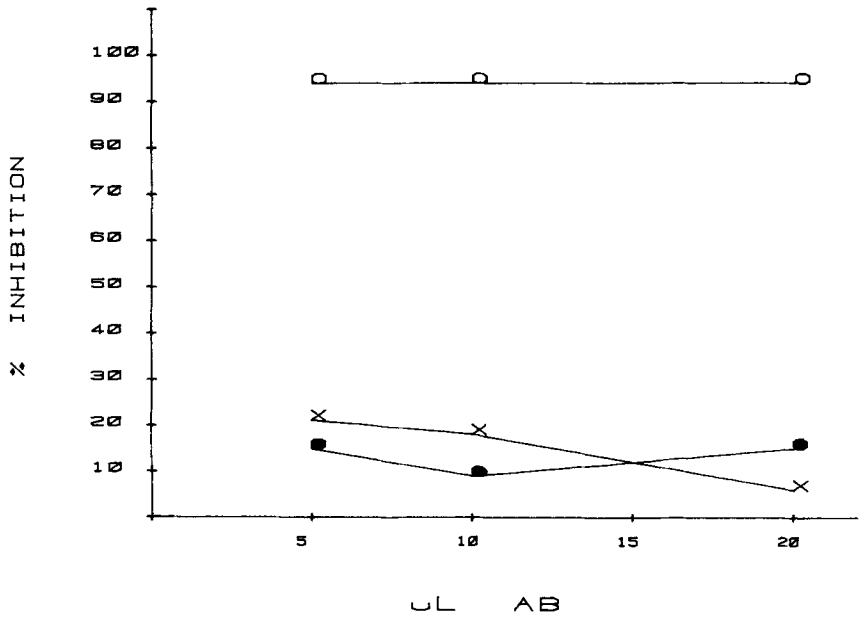

FIG. 6. Ability of monoclonal antibody to inhibit lysis of 8-5-VSV cells by anti-VSV CTL. VSV-infected 8-5 target cells were exposed to BALB/c anti-VSV effector cells in the presence of myeloma protein (control), (×), anti-H-2L^d (○), or anti-H-2D^d (●) monoclones. Net release of isotope in the absence of antibody was 32%. Percent inhibition calculated as in Fig. 3.

present study demonstrate that the BALB/c DNA clone 27.5, which encodes H-2L^d, is the major restricting element for anti-VSV CTL generated in *H-2^d* animals.

We previously demonstrated (9), using H-2-recombinant and mutant strains together with monoclonal antibodies, that *H-2L^d* is the restricting element for anti-VSV CTL in BALB/c animals. However, more recent evidence indicates that in addition to H-2D, L, M, and R, additional D region-controlled antigens can be detected in *H-2^d* mice (10, 11). These include L_q and L2^d. Further, it is conceivable that other H-2D^d region-controlled molecules will subsequently be discovered, especially as new monoclonal reagents are derived. Since some monoclonals would be expected to react with similar determinants on several different H-2 molecules, the use of monoclonal antibodies to block CTL recognition does not definitively identify the gene responsible for restricting activity. Therefore, to unequivocally demonstrate that the *H-2L^d* gene is responsible for restricting anti-VSV CTL, we used the technique of DNA-mediated gene transfer. The cells used for gene transfer were mouse C3H L (*H-2^k*) cells. These cells express H-2^k antigens and are recognized by anti-H-2^k alloreactive CTL. However, infection of these cells with VSV does not render them sensitive to lysis by BALB/c (*H-2^d*) anti-VSV effector cells. This demonstrates that the *H-2^k* haplotype does not control cross-reactive determinants that can be recognized by H-2^d-restricted anti-VSV CTL. Goodenow et al. (7) have cloned a single BALB/c *H-2* gene (27. 5) in λ bacteriophage and transferred this along with the HSV-1 tk gene into Ltk$^-$ cells by the technique of DNA-mediated gene transfer. One clone derived from this transfection, 8-5, expressed the BALB/c H-2L^d molecule, as determined by the reaction with anti-H-2L^d-specific monoclones. Additional evidence demonstrating that the new molecule expressed in the transformed cells was H-2L^d was provided by showing that

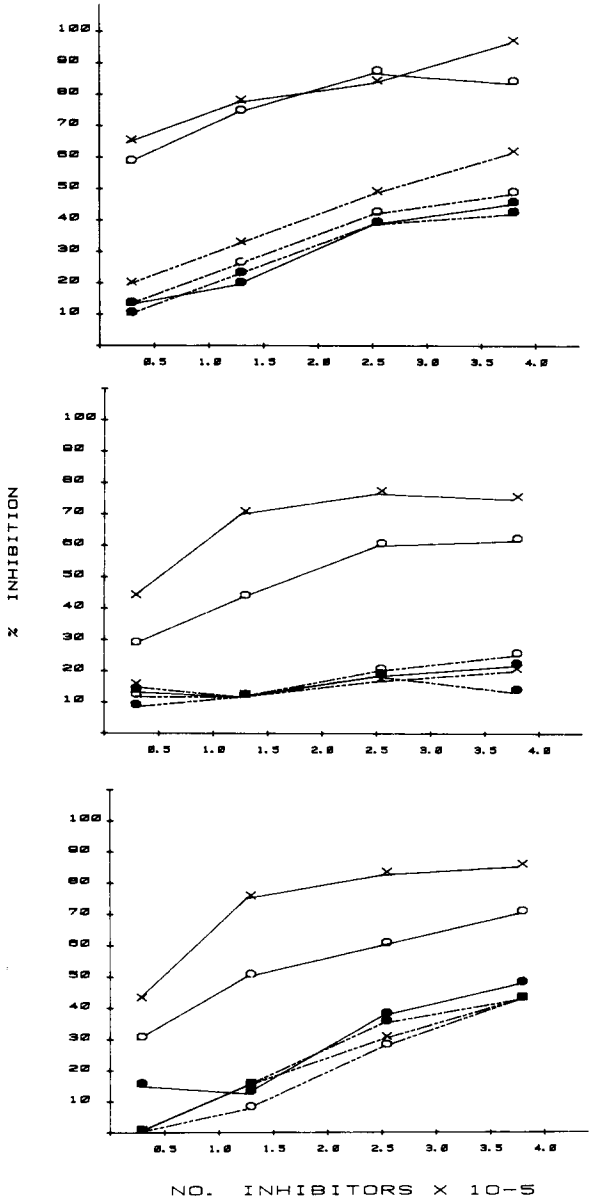

FIG. 7. Ability of unlabeled VSV infected cells to block target cell lysis. Uninfected (-----) or infected (——) unlabeled P815, (×), 8-5 (○), or tk^+ (●) inhibitor cells were added to CTL cultures containing P815-VSV labeled target cells along with BALB/c anti-VSV effectors. The three panels represent three different experiments. Net release of isotope in the absence of inhibitor cells in the experiment in the top panel is 31%; middle panel, 60%; and bottom panel, 39%. Percent inhibition = [1 − (percent net release in the presence of inhibitor cells)/(percent net release in the absence of inhibitor cells)] × 100.

the DNA sequence of the genomic clone (27.5) corresponded to the known protein sequence of $H\text{-}2L^d$ (14). Further, the molecule precipitated on 8-5 cells by anti-$H\text{-}2L^d$ monoclones is identical to $H\text{-}2L^d$ isolated from BALB/c splenocytes, as determined

by two-dimensional gel electrophoresis. Thus, these data demonstrate that 8-5 cells express H-2L^d but not other gene products derived from the *H-2^d* haplotype and therefore provide a unique tool for evaluating the role of this gene in *H-2*-restricted recognition by anti-viral CTL. Because VSV-infected 8-5 transformants were recognized and lysed by anti-VSV CTL generated in BALB/c animals, this unequivocally shows that H-2L^d is a restricting molecule for anti-VSV CTL. Further confirmation that H-2L^d and not an *H-2^k*-encoded gene product is responsible for this effect was demonstrated by the use of an anti-H-2L^d monoclone. When added to target cells, this antibody blocked the ability of CTL to cause lysis.

It has been previously shown that anti-influenza CTL are restricted by both *H-2D^d* and *H2L^d*. However, we previously demonstrated that monoclonal antibodies directed against H-2D^d do not block the cytolytic activity of anti-VSV CTL, ruling out a restricting role for this antigen. To further assess the ability of other H-2^d antigens to restrict anti-VSV CTL, we generated effector cells in BALB/c animals and tested them against P815-VSV (*H-2^d*)-infected target cells in the presence of unlabeled 8-5-VSV (*H-2L^d*) inhibitor cells. Similar to P815-VSV inhibitor cells, the 8-5-VSV cells also inhibited most of the CTL activity, although usually to a slightly lesser extent than P815-VSV cells. We interpret the lower inhibitory activity of the 8-5-VSV cells to be due to their lesser sensitivity to lysis, a factor that correlates with the ability of cells to inhibit in the competition assay (18), and not antigen density, since the expression of H-2L^d is actually greater on 8-5 cells than P815. Therefore, our data are most consistent with the postulate that H-2L^d is the only molecule expressed by H-2^d mice that restricts anti-VSV CTL. However, since in some experiments complete blocking of CTL activity was not seen by 8-5-VSV cells, it is still possible that a minor component of restricting activity is exhibited by class I gene products(s) other than *H-2K* or *-D*.

The homology between *H-2K* or *H-2D* alleles is no greater than that between *H-2K* or *H-2D* genes themselves (20). In most cases, H-2 molecules share ~75–85% of their sequence (20, 21). More recently, H-2L or L-like gene products have been described in several different mouse *H-2* haplotypes (22, 23). Maloy and Coligan (24) observed a 94% sequence homology between H-2L^d and H-2D^b, including no amino acid differences between residues 158 and 338. They raised the possibility that *H-2L^d* rather than *H-2D^d* is allelic to *H-2D^b*. However, as an alternative, *H-2L^d* may be a duplicated *H-2D^b* gene created by unequal crossing over, similar to that postulated for the two (duplicated) IgG_{2a} genes detected in mice of the species *Mus musculus molossinus* (25). Regardless of the similarities between H-2L^d and H-2D^b, the former and not the latter serves as a restriction element for anti-VSV CTL. Thus, the variant residues in the first two domains of these molecules play a critical role in controlling the generation of anti-VSV CTL.

The technique of DNA-mediated gene transfer has also been used by Örn et al. (17) and Mellor et al. (6), who demonstrated that H-2L^d restricts anti-lymphocytic choriomeningitis virus CTL and that H-2K^b restricts anti-influenza virus CTL, respectively. Thus, this technique provides a valuable method for analyzing the structure and function of individual *H-2* genes.

Summary

Mouse thymidine kinase (tk$^-$) C3H L (*H-2^k*) cells transformed by the technique of DNA-mediated gene transfer with the herpes simplex virus *tk* gene together with the

BALB/c *H-2L*^{d} gene express H-2L^d molecules indistinguishable from their counterparts on spleen cells. An established cloned cell line (8-5) was used to assess the function of the H-2L^d antigen in determining the specificity of alloreactive as well as anti-vesicular stomatitis virus (VSV) cytotoxic T cells (CTL).

Both anti-H-2^d and anti-H-2L^d CTL displayed a cytotoxic effect against 8-5 cells but not a control cell line transformed with the *tk* gene only (tk$^+$ cells). Further evidence that 8-5 cells express H-2L^d was provided by the finding that monoclonal anti-H-2L^d but not H-2D^d antibodies blocked target cell lysis by the effector cells.

Both BALB/c (*H-2*^{d}) and DBA/2 (*H-2*^{d}) animals generated anti-VSV CTL that lysed infected 8-5 but not tk$^+$ cells. To further establish that H-2L^d controlled the specificity of the effector cells, a monoclonal antibody directed against H-2L^d was shown to inhibit lysis of infected 8-5 target cells.

To determine whether other H-2^d-encoded gene products could serve as restricting antigens for anti-VSV CTL in BALB/c animals, unlabeled VSV infected 8-5 cells were tested for their ability to block lysis of ^{51}chromium-labeled P815 (*H-2*^{d})-infected target cells. The 8-5-VSV inhibitor cells inhibited lysis to a slighly lesser extent than unlabeled P815-VSV cells, indicating that H-2L^d plays a major if not exclusive role in restricting anti-VSV CTL in *H-2*^{d} animals.

Received for publication 27 August 1982 and in revised form 9 November 1982.

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