RMA/S Cells Present Endogenously Synthesized Cytosolic Proteins to Class I–restricted Cytotoxic T Lymphocytes

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Summary

RMA/S is a mutant cell line with decreased cell surface expression of major histocompatibility complex class I molecules that has been reported to be deficient in presenting endogenously synthesized influenza virus nucleoprotein (NP) to cytotoxic T lymphocytes (CTL). In the present study we show that RMA/S cells can present vesicular stomatitis virus nucleocapsid protein, and, under some conditions, NP, to K^b- and D^b-restricted CTL, respectively. Antigen presentation results from processing of cytosolic pools of endogenously synthesized proteins, and not the binding to cell surface class I molecules of antigenic peptides present in the virus inoculum or released from infected cells. Antigen processing of RMA/S differs, however, from processing by wild-type cells in requiring greater amounts of antigen, longer times to assemble or transport class I–peptide complexes, and in being more sensitive to blocking by anti-CD8 antibody. Thus, the antigen processing deficit in RMA/S cells is of a partial rather than absolute nature.

Mutant cells deficient in the presentation of endogenously synthesized antigens have become important tools for studying antigen processing: the process by which cells derive peptides from cytosolic proteins and deliver them across an internal membrane to MHC class I molecules. RMA/S cells were the first mutant cells appreciated to be deficient in antigen processing. Townsend et al. (1) reported that RMA/S cells failed to present influenza virus nucleoprotein (NP)[1] to H-2D^b-restricted, NP-specific CTL. RMA/S cells were originally selected from mutagenized RMA cells on the basis of low cell surface expression of class I molecules (2). The cells synthesize class I α chains and β_2-microglobulin at normal levels, but fail to efficiently assemble these components, resulting in the slow transit of class I molecules to the cell surface (1, 3). The stability of class I molecules can be enhanced in detergent extracts or at the surface of viable RMA/S cells by the addition of antigenic peptides able to bind to class I molecules (1, 4). Based on these findings, it was suggested that RMA/S cells are deficient either in the production of antigenic peptides from proteins, the transport of antigen from the cytosol, or in a factor required for determinants to efficiently associate with class I molecules (1). In the present study we have further characterized the capacity of RMA/S cells to present cytosolic viral antigens to CTL.

[1] *Abbreviations used in this paper:* BFA, brefeldin A; G, glycoprotein; N, nucleocapsid; NP, nucleoprotein; N-Vac, nucleocapsid/vaccinia virus recombinant; Vac, vaccinia virus; VSV, vesicular stomatitis virus.

Materials and Methods

Mice. 6–8-wk-old C57BL/6 (H-2^b), CBA/J (H-2^k), B10.HTG (H-2^g), and B10.D2(R107) (H-2^{r7}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Viruses and Peptides. A/PR/8/34 (H1N1) (PR8) influenza virus was used as infectious allantoic fluid. The Indiana strain of vesicular stomatitis virus (VSV) was grown in BHK-21 cells and used as infectious tissue culture supernatants. The infectivity of VSV stocks was determined by plaquing on BHK-21 cells. The construction and characterization of vaccinia virus (Vac) recombinant containing the Indiana VSV nucleocapsid (N) gene (N-Vac) has been described elsewhere (8). A peptide corresponding to residues 365–380 of the PR8 NP ($NP_{365-380}$) was synthesized by the Biological Resources Branch (NIAID). This peptide is recognized by NP-specific CTL in conjunction with D^b (5). The peptide corresponding to residues 52–59 of the VSV N protein (N_{52-59}) was kindly provided by Dr. Stanley Nathenson (Albert Einstein College of Medicine, Bronx, NY). This peptide is recognized by N-specific CTL in conjunction with K^b (6).

Generation of CTL. CTL from virus-primed animals were generated from splenocytes by in vitro stimulation with virus-infected autologous splenocytes as described (7, 8), or by adding antigenic peptides to the culture medium at a concentration of 1 μg/ml. In some instances, cultures were harvested after a 7-d incubation, washed, and cultured in fresh medium supplemented with 10% (vol/vol) of Con A–stimulated rat spleen cell supernatant for a further 3–5 d. CTL clone 33 specific for the N protein of VSV (9) was kindly provided by Dr. James Sheil (West Virginia University, Morgantown, WV).

Preparation of Target Cells. Before infection with PR8, cells were washed twice with PBS and suspended at 10^7 cells/ml in Autopow

MEM (Gibco Laboratories, Grand Island, NY) supplemented with 20 mM Hepes (pH 7.0) and 0.1% (wt/vol) BSA. 1 ml of PR8 in allantoic fluid (2,000 HAU/ml) was added, and cells incubated for 1 h at 37°C while constantly rotated. 9 ml of IMDM was added and cells incubated for further 1–3 h. Cells were then pelleted and resuspended in 50–100 μl of IMDM containing 100 μCi $Na^{51}CrO_4$ (^{51}Cr) and incubated for 1 h at 37°C. After washing twice with 5 ml ice-cold PBS, cells were resuspended in IMDM and used as target cells for microcytotoxicity assays. For VSV infection, cells were resuspended at 10^7 cells/ml in balanced salt solution containing 0.1% BSA. VSV was added to cells at a multiplicity of 1 PFU/cell unless otherwise stated. Cells were incubated for 1–2 h at 37°C and labeled with ^{51}Cr as described above. Alternatively, target cells were infected with influenza or VSV and ^{51}Cr labeled at the same time for 1.5 h before plating. Target cells were sensitized with antigenic peptides either during ^{51}Cr labeling, or by including peptides in the microcytotoxicity assay. Cells were treated with brefeldin A (BFA) at a concentration of 5 μg/ml during infection or peptide labeling and at 2 μg/ml during the cytotoxicity assay. The protein synthesis inhibitor cycloheximide was used at 30 μg/ml throughout the preparation of target cells and the cytotoxicity assay.

Cytotoxicity Assays. 4-h microcytotoxicity assays were performed as previously described (7, 8). Data are expressed as percent specific release defined as: 100× [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)]. In all experiments presented, the spontaneous release of target cells was <25% of total release values unless otherwise stated.

Results

VSV-infected RMA/S Cells Are Lysed by N-specific, K^b-restricted CTLs. To gauge the extent of the antigen processing deficit of RMA/S cells, we tested their ability to present VSV antigens to VSV-specific CTL. VSV was chosen since preliminary experiments indicated that RMA/S, while highly resistant to infection with vaccinia virus, and somewhat resistant to influenza virus, were highly susceptible to VSV infection. To our surprise, VSV-infected RMA/S cells were lysed at similar levels to VSV-infected RMA cells (Table 1). Recognition was antigen specific since PR8 NP-specific CTL lysed VSV-infected cells at only background levels and vice versa. The identity of RMA/S cells is confirmed by their failure to present NP after infection with PR8 in the same experiment.

Based on the findings of Van Bleek and Nathenson (6), we suspected that CTL recognition of VSV-infected RMA/S cells was predominantly, if not entirely, based on presentation of residues N_{52-59} in association with K^b (6). Indeed, N-specific CTL induced by VSV stimulation of splenocytes from mice primed with N-Vac (8) lysed VSV-infected RMA/S cells at comparable levels to VSV-infected RMA cells (Table 2). Similar results were obtained using splenocytes from B10.D2 (R107) mice (K^bD^d), while splenocytes from B10.HTG mice (K^dD^b) failed to lyse VSV-infected RMA or RMA/S cells, demonstrating that N was presented exclusively in association with K^b (Table 2). Further, CTL induced by stimulating splenocytes from VSV-primed animals with a synthetic peptide corresponding to the N_{52-59} sequence efficiently lysed VSV-infected RMA/S cells (Table 2), and a CTL clone previously shown to recognize N_{52-59} determinant in association with K^b (6) also efficiently recognized RMA/S cells (Table 2).

Table 1. *Presentation of Influenza Virus and VSV Antigens to CTL after Infection of RMA and RMA/S Cells*

				Percent specific ^{51}Cr release			
				Anti-NP		Anti-VSV	
Exp.	Cells	Virus	Time	1	2	3	4
	RMA						
A		PR8	90'	54	36	2	3
		VSV	90'	16	21	66	73
B		PR8	90'	57	51	0	
		PR8	180'	83	81	0	
	RMA/S						
A		PR8	90'	3	3	4	5
		VSV	90'	21	14	70	69
B		PR8	90'	0	0	0	
		PR8	180'	20	16	0	
		VSV	90'	0	0	53	

Cells were infected with PR8 or VSV for 90 or 180 min before testing in a 4-h cytotoxicity assay. Anti-NP and anti-VSV CTL were generated from spleen cells derived from mice infected with influenza virus or VSV and stimulated in vitro with $NP_{365-380}$ and VSV, respectively. Effector cells were incubated with target cells in Exp. A at ratios of 20:1 (1 and 3) or 10:1 (2 and 4), and in Exp. B at 40:1 (1) or 20:1 (2 and 3). Background values obtained using the same populations with uninfected cells have been subtracted from all values. These ranged from 1% to 12%.

RMA/S Cells Present Endogenously Synthesized N Protein. It was essential to demonstrate that recognition of RMA/S cells by N-specific CTL was based on presentation of endogenously synthesized N, and not antigenic peptides either present in the virus inoculum or released by infected cells during the assay. This was demonstrated in three ways.

First, we blocked viral gene expression by adding a protein synthesis inhibitor at the time of infection and maintaining the blockade throughout the preparation of target cells and the ^{51}Cr release assay. This completely blocked presentation of VSV antigens (Table 3). Second, we examined the effect of BFA on the ability of VSV-infected RMA/S cells to present N protein to CTL. BFA is known to prevent presentation of endogenously synthesized antigens, while not affecting presentation of exogenously provided peptides (10, 11). BFA completely inhibited presentation of N by VSV-infected cells (Table 3). As observed previously, this represents a specific effect on the presentation of endogenously synthesized antigens, since BFA did not affect presentation of N_{52-59}. Furthermore, the effect of BFA on presentation of N cannot be attributed to a blockade of virus penetration

Table 2. *VSV N Is Presented by RMA/S Cells in Association with K^b*

		Percent specific ^{51}Cr release									
		C57BL/6 (K^bD^b) N-VAC 1° VSV 2°		C57BL/6 (K^bD^b) N-VAC 1° N_{52-59} 2°		B10.HTG (K^dD^b) N-VAC 1° VSV 2°		B10.D2 (R107) (K^bD^d) N-VAC 1° VSV 2°		CLONE 33	
Cells	Virus	20:1	10:1	20:1	10:1	20:1	10:1	20:1	10:1	1:1	0.5:1
RMA	None	5	0	0	0	4	2	0	0	0	0
	VSV	77	73	55	49	11	3	72	74	67	61
RMA/S	None	0	0	0	0	0	0	0	0	0	0
	VSV	68	56	38	22	0	0	70	61	50	48

Target cells were infected with VSV for 2 h and tested in a 4-h cytotoxicity assay. N-specific CTL were derived from mouse strains with the designated MHC haplotypes by either priming mice with N-Vac and restimulating in vitro with VSV, or with N_{52-59} and were used at an E/T ratio of 20:1 or 10:1. Clone 33 is a K^b-restricted CTL clone specific for N_{52-59}, and was used at E/T ratios of 1:1 and 0.5:1.

Table 3. *Presentation of N Can Be Blocked by Cycloheximide or BFA*

		Percent specific ^{51}Cr release			
		RMA		RMA/S	
Exp.	Treatment	20:1	10:1	20:1	10:1
A	None	0	0	0	0
	VSV	64	59	56	47
	VSV + Ψ	14	11	1	4
B	None	0	0	0	0
	VSV	88	85	64	59
	VSV + BFA	0	1	2	4
	VSV + BFA ⇒ reversed	59	52	39	29
	VSV + Ψ in assay	56	43	57	46
	VSV + BFA ⇒ reversed + Ψ in assay	49	31	31	19
	N_{52-59}	70	60	61	56
	N_{52-59} + BFA	69	62	57	46

Cells were infected with VSV or treated with N_{52-59} at 5×10^{-6} M for 2 h and tested in a cytotoxicity assay. Anti-VSV CTL were generated from VSV-primed mice by in vitro stimulation with VSV and were used at E/T ratios of 20:1 or 10:1. Cycloheximide (Ψ) was added at the beginning of the infection in Exp. A, or at the beginning of the cytotoxicity assay in Exp. B. In Exp. B, BFA was added 15 min after VSV or peptide was added to cells, and was maintained throughout the remaining pre-assay incubation and the cytotoxicity assay. The BFA blockade was reversed by washing the cells before the cytotoxicity assay.

or protein synthesis since VSV-infected cells presented N after BFA was removed and cells were incubated with protein synthesis inhibitors to block any new synthesis of viral proteins (Table 3). Third, unlabeled VSV-infected cells were incubated with ^{51}Cr-labeled uninfected cells during a 6-h cytotoxicity assay (not shown). Although this resulted in sensitization of a significant fraction of cells (39%) for CTL lysis, this effect was completely blocked by addition of either protein synthesis inhibitors or BFA to the mixture of cells. In the same experiment, these inhibitors did not block sensitization by exogenous peptide. Additionally, sensitization was almost completely blocked by incubation with a mAb specific for VSV glycoprotein (G) with virus neutralizing activity, while a control mAb had little effect. Thus, the observed sensitization can be attributed to the release of infectious virions from RMA/S cells, and not the release of antigenic peptides in sufficient quantity to sensitize target cells for CTL lysis. Based on these findings, we conclude that RMA/S cells are able to process and present endogenously synthesized N protein to CTL in association with K^b.

Characteristics of RMA/S Presentation of N. We performed several additional experiments to characterize the ability of RMA/S cells to present N to CTL. First, we compared the amount of input VSV required to sensitize RMA and RMA/S cells for CTL recognition. After infection of cells at multiplicities of infection ranging from 0.004 to 2 PFU per cell, RMA and RMA/S cells demonstrated only minor differences in presentation of VSV antigens over the entire range tested (Fig. 1 *A*). Immunoperoxidase staining of the same cells used in the CTL assay indicated that RMA/S cells expressed similar quantities of N protein at a given multiplicity of infection (not shown).

We next examined the time required by RMA and RMA/S cells to present N to CTL. This was done by adding BFA to cells at various times after infection with VSV, and main-

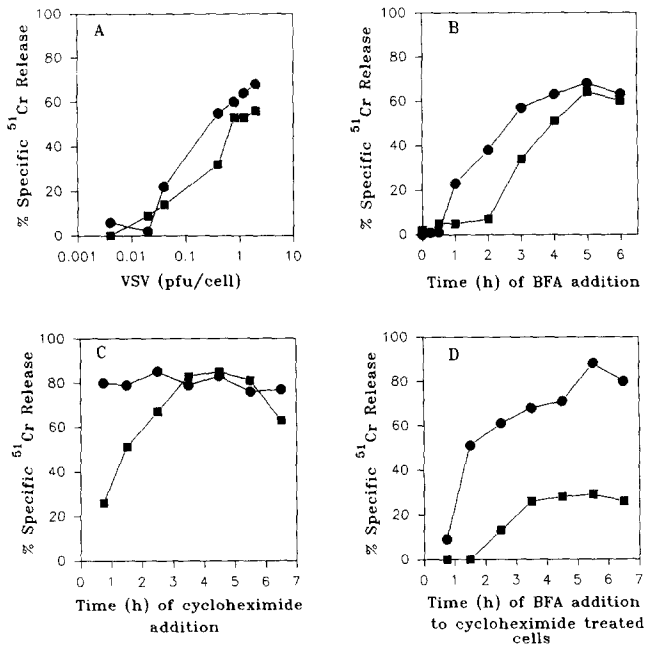

Figure 1. Characteristics of RMA/S processing of VSV N protein. Anti-N CTL generated by VSV in vitro stimulation of splenocyte-derived N-Vac-primed mice were used to characterize the antigen processing capacity of RMA (●) or RMA/S (■) cells. E/T ratios were 20:1 (*A*), 10:1 (*B*), and 15:1 (*C* and *D*). Percent of cytotoxicity of uninfected cells was subtracted from all values. (*A*) Amount of virus required for sensitization. Cell were infected with various PFU of VSV per cell, incubated for 2 h and tested in a 4-h cytotoxicity assay. (*B*) Time course of antigen presentation. Cells were infected for 2 h and tested in a 4-h cytotoxicity assay (6-h point in graph). The other points on the graph represent the times at which BFA was added to cells to block additional antigen processing. (*C*) Time of protein synthesis required for antigen presentation. Cells were infected for 2.5 h and tested in a 4-h cytotoxicity assay (6.5-h point in graph). The other points on the graph represent the times at which cycloheximide was added to cells to block additional synthesis of N protein. (*D*) Time of presentation required after blocking protein synthesis. RMA and RMA/S cells treated with cycloheximide after 45 min of infection in *C*, were treated with BFA after various times of additional incubation, and tested in a 4-h microcytotoxicity assay beginning 2.5 h after infection. This assay measures the rate of antigen presentation from a pool of N protein synthesized before the addition of cycloheximide.

taining BFA throughout the remaining incubations. As seen in Fig. 1 *B*, while RMA cells were sensitized for 50% of their maximal lysis by ~105 min, RMA/S cells required nearly twice as long to achieve a similar level of recognition. This difference could be due to: (*a*) a requirement for higher levels of protein, or (*b*) slower formation or transport of K^b-peptide complexes. To help distinguish between these mechanisms, we stopped the synthesis of VSV proteins by adding protein synthesis inhibitors to cells at various times after infection. Whereas RMA cells expressed sufficient N after 45 min of virus infection to sensitize cells at maximal levels, RMA/S cells required infection for at least an additional 210 min to achieve similar levels of sensitization, which indicates that greater amounts of N are required to load sufficient class I molecules for target cell sensitization (Fig. 1 *C*). In the same experiment, we examined the kinetics of presentation of a pool of N produced before the addition of cycloheximide by treating cells with BFA at various times after blocking protein synthesis 45 min post-infection. While RMA cells transported sufficient K^b-peptide complexes to reach half-maximal lysis within 45 min after adding cycloheximide, RMA/S cells required approximately twice as much time to achieve the same relative level of recognition (Fig. 1 *D*). This indicates that the formation or transport of class I–peptide complexes occurs more slowly in the mutants.

In additional experiments (not shown), we found that at least a 32-fold higher concentration of an anti-CD8 mAb (3.168 [12]) was required to block lysis of VSV-infected RMA cells compared with RMA/S cells by N-specific CTL when this antibody was included during the cytotoxicity assay. The specificity of inhibition was demonstrated by comparison to the low levels of inhibition obtained using an antibody specific for CD4.

These experiments indicate that while RMA/S cells are capable of processing N and presenting it to CTL, this requires more N protein, occurs more slowly than presentation by RMA cells, and has a greater dependence on CD8–class I interactions.

After Prolonged PR8 Infection, RMA/S Cells Present Endogenously Synthesized NP. Our finding that RMA/S cells were capable of presenting VSV N protein given sufficient levels of N and long enough time to process and present it suggested that the cells might be able to prevent influenza virus NP given a sufficiently long infection period before incubation with CTL. In their studies with RMA/S cells, Townsend et al. (1) infected cells for 1.5 h before their incubation with CTL in a 4-h assay. Under these conditions, we also found that PR8-infected RMA/S cells were not lysed by NP-specific CTL populations induced by priming mice with PR8 and restimulating splenocytes in vitro with $NP_{365-380}$ (Table 1). If, however, cells were infected for at least an additional 90 min before the assay, they were lysed at low but consistent levels by NP-specific CTL (Table 1). The specificity of recognition is shown by the reciprocal nature of recognition by NP- and VSV-specific CTL of PR8- and VSV-infected cells, respectively. Presentation of NP was blocked by BFA and also by protein synthesis inhibitors (not shown). Thus, as with N, RMA/S cells are able to process NP from a biosynthesized cytosolic pool of antigen and present it to CTL via the standard endogenous pathway.

Discussion

In contrast to prior reports, we find that the antigen processing deficit in RMA/S cells is partial rather than absolute in nature. Thus, RMA/S cells are able to present N or NP to CTL, but they require more antigen and more time to either generate the class I–peptide complex, or transport it to the cell surface (or both). The partial nature of the deficit could reflect an incomplete deficit in one of the steps of the normal class I–restricted pathway, or a total deficit that is

partially overcome by the operation of an alternative mechanism constitutively functioning at low efficiency.

The fact that RMA/S cells are capable of processing and presenting cytosolic antigens does not necessarily indicate that they create normal class I–peptide complexes. Indeed, our data are consistent with the possibility that the peptide–class I complexes on the surface of RMA/S cells are not as stable as normal complexes, which in a cell-free system are stable for many hundreds of hours (4). As the evidence strongly favors the idea that RMA/S cells express normal α chains and β_2-microglobulin (13), less stable complexes would have to result either from a deficiency in delivering peptides of the proper length to class I molecules (since longer or shorter peptides bind with a much lower affinity to class I molecules [14, 15]), or from a deficiency in a molecule required for class I assembly in vivo. That RMA/S cells might produce defective class I–peptide complexes is consistent with their increased sensitivity to blocking by anti-CD8 antibodies, first observed by Ohlen et al. (16) studying alloreactive recognition of RMA and RMA/S cells. Via HPLC analysis of antigenic peptides acid extracted from RMA/S cells (14), it should be possible to directly determine the nature of the peptide–class I complex in the cells.

Antigen processing mutants have attracted wide interest for determining the nature of class I molecules recognized by alloreactive CTL. Based on the recognition of RMA/S cells by alloreactive CTL clones, Ohlen et al. (16) proposed that some alloreactive CTL recognize peptides produced from proteins with endoplasmic reticulum insertion sequences. Our findings that two cytosolic proteins are presented by RMA/S cells indicate that there is no need to invoke this limitation to presentation of allo-determinants. It has also been proposed that a portion of the alloreactive response is directed solely to class I molecules, either in a peptide-free state or while bound to peptide. RMA/S cells and similar antigen processing mutants have been used as a means of identifying alloreactive clones that recognize "empty" class I molecules. Our findings indicate that RMA/S cells, at least, are not well suited for this purpose, as they clearly maintain some capacity to process and present proteins to CTL.

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