An Endogenous Antigenic Peptide Bypasses the Class I Antigen Presentation Defect in RMA-S

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Summary

The RMA-S cell line was derived from the Raucher virus–induced murine cell line RBL-5 by ethylmethane sulfonate mutagenesis and anti-H-2 antibody plus complement selection (Ljunggren, H.-G., and K. Karre. 1985. *J. Exp. Med.* 162:1745). RMA-S is defective in the ability to present endogenously synthesized antigens to class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL) (Townsend, A., C. Ohlen, J. Bastin, H.-G. Ljunggren, L. Foster, and K. Karre. 1989. *Nature [Lond.].* 340:443; Ohlen, C., J. Bastin, H.-G. Ljunggren, L. Foster, E. Wolpert, G. Klein, A. R. M. Townsend, and K. Karre. 1990. *J. Immunol.* 145:52). This defect has been attributed to the inability of RMA-S to deliver antigenic peptides derived from antigens in the cytosol into the endoplasmic reticulum (ER), where they can associate with class I MHC molecules (Townsend, A., C. Ohlen, J. Bastin, H.-G. Ljunggren, L. Foster, and K. Karre. 1989. *Nature [Lond.].* 340:443). We show that RMA-S can present at least one endogenous antigen, vesicular stomatitis virus nucleoprotein (VSV-N), to class I MHC-restricted CTL. RMA-S presents VSV-N to CTL both when infected with VSV or transfected with the VSV nucleoprotein gene. The natural antigenic VSV nucleoprotein peptides purified from either RMA or RMA-S are indistinguishable when analyzed by high performance liquid chromatography. We also show that the genetic defect responsible for the RMA-S phenotype maps to the murine chromosome 17. This chromosome encodes the murine class I MHC genes as well as two genes, HAM-1 and -2, with homology to the adenosine triphosphate–dependent transporter superfamily (Monaco, J. J., S. Cho, and M. Attaya. 1990. *Science [Wash. DC].* 250:1723). These results suggest that the system that delivers antigenic peptides from the cytosol to the ER in RMA-S may still be present and retain partial function.

Class I MHC molecules present short peptides derived from endogenous proteins to antigen-specific, class I–restricted T cells (1–4). These peptides are thought to be derived by an unknown mechanism in the cytosol and delivered by a peptide transport system from the cytosol into the lumen of the endoplasmic reticulum (ER),[1] where they can associate with class I MHC molecules (5). Evidence for the existence of a peptide transport system comes from analysis of several cell lines defective in the ability to present endogenous antigens to class I–restricted CTL. These cell lines include the human mutant LCL 721.174 and the murine mutant RMA-S. These cell lines were derived in a similar manner by either γ irradiation (LCL 721.174) or ethylmethane sulfonate (EMS) mutagenesis (RMA-S) followed by several rounds of selection with anti-MHC antibody plus complement (6, 7). They also display a phenotype that has several common features. First, they retain the majority of their class I MHC molecules in the ER in an incompletely assembled state (5, 8, 9). Treatment of these cell lines with IFN-γ does not result in increased cell surface expression of class I MHC molecules (6, 10). However, addition of large quantities of exogenous antigenic peptides to these cell lines induces increased cell surface expression of stable class I MHC molecules (5, 9, 11). A second common phenotypic feature of these mutants is that they are able to present exogenous antigenic peptides, but not endogenous antigens, to class I–restricted CTL, suggesting that their class I MHC molecules are fully functional (5, 9, 12, 13). Last, the genetic defect in these cell lines is either recessive or null in fusions between the mutants and normal cell lines and is not due to defects either in the class I heavy chains or β_2-microglobulin (β_2m) encoded by the mutant cell lines (10, 14). These observations have led to the hypothesis that these cell lines are defective in the ability to transport antigenic peptides into the lumen of the ER (5,

[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; EMS, ethylmethane sulfonate; ER, endoplasmic reticulum; HAU, hemagglutinating units; MOI, multiplicity of infection; NP, nucleoprotein.

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9, 13). Support for this hypothesis comes from cell fusion and selection experiments performed with the LCL 721.174 mutant. These experiments mapped the genetic location of the defect in LCL 721.174 to the human chromosome 6 (10). LCL 721.174 has a large deletion in the class II region of the MHC on chromosome 6 (15). Included within this deletion are genes with homology to members of the ATP-dependent transporter superfamily that may encode all or part of a peptide transport system (16, 17). Evidence that one of these genes (Y3) may encode at least part of a peptide transporter was provided by experiments showing that transfection of LCL 721.134 cells that lack Y3 expression with the Y3 cDNA restored high levels of HLA class I expression at the cell surface (18). The mutant LCL 721.134 was derived from the same series of mutagenesis and antibody plus complement selection experiments as LCL 721.174 (7). Transfection with the Y3 cDNA did not restore high levels of cell surface class I expression by the LCL 721.174 mutant cell line (18). The genetic defect responsible for the RMA-S phenotype is less well characterized than the defect in LCL 721.174. Like LCL 721.174, RMA-S has been shown to be unable to present several endogenous antigens to CTL (5, 12). We show here that RMA-S differs from LCL 721.174 in being capable of presenting at least one endogenous antigen, VSV-N, to class I–restricted CTL. We, and others, have previously reported that LCL 721.174 is unable to present any of several endogenous antigens, including VSV-N, to class I–restricted CTL (9, 13). We also provide evidence that the genetic defect responsible for the RMA-S phenotype maps to the murine chromosome 17, which encodes the murine MHC as well as the putative peptide transporter genes HAM-1 and -2 (19). It has been shown that RMA-S expresses mRNA for, and has therefore not deleted, the MHC-linked, putative peptide transporter genes HAM-1 and -2 (20). Based on these data, we speculate that the system that delivers antigenic peptides from the cytosol to the ER in RMA-S may still be present and retain partial function.

Materials and Methods

Mice. Young adult female C57Bl/6, C57Bl/10, AKR/J, and B10.BR mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cell Lines. The C57Bl/6 (H-2^b, B_2M^b, Thy-1.2)-derived, EMS-mutagenized cell line RMA and the mutagenized and antibody plus complement-selected cell line RMA-S were obtained from Dr. Linda Sherman (Research Institute of Scripps Clinic, La Jolla, CA) with the permission of Dr. Klaus Karre (6). The human cell lines, T2K^b and Jurkat/K^b, transfected with the murine class I H-2K^b gene were obtained as described previously (13). BW Lyt-2.4, an AKR/J (H-2^k, $\beta_2m^{non\text{-}a,b}$, Thy-1.1)-derived cell line transfected with the murine Lyt-2 gene, was obtained from Dr. Phillipa Marrack (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) (21).

Cytotoxic T Cells. The VSV-N-specific CTL line was derived from spleen cells from C57Bl/6 mice primed intraperitoneally with 10^6 PFU of VSV (Indiana) 7 d before restimulation in vitro as described previously (13). The chicken OVA-specific CTL clone B3 was derived from spleen cells from C57Bl/6 mice primed in vivo with the $OVA_{229\text{-}276}$ peptide and restimulated and cloned in vitro as described previously (22). The influenza nucleoprotein (NP)-specific CTL line was derived from C57Bl/6 mice primed intraperitoneally with 100 hemagglutinating units (HAU) of A/PR/8/34 influenza virus 7 d before restimulation in vitro. Spleen cells (3×10^7) from A/PR/8/34 virus primed mice were stimulated twice in vitro with 3×10^7 irradiated (3,000 rad) A/PR/8/34 virus–infected (1,000 HAU per spleen) syngeneic spleen cells in RP10 medium (RPMI 1640 containing 10% FCS and 50 μM 2-ME) in upright 25-cm^2 flasks at 37°C in 7% CO_2/air. Thereafter, $0.5\text{–}1.0 \times 10^5$ CTL were restimulated with 5×10^6 irradiated (3,000 rad) syngeneic spleen cells coated with synthetic $NP_{365\text{-}380}$ peptide (200 $\mu g/10^8$ cells) per well of a 24-well plate in RP10 medium containing 50 mM α-methyl mannoside and 5% supernatant from Con A–stimulated rat spleen cells at 37°C. The alloreactive C57Bl/6 (H-2^b) anti-B10.BR (H-2^k) CTL line was derived by primary in vitro stimulation of 3×10^7 C57Bl/6 splenocytes with 3×10^7 irradiated (3,000 rad) B10.BR spleen cells in 10 ml RP10 medium in upright 25-cm^2 flasks for 7 d at 37°C. The responding cells (5×10^6) were restimulated every 7 d with 3×10^7 irradiated (3,000 rad) B10.BR spleen cells in 10 ml RP10 medium containing 50 mM α-methyl mannoside and 5% supernatant from Con A–stimulated rat spleen cells in upright 25-cm^2 flasks at 37°C.

Chromium Release CTL Assays. Target cells (10^6) were infected with 200 HAU of either A/PR/8/34 or recombinant X31 influenza virus in RPMI 1640 with 200 μCi sodium [^{51}Cr]chromate for 1 h at 37°C. OVA was introduced into the cytoplasm of 10^6 sodium [^{51}Cr]chromate-labeled target cells by the method of the osmotic lysis of pinosomes with 100 μl of hypertonic medium containing 10 mg/ml OVA as previously described (23). Target cells (10^6) were infected with VSV(Indiana) at a multiplicity of infection (MOI) of 40 in HBSS containing 200 μCi of sodium [^{51}Cr]chromate for 1 h at 37°C. After washing with PBS, virus-infected or OVA-loaded target cells (10^4) were incubated with serial dilutions of effector CTL in wells of a round-bottomed 96-well plate with or without added $NP_{365\text{-}380}$ (10 μM), $OVA_{242\text{-}276}$ (10 μM), or $N_{53\text{-}63}$ (10 nM) synthetic peptides in a total volume of 200 μl of RP10 medium. After 3 h of incubation at 37°C, 100 μl of the supernatant was collected, and the amount of released chromium determined using a gamma counter (Beckman Instruments, Inc., Palo Alto, CA). Percent specific lysis = 100×[(release by CTL − spontaneous release)/(release by detergent − spontaneous release)]. Spontaneous release of chromium in the absence of CTL was <20% of detergent release in all experiments.

Transfection of RMA and RMA-S. The pSV2neo-RSV-N vector for expression of VSV(Indiana) NP in mammalian cells was obtained from Dr. Leo LeFrancois (Upjohn, Kalamazoo, MI) (24). RMA (5×10^7) and RMA-S (2×10^7) cells were transfected with 10 μg of circular plasmid in 1 ml PBS by electroporation at 375 V, 50 ms, and 1,400 μF using an X-Cell electroporater (Promega Biotec, Madison, WI). Surviving cells were cultured in RP10 medium at 37°C overnight. The cells were then plated at 10^4 per well in flat-bottomed 96-well plates in RP10 medium containing 600 μg/ml G418 (effective concentration) (Gibco Laboratories, Grand Island, NY). After 14–21 d, G418-resistant colonies were transferred to individual wells of a 24-well plate containing RP10 with 600 μg/ml G418. Clones testing positive for recognition by VSV-N-specific CTL, including RMA/N.4 and RMA-S/N.4, were maintained in culture in RP10 medium containing 600 μg/ml G418.

FACS® Analysis of Cell Lines. Cells ($0.3\text{–}1.0 \times 10^6$) were first incubated with either 100 μl of antibody-containing ascites diluted to the appropriate concentration in PBS/1% BSA (wt/vol), 200

μl of hybridoma supernatants, or 100 μl PBS/1% BSA alone for 30 min on ice. The cells were washed once with ice-cold PBS and incubated with 100 μl of either FITC-labeled antibody against mouse immunoglobulin Fc (Cappel Laboratories, Mulvern, PA) diluted 1:50 in PBS/1% BSA. FITC-labeled 30H12 antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:20 in PBS/1% BSA, or PBS/1% BSA alone for 30 min on ice. The cells were then washed once with ice-cold PBS and resuspended in PBS containing 1% formaldehyde (vol/vol). Analysis of 10,000 gated events per sample of stained cells was performed on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Purification of Naturally Processed VSV-N Peptides. Cell lysates of RMA (5×10^8), RMA-S (5×10^8), RMA/N.4 (5×10^8), RMA-S/N.4 (6×10^8), and both RMA and RMA-S infected with VSV (10^8 each; MOI = 40) were prepared in 0.1% TFA (vol/vol) as previously described (25). The lysates were lyophilized, resuspended in 0.1% TFA, and passed over a Sephadex G-25 column and eluted with 0.1% TFA. The included column volume was collected, lyophilized, resuspended in 1.0 ml of 0.1% TFA, and chromatographed by reverse-phase HPLC on a C18 column (Delta Pak; Waters Associates, Milford, MA) using a 0–60% acetonitrile gradient. 1-ml fractions were collected, lyophilized, and resuspended in 250 μl RPMI 1640. 50 μl of RP10 medium containing 10^4 sodium [^{51}Cr]chromate-labeled RMA-S target cells that had been previously cultured at 31°C overnight was added to 50 μl of each fraction in wells of round-bottomed 96-well plates, and the plates were incubated at 31°C for 1 h. Anti-VSV CTL were then added to the wells, bringing the total volume of RP10 medium per well to 200 μl, and the plates were incubated at 37°C for 4 h.

Fusion of RMA-S with BW Lyt-2.4 and Subsequent CTL Selection. RMA-S (G418^s, HAT^r; 5×10^6 cells) and BW Lyt-2.4 (G418^r, HAT^s; 5×10^6 cells) were combined in a 15-ml conical tube, washed once with warm (37°C) RPMI 1640, pelleted, and all RPMI was removed from the cells. 0.5 ml of warm 50% (vol/vol) polyethylene glycol 1000 in RPMI 1640 was slowly added to the cell pellet over a 2-min period. The cells were then pelleted for 2 min and incubated for an additional 2 min at room temperature. Warm RPMI 1640 (10 ml) was then slowly added to the cells over a 2-min period. The cells were pelleted and gently resuspended in warm RP10 medium and cultured at 37°C overnight. The following day, the cells were plated at 10^4 per well in flat-bottomed 96-well plates in RP10 medium containing 600 μg/ml G418 (effective concentration) and 1× HAT (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine; Sigma Chemical Co., St. Louis, MO). After ~14 d of incubation at 37°C, viable clones were transferred to individual wells of 24-well plates containing RP10 medium with 600 μg/ml G418 and 1× HT (100 μM hypoxanthine, and 16 μM thymidine; Sigma Chemical Co.). 40 individual clones were screened by FACS® analysis for expression of H-2K^b and H-2K^k class I MHC molecules and clone RxB.6 chosen for further analysis. Selection of RxB.6 was performed by mixing 10^7 RxB.6 cells with C57BL/6 anti-B10.BR CTL at E/T ratios of either 0.3:1 (Sel 3 and 5) or 1:1 (Sel 4) in 20 ml of RP10 medium containing 50 mM α-methyl mannoside and 5% supernatant from Con A–stimulated rat spleen cells in upright 25-cm^2 flasks. After incubation at 37°C for 24 h, the cells from each culture were pelleted, resuspended in 5 ml RP10 medium, and cultured in a single well of a six-well plate. Each culture was harvested after 7 d at 37°C. Dead cells and cell debris were removed with Ficoll and the remaining viable cells expanded and maintained in culture in RP10 medium at 37°C.

Southern Blot Analysis. Genomic DNA was prepared by overnight incubation at 37°C of 10^7 tumor cells or 10^8 spleen cells in 0.5 ml Lysis Buffer (1 M Tris, 5 M NaCl, 0.5 M EDTA, 10% SDS [wt/vol]) containing 100 μg/ml proteinase K and 20 μg/ml RNAse. The next day, 125 μl of a saturated NaCl solution was added to each lysate. The lysates were vortexed vigorously and then spun in a microfuge for 15 min. The supernatants were extracted with phenol and the DNA was precipitated by the addition of 1 ml of ethanol. 2 μg of HindIII-digested DNA from each cell line was subjected to electrophoresis on a 0.8% agarose gel in TBE buffer (45 mM Tris-Borate, 1 mM EDTA) at 40 V overnight. The DNAs were then blotted onto a nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, NH), crosslinked to the membrane with UV irradiation (120 millijoules) using a Stratalinker (Stratagene, La Jolla, CA), and the blot probed with a purified 3-kb EcoRI-HindIII fragment from the genomic 5′ promoter of H-2K^b in a 0.25 M NaH_2PO_4, 0.25 M Na_2HPO_4, 2% SDS (wt/vol), 1% BSA (wt/vol) solution at 65°C overnight. The blot was then washed with a 0.5× SSC/0.1% SDS (wt/vol) solution at 65°C for 1.5 h and autoradiographed.

Results

Presentation of Endogenous Antigens by RMA and RMA-S. The RMA-S cell line has been reported to be unable to present several endogenous antigens, including tumor-specific antigens, minor histocompatibility antigens, and influenza NP, to class I–restricted CTL (5, 12). Of these antigens, only the influenza NP epitope has been identified and its class I MHC restriction element defined (26). To further characterize the RMA-S defect, we compared the presentation of influenza NP with that of two well-characterized H-2K^b-restricted antigens, OVA and VSV-N.

As previously reported, A/PR/8/34 influenza virus–infected RMA, but not infected RMA-S, target cells were recognized and lysed by the NP-specific, H-2D^b-restricted anti-PR8 CTL line (Table 1). Uninfected control RMA and RMA-S target cells were not lysed by these CTL. As expected, both RMA and RMA-S target cells were sensitized for lysis by these CTL in the presence of exogenous antigenic $NP_{365-380}$ peptide.

Similar observations were made with the H-2K^b-restricted antigen OVA. After cytoplasmic loading with OVA by the osmotic lysis of pinosomes, RMA, but not RMA-S, target cells were recognized and lysed by the OVA-specific, H-2K^b-restricted CTL clone B3. Unloaded control RMA and RMA-S target cells were not lysed by this CTL clone. Both RMA and RMA-S target cells were sensitized for lysis by this CTL clone in the presence of exogenous antigenic $OVA_{242-276}$ peptide.

However, after infection with VSV, both RMA and RMA-S target cells were recognized and lysed by the H-2K^b-restricted, VSV-N-specific CTL line (24). VSV-infected RMA-S cells were consistently recognized and lysed by these CTL in numerous experiments, with the level of lysis ranging from at least 60% to as high as 100% that of VSV-infected RMA cells at the same E/T ratios. Both RMA and RMA-S target cells were sensitized for lysis by these CTL in the presence of exogenous antigenic VSV-N_{53-63} peptide. Neither a difference in VSV-N expression nor an increase in H-2K^b cell surface expression by VSV-infected RMA-S targets could explain their recognition by CTL. Both VSV-infected RMA and

Table 1. *RMA-S Is Able to Present One Endogenous Class I-restricted Antigen to Specific CTL*

APC	Antigen	Peptide	Percent specific lysis by CTL at E/T of:	
		Anti-PR8 CTL line:	10:1	1:1
RMA	–	–	3	2
RMA	–	$NP_{365-380}$	70	48
RMA	A/PR/8/34	–	49	33
RMA-S	–	–	0	0
RMA-S	–	$NP_{365-380}$	62	32
RMA-S	A/PR/8/34	–	2	0
		Anti-OVA B3 CTL clone:	10:1	1:1
RMA	–	–	0	0
RMA	–	$OVA_{242-276}$	77	40
RMA	OVA	–	44	30
RMA-S	–	–	5	2
RMA-S	–	$OVA_{242-276}$	78	46
RMA-S	OVA	–	7	5
		Anti-VSV CTL line:	10:1	1:1
RMA	–	–	0	0
RMA	–	N_{53-63}	60	37
RMA	VSV	–	61	41
RMA-S	–	–	1	0
RMA-S	–	N_{53-63}	86	71
RMA-S	VSV	–	64	44

RMA-S cells synthesized quantitatively similar amounts of the VSV-N protein as determined by SDS-PAGE (data not shown). FACS® analysis of VSV-infected RMA-S cells revealed that cell surface expression levels of H-2K^b molecules did not increase for up to 6 h after infection with VSV (data not shown). One curious observation was that RMA-S cells required approximately eightfold more VSV virus to become targets for CTL recognition than did RMA cells (Fig. 1 *A*).

The requirement for a higher MOI in the case of RMA-S cannot be explained by the presence of free targeting peptide in the viral preparation for a number of reasons. (*a*) Up to 10-fold more virus does not lead to lysis of the human, antigen presentation–defective cell line T2K^b, which is lysed by the CTL in the presence of synthetic VSV-N_{53-63} peptide (Fig. 1 *B*). A control human cell line expressing H-2K^b is lysed after VSV infection. (*b*) Fixation of RMA or RMA-S cells before incubation with VSV prevents their sensitization for lysis by these CTL (data not shown). (*c*) Brefeldin A treatment of RMA and RMA-S cells blocks presentation of the of the VSV-N epitope after viral infection while allowing presentation of exogenous peptide to occur (data not shown).

Presentation of VSV-N by Transfected Cells. To rule out possible nonspecific effects of VSV infection on RMA-S, we stably transfected RMA and RMA-S cells with the pSV2neo-RSV-N plasmid, which contains both the selectable G418 resistance gene and the gene encoding VSV-N (24). Of the resulting G418-resistant clones, 4 of 22 RMA and 1 of 4 RMA-S clones were recognized and lysed by VSV-N-specific CTL (data not shown). A representative experiment (Table 2) shows that in the absence of exogenous antigenic N_{53-63} peptide, the transfected clones RMA/N.4 and RMA-S/N.4, but not untransfected RMA or RMA-S cells, are recognized and lysed by these CTL. Similar to our observations with VSV-infected target cells, RMA-S/N.4 targets were recognized and lysed by VSV-N-specific CTL at levels >50% of RMA-N.4 targets at the same E/T ratios. Sensitization of RMA-S/N.4 for CTL recognition was not due to secretion of antigenic VSV-N peptides since supernatants from high

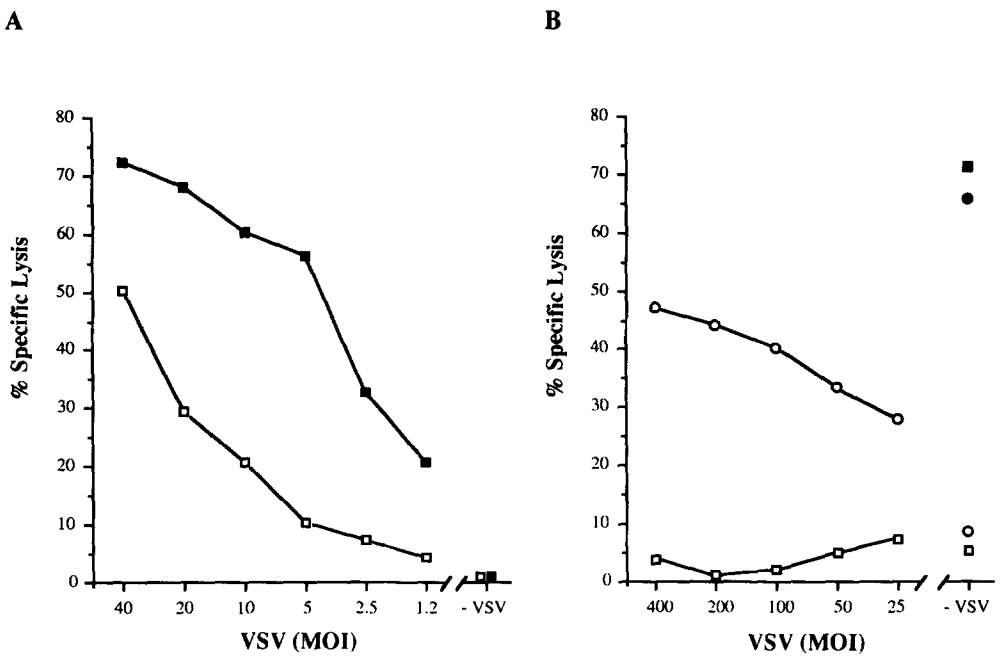

Figure 1. A higher MOI of VSV is required for sensitization of RMA-S for CTL recognition than for RMA. (*A*) RMA (■) and RMA-S (□) or (*B*) T2K^b (□) and Jurkat/K^b (○) cells were infected with VSV at the indicated MOI or left uninfected. In addition, 10 nM N_{53-63} peptide was added to wells containing uninfected T2K^b (■) and Jurkat/K^b (●) cells during the assay. Points represent percent specific lysis of these cells by the VSV-N-specific CTL line at an E/T of 3:1.

density cultures of these cells did not sensitize RMA or RMA-S cells for lysis by CTL (data not shown). Recognition of RMA-S/N.4 by CTL could also not be explained by an increase in cell surface class I MHC expression since its expression of H-2K^b and D^b molecules was only slightly higher than that of RMA-S cells when analyzed by FACS® (Table 3).

RMA-S/N.4 Remains Unable to Present Endogenous Antigens to CTL. To rule out the possibility that the RMA-S/N.4 transfectant was somehow altered relative to RMA-S in its ability to present endogenous antigens, we examined the ability of RMA-S/N.4 to present endogenous influenza NP and OVA to specific CTL. Upon infection with the recombinant influenza virus X31, both RMA and RMA/N.4 target cells were recognized and lysed by the NP-specific CTL (Table 4). In contrast, X31-infected RMA-S and RMA-S/N.4 target cells were both unable to present endogenous influenza NP to these CTL. All target cells were sensitized for lysis by these CTL in the presence of exogenous antigenic $NP_{365-380}$ peptide.

Again, similar results were obtained with the H-2K^b-restricted antigen OVA. OVA-loaded RMA and RMA/N.4, but not RMA-S and RMA-S/N.4, target cells were recognized and lysed by the OVA-specific CTL (Table 4). Endogenous expression of the H-2K^b-restricted antigen VSV-N does not interfere with presentation of OVA since OVA-loaded RMA/N.4 target cells are recognized by the OVA-specific CTL. All cells were sensitized for lysis by these CTL in the presence of exogenous antigenic $OVA_{242-276}$ peptide. These

Table 2. *VSV-N-specific CTL Recognize RMA-S Transfected with the VSV-N Gene*

APC	Peptide	Percent specific lysis by anti-VSV CTL at E/T of: 10:1	1:1
RMA	-	0	0
RMA	N_{53-63}	72	67
RMA/N.4	-	70	59
RMA/N.4	N_{53-63}	59	57
RMA-S	-	4	1
RMA-S	N_{53-63}	82	66
RMA-S/N.4	-	51	36
RMA-S/N.4	N_{53-63}	63	57

Table 3. *FACS® Analysis of Class I MHC Expression by VSV-N Transfectants*

APC	Antibody used to stain cells: None	NFA*	Y3 (H-2K^b)	28.14.8 (H-2D^b)
RMA	5.0‡	8.0	208.0	145.0
RMA/N.4	5.0	8.0	180.0	110.0
RMA-S	5.0	8.0	17.0	10.0
RMA-S/N.4	6.0	8.0	21.0	12.0

* No first antibody. The secondary antibody was FITC-labeled antibody against mouse immunoglobulin Fc.
‡ Numbers represent the mean relative fluorescence of 10,000 gated events using a FACScan® flow cytometer.

Table 4. *RMA-S/N.4 Is Unable to Present Endogenous Influenza NP and OVA to Specific CTL*

APC	Antigen	Peptide	Percent specific lysis by CTL at E/T of:	
		Anti-PR8 CTL line:	10:1	1:1
RMA	–	–	5	1
RMA	–	$NP_{365-380}$	59	35
RMA	X31	–	65	50
RMA/N.4	–	–	0	0
RMA/N.4	–	$NP_{365-380}$	63	45
RMA/N.4	X31	–	61	44
RMA-S	–	–	2	0
RMA-S	–	$NP_{365-380}$	66	40
RMA-S	X31	–	7	5
RMA-S/N.4	–	–	0	0
RMA-S/N.4	–	$NP_{365-380}$	39	21
RMA-S/N.4	X31	–	8	5
		Anti-OVA B3 CTL clone:	10:1	1:1
RMA	–	–	1	3
RMA	–	$OVA_{242-276}$	55	22
RMA	OVA	–	37	21
RMA/N.4	–	–	2	0
RMA/N.4	–	$OVA_{242-276}$	55	24
RMA/N.4	OVA	–	53	26
RMA-S	–	–	5	2
RMA-S	–	$OVA_{242-276}$	78	46
RMA-S	OVA	–	7	5
RMA-S/N.4	–	–	5	0
RMA-S/N.4	–	$OVA_{242-276}$	43	10
RMA-S/N.4	OVA	–	0	0

results demonstrated that the RMA-S/N.4 transfectant remained defective in the ability to present endogenous antigens to specific CTL and had therefore not reverted to the antigen presentation phenotype of RMA.

HPLC Analysis of Natural VSV-N Peptides Produced by RMA and RMA-S. It was theoretically possible that antigenic VSV-N peptides were being produced in the cytosol of RMA-S but delivered to H-2K^b molecules in some nonphysiological manner. If this were the case, it was possible that the natural antigenic VSV-N peptides produced by RMA and RMA-S might differ, and that this difference might be detected by HPLC analysis. TFA extracts of cell lysates were made from both RMA and RMA-S cells either untransfected, transfected with the VSV-N gene, or infected with VSV. These extracts were chromatographed on a C18 HPLC column and fractions tested for the presence of antigenic VSV-N peptides by their ability to sensitize RMA-S target cells cultured at 31°C overnight (RMA-S [31°C]) for recognition by VSV-N-specific CTL. Neither extracts from RMA nor RMA-S cells sensitized the RMA-S (31°C) targets for lysis by the VSV-N-specific CTL (Fig. 2). Extracts from both RMA and RMA-S cells either transfected with the VSV-N gene or infected with VSV all sensitized RMA-S (31°C) targets for lysis by these CTL. The sensitizing activity from each of these extracts eluted from the HPLC column primarily in fraction 30, thus, the natural antigenic VSV-N peptides contained in these extracts are essentially indistinguishable by HPLC. The synthetic peptide N_{52-59}, which corresponds to the natural antigenic VSV-N

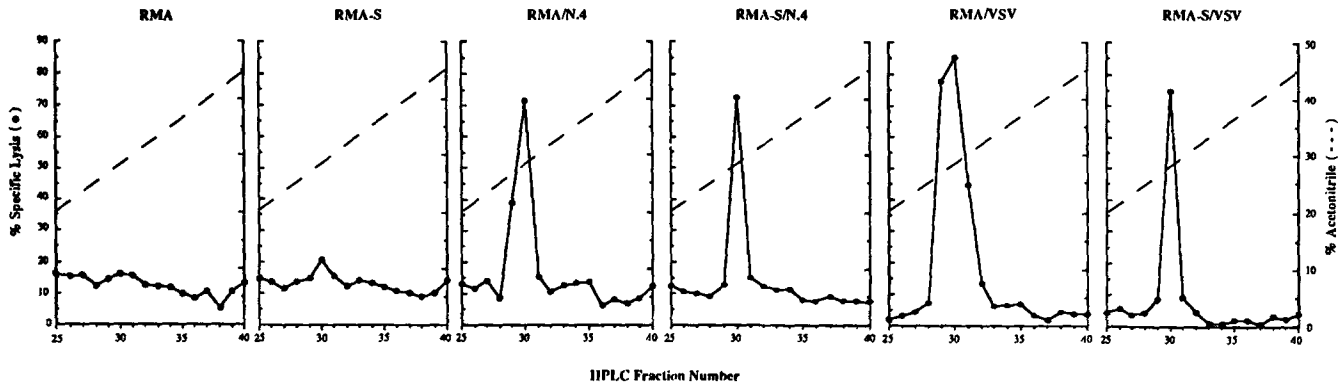

Figure 2. HPLC analysis of the natural VSV-N peptides in RMA and RMA-S extracts. TFA extracts were prepared from cells either untreated (RMA, RMA-S), transfected with the VSV-N gene (RMA/N.4, RMA-S/N.4), or infected with VSV at an MOI of 40 (RMA/VSV, RMA-S/VSV). These extracts were chromatographed by reverse-phase HPLC and the collected fractions tested for their ability to sensitize RMA-S cells grown at 31°C for lysis by the VSV-N specific CTL line at an E/T ratio of 3:1.

peptide produced by VSV infected cells (27), also eluted from this HPLC column in fraction 30 under the conditions used in this experiment (data not shown).

Mapping the Defect in RMA-S by Cell Fusion. We next asked if the RMA-S phenotype could be attributed to a defect on chromosome 17, which encodes the murine MHC. We first fused RMA-S (H-2^b, B_2M^b, Thy-1.2) to the nonmutant cell line BW Lyt-2.4 (H-2^k, $B_2M^{non-a,b}$, Thy-1.1) and isolated individual fusion clones for analysis. One of these clones, RxB.6, was subsequently subjected to selection by an alloreactive anti-H-2^k CTL line for loss of the BW Lyt-2.4–derived chromosome 17. The resulting cell populations Sel 3, Sel 4, and Sel 5 were each products of a single round of CTL selection in individual cultures. The RxB.6 fusion clone and the selected cell populations were then analyzed for cell surface class I MHC expression phenotype, for the ability to present endogenous influenza NP to specific CTL, and for presence of the BW Lyt-2.4–derived chromosome 17. Consistent with the results of a previously reported fusion of RMA-S with a nonmutant fibroblast cell line (14), fusion of RMA-S with BW Lyt-2.4 resulted in elevated expression of H-2^b class I MHC molecules and β_2m^b at the cell surface relative to the levels expressed by RMA-S (Table 5). This observation is interpreted to mean that the low level of class I MHC cell surface expression by RMA-S is not caused by defects in either the class I heavy chains or β_2m. The RxB.6 clone also ex-

Table 5. *Sel 3, 4, and 5 Display the RMA-S Class I MHC Surface Expression Phenotype*

		Antibody used to stain cells								
Exp.	APC	None	NFA*	AF6-88.5.3 (H-2K^b)	B22.049 (H-2D^b)	AF3-12.1.3 (H-2K^k)	15.5.5S (H-2D^k)	S19.8 (B2M^b)	19E12 (Thy-1.1)	FITC-30H12 (Thy-1.2)
1	RMA	3‡	15	309	175	22	19	298	19	104
	RMA-S	3	20	45	28	24	23	44	21	126
	BW Lyt-2.4	2	19	19	22	232	65	25	535	9
	RxB.6	3	13	171	90	141	64	201	1,583	90
	Sel 3	3	20	38	21	18	19	16	989	57
2	RMA	3	5	154	91	6	6	111	5	72
	RMA-S	3	5	13	7	6	5	8	6	72
	BW Lyt-2.4	2	5	6	6	136	34	6	361	4
	RxB.6	3	5	48	38	77	22	50	617	51
	Sel 4	3	5	10	7	5	5	6	500	41
	Sel 5	3	4	13	5	5	5	6	369	34

* No first antibody. The secondary antibody was FITC-labeled antibody against mouse immunoglobulin Fc.
‡ Numbers represent the mean relative fluorescence of 10,000 gated events using a FACScan® flow cytometer.

Table 6. *Sel 3, 4, and 5 are Defective for Presentation of Endogenous Influenza NP to Specific CTL*

APC	Antigen	Peptide	Percent specific lysis by anti-PR8 CTL at E/T of: 10:1	1:1
RMA	-	-	0	0
RMA	-	$NP_{365-380}$	72	20
RMA	X31	-	58	23
RMA-S	-	-	2	3
RMA-S	-	$NP_{365-380}$	43	13
RMA-S	X31	-	1	0
BW Lyt 2.4	-	-	22	3
BW Lyt 2.4	-	$NP_{365-380}$	30	4
BW Lyt 2.4	X31	-	28	9
RxB.6	-	-	2	2
RxB.6	-	$NP_{365-380}$	59	23
RxB.6	X31	-	45	16
Sel 3	-	-	18	0
Sel 3	-	$NP_{365-380}$	55	28
Sel 3	X31	-	21	4
Sel 4	-	-	10	0
Sel 4	-	$NP_{365-380}$	48	12
Sel 4	X31	-	14	1
Sel 5	-	-	9	1
Sel 5	-	$NP_{365-380}$	65	19
Sel 5	X31	-	15	3

pressed the BW Lyt-2.4–derived H-2^k class I MHC and Thy-1.1 molecules at its cell surface, indicating that it was the product of a fusion event between RMA-S and BW Lyt-2.4. Also consistent with previous fusion experiments performed with RMA-S (14) was the observation that RxB.6 was capable of presenting endogenous influenza NP, as well as exogenous antigenic $NP_{365-380}$ peptide, to NP-specific CTL (Table 6).

The CTL-selected cell populations Sel 3, 4, and 5 phenotypically resembled RMA-S in being homogeneous for low cell surface levels of H-2^b class I MHC molecules and defective in the ability to present endogenous influenza NP to NP-specific CTL (Tables 5 and 6). Sel 3, 4, and 5 were sensitized for specific CTL lysis in the presence of exogenous antigenic $NP_{365-380}$ peptide, indicating that they had not been selected for resistance to lysis by CTL. However, unlike RMA-S, they were positive for cell surface expression of BW Lyt-2.4–derived Thy-1.1 molecules (Table 5), indicating that they were derived from the RxB.6 fusion clone and not from residual RMA-S cells in the cultures. Sel 3, 4, and 5 appeared to have lost the BW Lyt-2.4 chromosome 17 since they were negative for H-2^k expression by FACS® analysis (Table 5). Loss of the BW Lyt-2.4–derived chromosome 17 by these populations was confirmed by Southern blot analysis using a probe spanning the genomic H-$2K^b$ promoter. This probe hybridized to two polymorphic bands in DNA from each of the control H-2^k haplotype cells tested, one of which is distinctly different from the four polymorphic bands detected in DNA from control H-2^b haplotype cells (Fig. 3). RxB.6 DNA contains both the H-2^b- and H-2^k-derived bands, indicating that it contains both the RMA-S and BW Lyt-2.4–derived chromosomes 17. DNA from each of the selected cell populations contains only the H-2^b-derived polymorphic bands, confirming that each of these populations have lost the BW Lyt-2.4–derived chromosome 17. Thus in three of three independent cases, we observed that loss of the BW Lyt-2.4–derived chromosome 17 by RxB.6 was associated with reversion to the RMA-S phenotype. These data imply that the defect responsible for the RMA-S phenotype is likely to reside on chromosome 17.

Discussion

The murine RMA-S and human LCL 721.174 mutant cell lines display similar defective phenotypes. Both of these cell lines retain the majority of their class I MHC molecules in the ER and both are defective in the ability to present endogenous class I MHC-restricted antigens to CTL (5, 9, 12, 13). The LCL 721.174 phenotype has been attributed to the deletion of genes encoded within the class II region of the MHC on human chromosome 6 (9, 10, 15). Included within this deletion is Y3, a putative peptide transporter gene, which has been shown to restore high levels of cell surface class I MHC expression by a second human mutant cell line, LCL 721.134, lacking Y3 expression (18). The Y3 gene does not restore high levels of cell surface class I expression by LCL 721.174, indicating that expression of other genes encoded within the deleted region of the MHC in these cells may also be necessary to correct the LCL 721.174 defect (18).

The RMA-S cell line has been observed to express mRNA for, and thus has not deleted, its MHC-encoded putative peptide transporter genes HAM-1 and -2 (20). Our data demonstrate that the RMA-S phenotype can be attributed to a defect that maps to the murine chromosome 17, which encodes the MHC. Fusion of RMA-S with BW Lyt-2.4 resulted in restoration of the wild-type RMA phenotype. This was also true when RMA-S was fused with a nonmutant fibroblast cell line (14). Thus, the RMA-S phenotype, like that of LCL 721.174, is recessive rather than dominant in cell fusion experiments. Loss of the BW-derived chromosome 17 resulted in reversion of the RxB.6 hybrid to the RMA-S phenotype in three of three independent selected cell populations. Taken together with the observation that LCL 721.174 and RMA-S display similar phenotypes, our data, which map the RMA-S defect to chromosome 17, suggest that the defect in RMA-S is likely to reside within the MHC.

The defect in RMA-S and LCL 721.174 results in the ina-

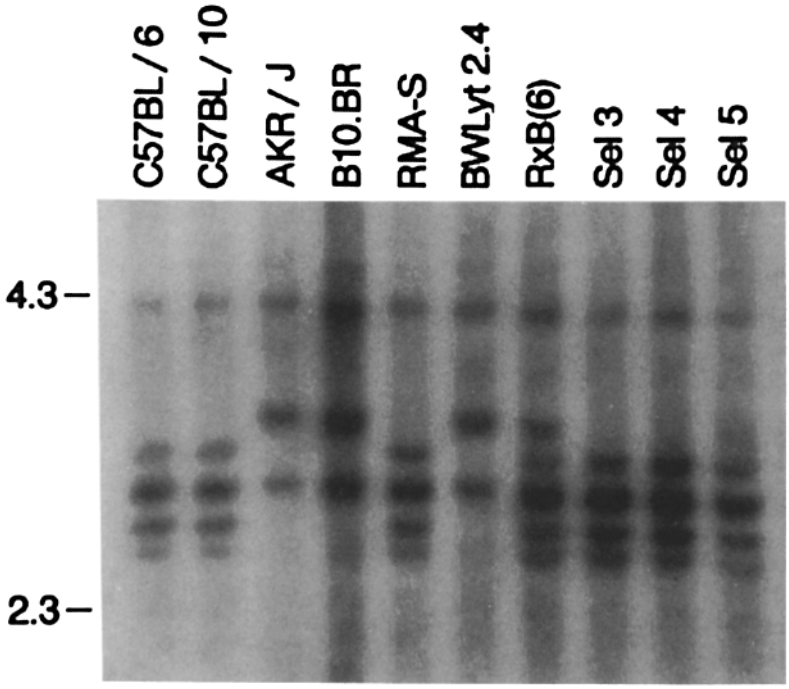

Figure 3. The CTL-selected populations Sel 3, 4, and 5 have lost the BW Lyt-2.4-derived chromosome 17. Genomic DNA from the indicated spleen or tumor cells was subjected to Southern blot analysis. Each lane contains 2 μg of HindIII-digested genomic DNA. The blot was probed with a purified 3-kb EcoRI-HindIII fragment spanning the genomic H-2K^b promoter.

bility of these cell lines to present several endogenous class I–restricted antigens (5, 9, 12, 13). However, we have found that they differ significantly in their ability to present at least one endogenous antigen, VSV-N. We have previously reported that LCL 721.174 cells expressing H-2K^b (T2K^b) were unable to present endogenous VSV-N to VSV-N-specific CTL (13). Even a very high MOI with VSV does not lead to the appearance of the H-2K^b-restricted CTL determinant on T2K^b cells (Fig. 1 *B*). The inability of T2K^b cells to present VSV-N was not due to a failure to infect these cells with VSV since VSV-N expression by VSV-infected T2K^b cells can be detected by SDS-PAGE (data not shown). We have shown here that RMA-S was able to present endogenous VSV-N to specific CTL. This was true whether RMA-S was infected with VSV or transfected with the VSV-N gene, indicating that presentation of VSV-N was not due to nonspecific effects of viral infection. CTL recognition of RMA-S cells expressing VSV-N was generally less efficient than recognition of RMA cells expressing VSV-N, although specific lysis of RMA-S cells was always >50–60% that of RMA cells. This was not due to differences in expression of VSV-N since both infected and transfected RMA-S cells expressed equal or greater amounts of VSV-N compared with similarly treated RMA cells, as determined by SDS-PAGE (data not shown).

There are several possible explanations for the ability of RMA-S, but not T2K^b, to present endogenous VSV-N to specific CTL. First, it is possible that the endogenous VSV-N peptide is delivered to H-2K^b molecules in RMA-S in some nonphysiological manner. It is possible that the VSV-N protein has access to and is degraded in the ER in RMA-S, but this is unlikely given that VSV-N lacks a signal sequence and has been observed to be localized in the cytosol of mammalian cells either infected with VSV or transfected with the VSV-N gene (28, 29). It is also possible that other nonphysiological transport mechanisms, such as the signal sequence–mediated transport mechanism, could deliver the VSV-N peptide to H-2K^b molecules in RMA-S. We feel that this explanation is unlikely in that these mechanisms would have to operate only in RMA-S and not in T2K^b cells. Furthermore, if the VSV-N peptide were being loaded onto H-2K^b molecules by some nonphysiological pathway in RMA-S, one might expect to find that the peptide might differ from the natural octamer expressed by wild-type cells. In fact, we found that the peptides in extracts from RMA and RMA-S cells expressing VSV-N were indistinguishable by HPLC.

We favor a second possible explanation: that the system that transports antigenic peptides from the cytosol to the ER in wild-type cells may still be present in RMA-S and retain partial function. This explanation would be consistent with the idea that the RMA-S phenotype is due to a point mutation rather than a genomic deletion like that observed in LCL 721.174 (30). It would also be consistent with the observation that HAM-1 and -2 mRNA expression can be detected in RMA-S (20). A partially defective system, if present in RMA-S, might inefficiently transport all cytosolic antigenic peptides produced. If this were the case, then lack of RMA-S recognition by CTL might be explained by differences in antigen density requirements for recognition and lysis of these targets by different antigen-specific CTL (12). Alternatively, this crippled system might transport only a subset of the cytosolic antigenic peptides produced by RMA-S. In either case, this explanation would predict that the class I molecules expressed stably by RMA-S at the cell surface are not empty, but are occupied by a subset of self peptides.

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