Brief Definitive Report

Endogenously Synthesized Peptide with an Endoplasmic Reticulum Signal Sequence Sensitizes Antigen Processing Mutant Cells to Class I-restricted Cell-mediated Lysis

By K. Anderson,* P. Cresswell,* M. Gammon,‡ J. Hermes,‡
A. Williamson,‡ and H. Zweerink‡

From the *Department of Microbiology and Immunology, Duke University Medical Center,
Durham, North Carolina 27710; and the ‡Department of Autoimmune Disease Research,
Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065*

Summary

The HLA-A2-positive human mutant cell line T2 is not lysed by influenza virus–specific HLA-A2-restricted cytotoxic lymphocytes after virus infection. However, lysis does occur when cells are incubated with the antigenic influenza matrix protein-derived peptide M57-68. To examine the nature of this defect, T2 cells were transfected with two different plasmids. One plasmid encoded the peptide M57-68, and the other encoded the same peptide preceded by an endoplasmic reticulum translocation signal sequence. Mutant T2 cells expressing the M57-68 peptide without the signal sequence were not susceptible to lysis by M57-68-specific HLA-A2-restricted cytotoxic T lymphocytes, whereas T2 cells expressing the M57-68 peptide plus signal sequence were lysed effectively. Lysis of parental T1 cells with either plasmid was equally effective. These results suggest that the T2 mutant cells are defective in the transport of antigenic peptides from the cytosol into the secretory pathway.

Three mutant cell lines (RMA-S, .174, and T2) have been described that are not lysed by class I–restricted CTL after virus infection but are lysed after exposure to synthetic peptides corresponding to antigenic determinants of the viral proteins (1–3). Furthermore, these mutants synthesize class I heavy chains, but the majority of detectable heavy chain remains intracellular and associates poorly with β_2-microglobulin (1, 4, 5).

The mouse cell line RMA-S was derived by EMS mutagenesis and selection for loss of H-2 expression (6). The human cell line .174 with a homozygous deletion of the class II region of the MHC fails to express HLA-B5 and expresses HLA-A2 at reduced levels (20–30%; reference 7). A hybrid cell line, T1, produced by fusing .174 cells to T lymphoblastoid cells (CEM), phenotypically resembles wild-type cells: high levels of HLA-A2 and -B5 are expressed and whole antigens as well as peptide are processed and presented in the context of HLA-A2 (1, 5). A derivative cell line (T2) without the two intact copies of chromosome 6 derived from CEM cells is similar to .174 with low or absent expression of HLA-A2 and -B5, and it fails to process and present antigen after virus infection (1, 5, 8). These observations suggest that a *trans*-acting factor encoded by a gene in the MHC controls the mutant phenotype.

The defect in the mutant cell lines may involve antigen processing, transport of antigen or antigen-derived peptides from the cytoplasm to a class I containing compartment, or the intracellular association of peptides with class I molecules and their subsequent transport to the cell surface. A defective antigen or peptide transport mechanism was originally proposed by Townsend and coworkers (1, 2) to explain the functional deficiencies of the mutant cell lines and the ability to correct them by supplying exogenous peptides. The recent finding that genes with sequences related to ATP-dependent transport proteins are present in the region of the MHC deleted in .174 and T2 (9–12) is consistent with this mechanism. To investigate this possibility experimentally, expression vectors encoding the influenza matrix peptide M57-68 with or without an endoplasmic reticulum translocation signal sequence were transfected into T2 cells, and presentation of that peptide to human CTL in the context of HLA-A2 was examined.

Materials and Methods

Cell Lines. The derivation of the cell lines C1R, T1, and T2 are described elsewhere (13). C1R:A2 is C1R that expresses a transfected genomic clone of HLA-A2.1 (14). C1R, C1R:A2, T1, and

T2 cells were maintained in IMDM (Gibco Laboratories, Grand Island, NY) or RPMI 1640 with 10% FCS (IMDM/FCS, RPMI/FCS) and 20 μg/ml gentamicin.

CTL lines specific for the influenza virus type A matrix peptide 57–68 (M57-68) were generated essentially as described (14), using 2 U/ml rIL-2 (Amgen Biologicals, Thousand Oaks, CA) and peptide-pulsed PBMC as stimulators.

Construction of Episomal Vectors. The expression plasmids p8901-M1 and p8901-SM1 were constructed by ligation of oligonucleotide duplexes, coding for the appropriate amino acid residues, into the unique BamHI site of p8901 (kindly provided by Dr. G.E. Mark, Merck Sharp and Dohme Research Laboratories). Plasmid p8901-M1 encodes a peptide of the influenza virus matrix protein (residues 57–68; M57-68), and plasmid p8901-SM1 encodes peptide M57-68 preceded by the adenovirus E3/19-kD protein signal sequence (15), as shown in Fig. 1.

Transfection of Episomal Minigene Expression Vectors. C1R and C1R:A2 cells were electroporated at 230 mV, 960 μFarads, and T1 and T2 cells at 210 mV, 500 μFarads, with 20 μg DNA as described (13). 24 h after electroporation, cells were resuspended in 24 ml selective (hygromycin-containing) medium (Boehringer Mannheim Biochemicals, Indianapolis, IN; 300 μg/ml for C1R, 450 μg/ml for T1 and T2 cells) and plated at 1 ml/well in 24-well plate. Three plasmids, referred to as p8901 (control plasmid without insert), p8901-M1 (with peptide M57-68 minigene), and p8901-SM1 (M57-68 minigene plus signal sequence), were transfected into C1R, C1R:A2, T1, and T2 cells. Transfected cells will be referred to as C1R(p8901), C1R(M1), C1R(SM1), etc.

Results

Intracellular Expression of a Peptide with a Leader Sequence Renders T2 Cells Sensitive to Lysis by HLA-A2-restricted CTL. Cytotoxic assays were performed with transfected C1R, T1, and T2 cells using HLA-A2-restricted CTL specific for matrix peptide M57-68 (Fig. 2). Cells with control plasmid p8901 (Fig. 2, A–C) were not susceptible to lysis and HLA-A2-negative C1R(M1) and C1R(SM1) cells were not lysed either (Fig. 2 A). Both T1(M1) and T1(SM1) cells were lysed with equal efficiency (Fig. 2 B); similar results (data not shown) were obtained with wild-type C1R:A2(M1) and C1R:A2 (SM1) cells. Mutant T2(M1) cells were lysed poorly in contrast to T2(SM1) cells, which were very susceptible to lysis (Fig. 2 C).

In this particular experiment, lysis of T2(p8901), T2(M1) and T2(SM1) was 3, 11, and 62%, respectively, at an E/T ratio of 5. Six additional experiments were carried out with these cells using CTL from two donors. Average lysis (E/T = 5) was 3 (T2[p8901]), 14 (T2[M1]) and 64% (T2[SM1]); lysis of T1 cells in four experiments was 14 (T1[p8901]), 53 (T1[M1]), and 42% (T1[SM1]). Additional control experiments established that all HLA-A2-positive cells that were not lysed in the experiments described above (i.e., T1[p8901], T2[p8901], and T2[M1]) were susceptible to lysis when assayed in the presence of 1 μM synthetic peptide M57-68 (45–75% at E/T = 5).

Extracellular Association of Plasmid-encoded Peptide with HLA-A2 Was Not Detectable. The most likely explanation for these results is that peptide M57-68, after synthesis in the cytoplasm, is 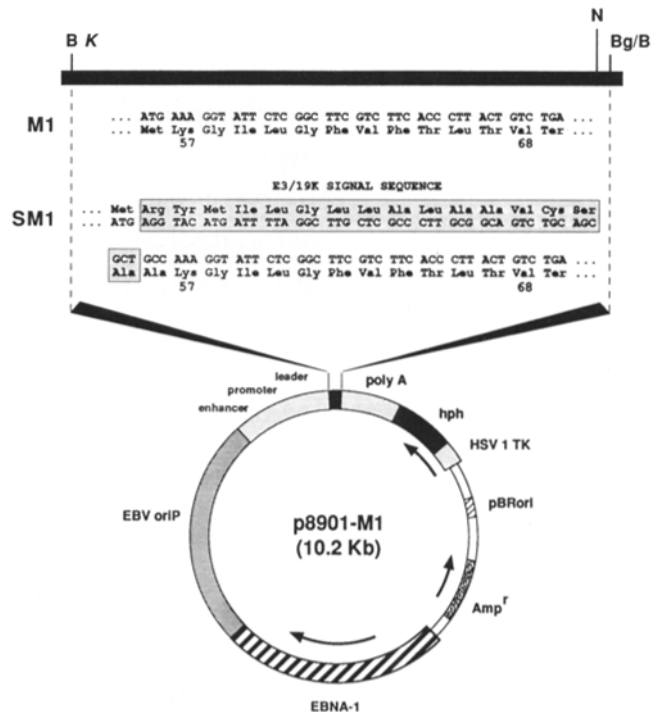translocated into the endoplasmic reticulum in T1 and wild-type C1R:A2 cells where it associates with HLA-A2 molecules. After transport to the cell surface, these complexes are recognized by CTL (16–18). In the mutant T2 cells, peptide M57-68 is translocated very inefficiently but the defect is reversed when the peptide also contains a signal sequence. A potential alternative explanation for the results is that p8901-SM1-derived peptides are translocated into the endoplasmic reticulum, secreted into the medium, and subsequently associate with HLA-A2 molecules at the cell surface.

Table 1 shows that such extracellular sensitization cannot be detected. T2(p8901) cells were cocultivated with C1R(SM1) cells such that both cell types were present after 7 d in equal amounts as judged by FACS® analysis with the HLA-A2-specific mAb MA2.1. Peptide M57-68, if secreted in significant amounts by HLA-A2-negative C1R(SM1) cells, should have sensitized bystander HLA-A2-positive T2(p8901) cells. However, lysis was not observed. Additional experiments (data not shown) established that the addition of supernatants of C1R(SM1), T1(SM1), and T2(SM1) cells to T1(p8901) and T2(p8901) cells did not render the latter susceptible to lysis.
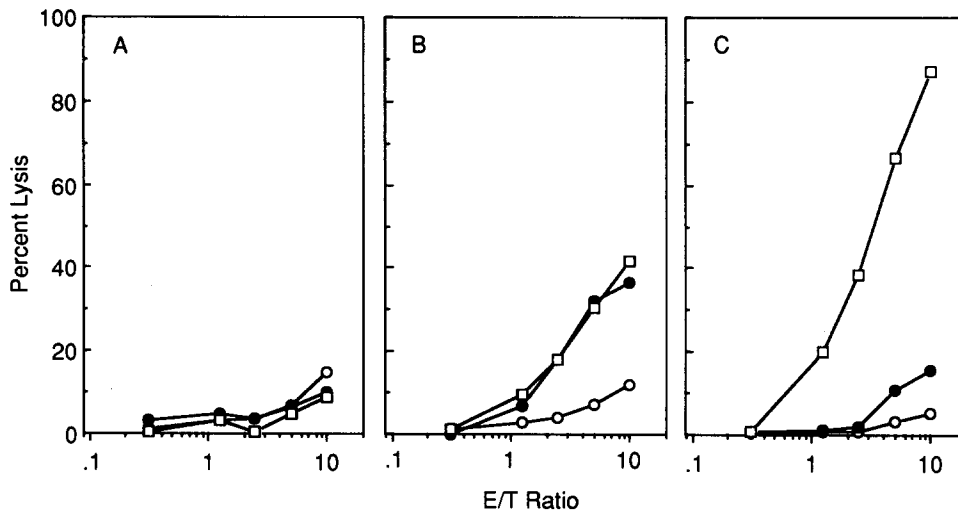

Figure 1. Episomal expression vectors. To construct the inserts for p8901-M1, the following oligonucleotides were annealed, extended with Klenow and dNTPs, and amplified by PCR: 5′-ATAGGATCCCCACCATGAAA-GGTATTCTCGGCTTCGTCTCC-3′ and 3′-GAGCCGAAGCAGAAG-TGGGAATGACAGACTCCATGGCGATCGCGC CGGCGTCTAGATAT-5′. The oligos for p8901-SM1 were 5′-ATAGGATCCCCACCATGAGG-TACATGATTTTAGGCTTGCTCGCCCTTGCGGCAGTCTGCAGCG-CTGCCAAAGGTATTCTCGGCTTCGTCTTC-3′ and 3′-GAGCCGAA-GCAGAAGTGGGAATGACAGACTCGCCGGCGTAT-5′. Note that the oligos will construct a fragment containing BamHI (B), NotI (N), and BglII (Bg) restriction sites, and a Kozak consensus sequence (K).

Figure 2. Lysis of minigene transfectants by HLA-A2-restricted M57-68-specific CTL. Target cells were labeled overnight with ^{51}Cr, and effectors were added at varying ratios in standard 4-h cytolytic assays (14). (*A*) C1R transfectants; (*B*) T1 transfectants; (*C*) T2 transfectants. (O) Cells with plasmid p8901; (●) with p8901-M1; (□) with p8901-SM1.

Table 1. *Assay for Peptide Secretion by CTL Lysis at a Mixed Cell Population*

Cell type	Percent lysis	
	No peptide	Plus M57-68*
T2(p8901) plusC1R(SM1)	4	46
T2(p8901)	4	54
C1R(SM1)	3	6
C1R:A2(p8901)	5	64
T2(SM1)	42	59

HLA-A2-positiveT2(p8901) cells were mixed with HLA-A2-negative C1R(SM1) cells at a ratio of 70–30%. After 1 wk of cocultivation, both cell types were present at equal amounts as judged by FACS® analysis using mAb MA2.1. This mixed culture as well as control cells were labeled with ^{51}Cr, and susceptibility to lysis by M57-68-specific CTL was determined at an E/T ratio of 5 in the presence and absence of synthetic peptide M57-68.
* At 1 μM.

Discussion

Their mutant antigen processing phenotype raises the possibility that T2 cells are deficient in peptide transport from the cytoplasm into the endoplasmic reticulum (1, 2). Consequently, complexes of class I molecules and peptides are not formed and expressed on the cell surface. If this were the case, then one should be able to reverse the mutant phenotype by using an alternative peptide transport pathway.

The results described in this paper showed that this reversal can be achieved. Both wild-type T1 and mutant T2 cells were transfected with expression plasmids containing minigenes

coding for the influenza virus matrix protein-derived peptide M57-68 with or without an NH$_2$-terminal signal sequence. Peptides with the signal sequence should cross the endoplasmic reticulum membrane by the normal signal recognition particle (SRP)-dependent translocation pathway (16). We observed that the addition of a leader sequence to the minigene-encoded matrix peptide dramatically improved recognition of T2 cells by HLA-A2-restricted M57-68-specific CTL. In contrast, T1 and wild-type C1R:A2 cells presented both minigene products equally well. These results are consistent with the hypothesis that T2 lacks the ability to translocate peptides across a membrane by an SRP-independent mechanism, the physiological mechanism used by wild-type cells. Our results add weight to the proposal that the putative ATP-dependent transport proteins that map to the MHC could provide the physiological step missing in T2 cells (9–12).

RMA-S, .174, and T2 were initially identified because of the shared phenotype of low surface expression of class I molecules. This low expression is believed to reflect either impaired transport (2) or reduced stability (17) of class I heavy chain β$_2$-microglobulin dimers that lack associated peptides. The addition of synthetic peptides to these cells increased class I cell surface expression (1, 2). Therefore, we examined the transport and surface expression of HLA-A2 heavy chain β$_2$-microglobulin dimers in the minigene transfectants by ^{35}S methionine pulse-chase experiments and FACS® analysis (see references 1 and 2). No differences were observed in T2 cells expressing either construct. One should note, however, that since T cell–mediated lysis requires few peptides bound to class I molecules at the cell surface (18, 19), peptide transport into the endoplasmic reticulum and the expression of HLA-A2-peptide complexes could be sufficient for detection by CTL but insufficient for detection by biochemical means.

References

1. Cerundolo, V., J. Alexander, K. Anderson, C. Lamb, P. Cresswell, A. McMichael, F. Gotch, and A. Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature (Lond.).* 345:449.
2. Townsend, A., C. Ohlen, J. Bastin, H.-G. Ljunggren, L. Foster, and K. Karre. 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature (Lond.).* 340:443.
3. Hosken, N.A., and M.J. Bevan. 1990. Defective presentation of endogenous antigen by a cell line expressing class I molecules. *Science (Wash. DC).* 248:367.
4. Ljunggren, H.-G., S. Paabo, M. Cochet, G. Kling, P. Kourilsky, and K. Karre. 1989. Molecular analysis of H-2-deficient lymphoma lines. Distinct defects in biosynthesis and association of MHC class I heavy chains and β_2-microglobulin observed in cells with increased sensitivity to NK cell lysis. *J. Immunol.* 142:2911.
5. Salter, R.D., and P. Cresswell. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:943.
6. Ljunggren, H.-G., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. *J. Exp. Med.* 162:1745.
7. DeMars, R., R. Rudersdorf, C. Chang, J. Petersen, J. Strandtmann, N. Korn, B. Sidwell, and H. Orr. 1985. Mutations that impair a posttranscriptional step in expression of HLA-A and -B antigens. *Proc. Natl. Acad. Sci. USA.* 82:8183.
8. Salter, R.D., D.N. Howell, and P. Cresswell. 1985. Genes regulating HLA class I antigen expression in T -B lymphoblast hybrids. *Immunogenetics.* 21:235.
9. Deverson, E.V., I.R. Gow, W.J. Coadwell, J.J. Monaco, G.W. Butcher, and J.C. Howard. 1990. MHC class II region encoding proteins related to the multidrug resistance family of transmembrane transporters. *Nature (Lond.).* 348:738.
10. Trowsdale, J., I. Hanson, I. Mockridge, S. Beck, A. Townsend, and A. Kelly. 1990. Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters. *Nature (Lond.).* 348:741.
11. Spies, T., M. Bresnahan, S. Bahram, D. Arnold, G. Blanck, E. Mellins, D. Pious, and R. DeMars. 1990. A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. *Nature (Lond.).* 348:744.
12. Monaco, J.J., S. Cho, and M. Attaya. 1990. Transport protein genes in the murine MHC: possible implications for antigen processing. *Science (Wash. DC).* 250:1723.
13. Alexander, J., J.A. Payne, B. Shikegawa, J. Frelinger, and P. Cresswell. 1990. The transport of class I major histocompatibility complex antigens is determined by sequences in the α_1 and α_2 protein domains. *Immunogenetics.* 31:169.
14. Hogan, K., N. Shimojo, S. Walk, V. Engelhard, W. Maloy, J. Coligan, and W. Biddison. 1988. Mutations in the $\alpha2$ helix of HLA-A2 affect presentation but do not inhibit binding of influenza virus matrix peptide. *J. Exp. Med.* 168:725.
15. Persson, H., H. Jornvall, and J. Zabielski. 1980. Multiple mRNA species for the precursor to an adenovirus-encoded glycoprotein: identification and structure of the signal sequence. *Proc. Natl. Acad. Sci. USA.* 77:6349.
16. Walter, P., and V.R. Lingappa. 1986. Mechanism of protein translocation across the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 2:499.
17. Ljunggren, H.-G., N.J. Stam, C. Ohlen, J.J. Neefjes, P. Hoglund, M.-T. Heemels, J. Bastin, T.N.M. Schumacher, A. Townsend, K. Karre, and H. Ploegh. 1990. Empty MHC class I molecules come out in the cold. *Nature (Lond.).* 346:476.
18. Rotzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.).* 348:252.
19. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature (Lond.).* 348:213.