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Possible Mechanisms by Which the *H-2K*^{bm3} Mutation May Decrease Cytotoxic T-Lymphocyte Recognition of Vesicular Stomatitis Virus Nucleoprotein Antigen

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Spleen cells from C57BL/6 (B6) mice generate a strong in vitro cytotoxic T-lymphocyte (CTL) response specific for vesicular stomatitis virus (VSV). Spleen cells from VSV-primed B6-*H-2*^{bm3} (*bm3*) mice, which have a mutation in *H-2K*^{b}, require approximately 10-fold more UV-inactivated VSV to generate in vitro secondary anti-VSV CTL, compared with spleen cells from primed B6 mice. Anti-VSV CTL elicited in both *bm3* and B6 mice are primarily specific for the viral nucleocapsid protein (N protein), as demonstrated by using recombinant vaccinia viruses that express the VSV N protein. *bm3* CTL were found to exhibit only a very low level of lytic activity when tested against autologous VSV-infected concanavalin A spleen cell blasts as well as several *H-2*^{b} tumor cell lines. The weak anti-VSV response of *bm3* CTL was found to be the result of a combination of inefficient recognition of VSV-infected target cells and decreased elicitation of secondary effector cells. VSV-infected *bm3* target cells were not killed as well as B6 targets by either *bm3* or B6 effectors. This is because of the inefficient recognition of targets, as demonstrated by the fact that VSV-infected *bm3* cells were unable to competitively inhibit the lysis of VSV-infected B6 target cells by either *bm3* or B6 effectors. By using cells from recombinant mice, it was shown that the CTL response restricted by *H-2K*^{b} was low in the *bm3* mice, compared with that of the B6 mice. However, the *H-2D*^{b}-restricted CTL activity was similarly low in both the B6 and *bm3* mice. The possibility that the low response to VSV-infected *bm3* cells is caused by differences between the *bm3* and B6 cells in expression of either viral antigens or *H-2K* was investigated by radiolabeling and immunoprecipitation. VSV-infected B6 and *bm3* cells were found to express equivalent levels of both viral antigens and *H-2K*. These results indicate that the *bm3* mutation alters a functional site on the *H-2K*^{b} molecule that is involved in the recognition of VSV-infected cells. The observation that elicitation of *bm3* CTL can occur at high antigen doses further suggests that the *bm3* mutation results in a lower affinity of *H-2K* either for viral antigen or for receptor sites on the CTL.

Antiviral cytotoxic T lymphocytes (CTL) recognize and lyse virus-infected cells only when viral antigens are expressed on the cell surface with the appropriate host antigen of the major histocompatibility complex (MHC). Recently it has been shown that the specificity of CTL for viral gene products primarily involves surface antigens derived from nucleocapsid proteins for both anti-influenza virus CTL (24, 25, 28) and also CTLs against vesicular stomatitis virus (VSV) (18, 27). Other viral antigens, such as viral surface glycoproteins, are also recognized by minor populations of antiviral CTL.

The specificity of CTL for MHC gene products primarily involves the class I antigens, which in the mouse are the *H-2K*, *H-2D*, and (in some strains) *H-2L* molecules. Different allelic forms of class I gene products vary in their ability to elicit a CTL response against several viruses. For instance, the *H-2*^{k} haplotype is associated with a low CTL response to VSV, and *H-2*^{b} or *H-2*^{d} is associated with a high response (19). In contrast, with influenza virus-specific CTL, the *H-2*^{b} haplotype is linked to a low response and *H-2*^{k} is linked to a high response (1, 6). Furthermore, within a given haplotype, there is often a preference for one *H-2* locus over another. For instance, in *H-2*^{d} mice most anti-VSV CTL are restricted by *H-2L*^{d}, and in *H-2*^{b} mice the anti-VSV CTL response is restricted primarily by *H-2K*^{b} (3, 7, 8). These observations provide evidence that some class I molecules are more effective than others in eliciting CTL responses against a given virus.

The effects of mutations in class I genes provide further support for the idea that class I molecules act as differential regulators of CTL activity. Most of the mutations in murine class I MHC antigens appear to be caused by recombinational mechanisms such as gene conversion, in which short sequences from another MHC-like gene are substituted into the sequence for wild-type MHC antigens (16). Thus, two or more amino acid substitutions frequently occur as a result of the mutation. The number of such "donor" genes is relatively large and is thought to be the source for generation of polymorphism in class I MHC antigens. Thus, the effects of mutations in class I MHC antigens on functional immune responses, such as CTL reactivity, should resemble the differential recognition of different allelic forms of class I MHC antigens. Indeed, the pattern that emerges from the numerous studies of the antiviral CTL reactivity of class I MHC antigen mutants is that whether the CTL response is altered by a particular mutation depends on the virus being studied. For example, the *H-2K*^{bm3} mutation, examined in this study, results phenotypically in a low CTL response to VSV (4) and Sendai virus (4, 5), but does not alter the response to Moloney leukemia virus (22, 23), vaccinia virus (10, 29), or simian virus 40 (17).

There are a number of possible mechanisms by which sequence variation in class I MHC antigens could lead to variability in CTL reactivity with a particular virus. Among

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the most frequently proposed mechanisms are alteration in affinity of the MHC antigen either for viral antigens or for the CTL receptor for antigen. Other possible mechanisms include alteration in antigen specificity, variation in the levels of antigen expression, and indirect effects of class I antigens on other components of the immune response. The goal of the present study was to investigate in detail the mechanism of the low CTL response to VSV in the *H-2K*^{bm3} mutant. The data obtained provide support for mechanisms involving a lower affinity of *H-2K*^{bm3} for either viral antigens or T-cell receptors compared with *H-2K*^{b}.

MATERIALS AND METHODS

Mice. Mice of the following strains were used: C57BL/6 (B6 mice) (*b*, *b*), B6-*H-2*^{bm3}/EgAoEg (*bm3* mice) (*bm3*, *b*), BALB/c (*d*, *d*), HTI/Go (*b*, *d*), and HTG/Go (*d*, *b*). These mice were either purchased from Jackson Laboratory (Bar Harbor, Maine), obtained from Bernard Amos (Duke University, Durham, N.C.), or raised in our own breeding colony from stock originally obtained from Jackson Laboratory or from Dr. Amos. The letters in parentheses indicate the *H-2K* and *H-2D* alleles. In all experiments, sex-matched mice were used.

Cultured cell lines. The following cell lines were used: EL-4 (*b*, *b*), a thymus lymphoma of B6 origin; MC57G (*b*, *b*), a macrophagelike cell line of B6 origin; and P815 (*d*, *d*), a mastocytoma of DBA/2 origin. All of these cells were cultured in supplemented RPMI 1640. Supplements included 10% fetal bovine serum (heat inactivated; 56°C, 1 h); 0.03% glutamine, 2-mercaptoethanol (50 μM), penicillin (Flow Laboratories, Inc., McLean, Va.), and streptomycin (Flow Laboratories).

Virus. Stocks of VSV were prepared by infecting baby hamster kidney cells with VSV (Indiana serotype) at a multiplicity of infection of 0.02. After overnight incubation, virus was purified from culture fluid by differential centrifugation and isopycnic centrifugation through a sucrose gradient as previously described (21). Viral protein concentration was determined by the method of Lowry et al. (14). Vaccinia virus recombinants that express the N protein or G protein of VSV (Indiana serotype) were obtained from Bernard Moss (National Institutes of Health, Bethesda, Md.) through Jonathan Yewdell (Wistar Institute of Anatomy and Biology, Philadelphia, Pa.). Virus stocks were prepared by infecting HeLa cells at a multiplicity of infection of 0.1. After incubation for 48 h, cells were harvested and lysed by sonication. Virus titers were determined by plaque assay on CV1 cells.

Monoclonal antibodies. The monoclonal antibodies specific for VSV G protein (clone I1) or N protein (clone 10G4) (13) and clone 34-4-20S, specific for *H-2K*^{b} (obtained from the American Type Culture Collection, Rockville, Md.), were purified from culture supernatant on a protein A-Sepharose column (Pharmacia, Inc., Piscataway, N.J.) (9).

Generation of effector cells. Mice were primed with 1.0×10^6 PFU of VSV in 0.1 ml of phosphate-buffered saline by intracardial injection. After at least 4 weeks, spleens were removed from these mice and cleared of erythrocytes with Tris-NH_4Cl (15), and 8×10^6 spleen cells were cultured with various amounts of UV-inactivated VSV in 2.0 ml of supplemented RPMI in 24-well plates (Linbro, Hamden, Conn.) at 37°C in an atmosphere of 6% CO_2 and 94% air. The cells were tested for their ability to lyse ^{51}Cr-labeled target cells after 6 days in culture. The effector cells elicited in the secondary culture were *H-2* restricted, were virus specific, and were found to express the phenotype of Thy-1$^+$, Lyt-1^{low}, Lyt-2,3$^+$ by removal of lytic activity with antisera and complement (Cedarlane Laboratories, Ltd., Westbury, N.Y.) (data not shown).

Production of conA-induced spleen cell blasts. Spleens were removed from mice and cleared of erythrocytes with Tris-NH_4Cl, and the spleen cells were adjusted to 1.0×10^7 cells per ml in supplemented RPMI and added to 60-mm-diameter tissue culture dishes. Concanavalin A (conA) in RPMI media with 5×10^{-5} M 2-mercaptoethanol was added to spleen cell cultures to give a final concentration of 2.0 μg/ml. The cultures were incubated for 48 h at 37°C in an atmosphere of 6% CO_2 and 94% air.

Target cells. Target cells were prepared by infecting 2.5×10^6 EL-4-, P815-, MC57G-, or conA-induced blasts from various mouse strains in 0.5 ml of supplemented RPMI with VSV or vaccinia virus at a multiplicity of infection of 30 to 50. Infection was allowed to occur for 1 h at 37°C in an atmosphere of 6% CO_2 and 94% air. Cells were then washed once in supplemented RPMI and, in the case of vaccinia virus infection, incubated for an additional 6 h at 37°C. Cells were then labeled with 200 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.) in 0.5 ml of supplemented RPMI for 1.5 h at 37°C.

Cytotoxic assay. For determination of cytotoxic activity, 5.0×10^3 ^{51}Cr-labeled target cells were mixed with serial dilutions of effector cells in a total volume of 200 μl of supplemented RPMI in 96-well round-bottom plates (Linbro). These mixtures were incubated for 4 h at 37°C in an atmosphere of 6% CO_2 and 94% air. The assay was stopped by centrifugation (500 × *g*, 10 min) in the cold, and the radioactivity in 100 μl of the supernatant was determined. Percent specific release was calculated as 100(E − C/T − C), in which E is the radioactivity released by the target cells in the presence of antigen-stimulated effector cells, C is the radioactivity released by target cells in the presence of medium only, and T is the total radioactivity determined either by counting the radioactivity in both the target cells and the supernatant or by quantitating the radioactivity released by incubation of target cells with 10% Triton X-100.

Cold target inhibition assay. A constant number of effector cells was mixed with serial dilutions of unlabeled target cells in a total volume of 100 μl of supplemented RPMI and incubated for 30 min at 4°C. A constant number of ^{51}Cr-labeled target cells in 100 μl of supplemented RPMI was then added to these mixtures and incubated for 4 h at 37°C in an atmosphere of 6% CO_2 and 94% air. The assay was stopped by centrifugation in the cold, and the radioactivity in 100 μl of the supernatant was determined. The percent inhibition was calculated as 100(I/U), in which I is the specific release in the presence of unlabeled target cells and U is the specific release in the absence of unlabeled target cells.

Radiolabeling and immunoprecipitation of viral and *H-2K* antigens. ConA blasts (1.0×10^7) were infected in 0.5 ml of supplemented RPMI with VSV at a multiplicity of infection of 30 or else were mock infected for 4.0 h at 37°C in an atmosphere of 6% CO_2 and 94% air. These cells were washed in PBS and either labeled with [^{35}S]methionine (13) for 1 h or were iodinated with ^{125}I for 45 min at 4°C by using a commercial lactoperoxidase radioiodination system (New England Nuclear). The labeled cells were washed twice in PBS and then lysed with RIPA buffer (10 mM Tris [pH 7.4], 0.15 M NaCl, 1.0% deoxycholate, 1.0% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1.0 mM phenylmethylsulfonyl fluoride) and immunoprecipitated with either anti-G protein, anti-N protein, or anti-*H-2K*^{b} monoclonal antibodies and protein-A Sepharose (Sigma Chemical Co., St. Louis,

Mo.), as described previously (13). The immunoprecipitates were analyzed by SDS gel electrophoresis, as described by Laemmli (12). The gels were visualized by exposing Kodak SB-5 film (Eastman Kodak Co., Rochester, N.Y.) with the aid of a Cronex Lightning-Plus intensifying screen (Du Pont Co., Wilmington, Del.) at −70°C.

RESULTS

Quantitation of the anti-VSV CTL response in the *bm3* mutant. B6 and *bm3* mice were primed by intracardial injection of VSV. After at least 4 weeks, spleen cells were cultured with UV-inactivated VSV for 6 days and then assayed for CTL activity against infected syngeneic, conA-stimulated spleen cell blasts over a range of effector:target (E:T) ratios (Fig. 1). UV-inactivated VSV was used only for the secondary elicitation, whereas infectious virus was used for priming and for generation of targets in the ^{51}Cr release assay. The dose of virus used in the secondary elicitation was that giving the maximal difference in CTL activity between B6 and *bm3* (1.0 μg). Titration of the lytic activity of B6 effectors shows a maximum of about 30% specific release, similar to previous results with VSV-infected conA-stimulated spleen cell blasts as CTL targets (e.g., reference 3), indicating that only a portion of these cells is susceptible to lysis. The data show that *bm3* CTL exhibit a low level of lysis against *bm3* targets at all E:T ratios, compared with lysis of B6 targets by B6 CTL. Because syngeneic target cells were used, this result indicates that the low response of the mutant is not caused by the presence of *bm3* anti-VSV CTL, which are uniquely restricted by $H\text{-}2^{bm3}$ and are therefore incapable of recognizing VSV antigens in the context of $H\text{-}2^{b}$. Instead, this result suggests a defect in the generation of *bm3* anti-VSV CTL in vitro.

In theory, if the low anti-VSV CTL response of the *bm3* mutant is caused by a decrease in the affinity of $H\text{-}2K^{bm3}$ compared with $H\text{-}2K^{b}$ for either viral antigens or T-cell receptors, the defect should be at least partially overcome by

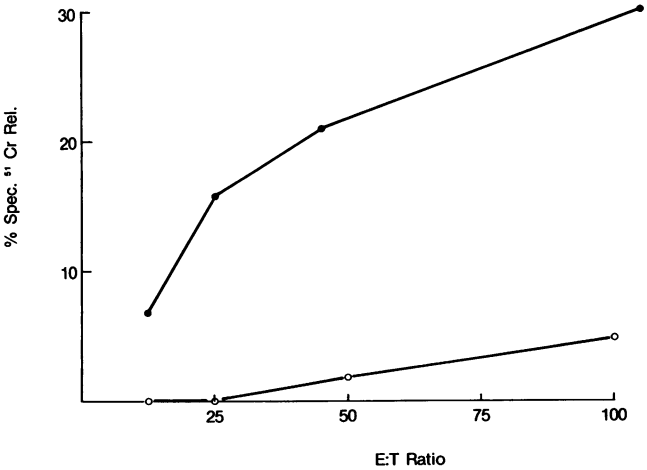

FIG. 1. Lysis of syngeneic VSV-infected conA spleen cell blasts by B6 or *bm3* secondary anti-VSV CTL. Spleen cells from B6 (●) or *bm3* (○) mice primed with VSV at least 4 weeks earlier were secondarily stimulated in vitro with 1.0 μg of UV-inactivated VSV per 8 × 10^6 cells for 6 days. These secondary anti-VSV CTL were tested against ^{51}Cr-labeled VSV-infected syngeneic conA blast cells. The spontaneous release levels for B6 and *bm3* were 21.1 and 22.7%, respectively. The points represent the means of triplicate determinations, with the standard deviation never exceeding 9.5% of the mean. Spec., Specific; Rel., release.

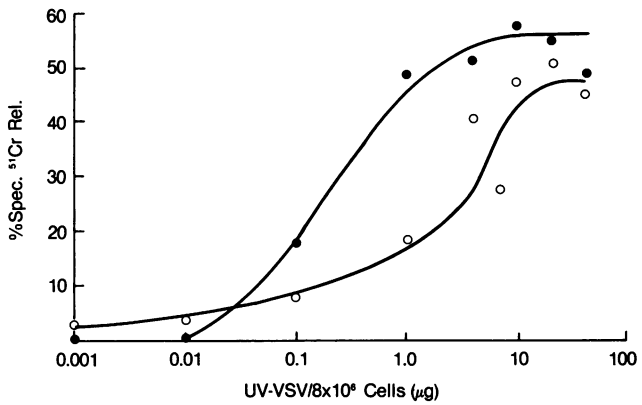

FIG. 2. Titration of dose of UV-inactivated VSV required to elicit secondary anti-VSV CTL in vitro. B6 (●) or *bm3* (○) mice were immunized with VSV. After at least 4 weeks, the spleen cells were removed and stimulated in culture with various doses of UV-inactivated VSV for 6 days. The secondary anti-VSV CTL activity was tested against ^{51}Cr-labeled VSV-infected EL-4 cells (multiplicity of infection, 30) at an E:T ratio of 50:1. The percent spontaneous ^{51}Cr release was 11.5%. The points represent the means of triplicate determinations, with the standard deviation never exceeding 11.1% of the mean. Spec., Specific; Rel., release.

using higher concentrations of antigen. Such a result is shown in Fig. 2. Primed spleen cells from either B6 or *bm3* mice were cultured in vitro with various doses of UV-inactivated VSV. The resulting effector populations were assayed for CTL activity against VSV-infected EL-4 ($H\text{-}2^{b}$) targets. When EL-4 cells are used as targets, higher levels of specific lysis and lower levels of spontaneous release are obtained than with conA-stimulated spleen cell blast targets. B6 CTL achieved 50% of maximal elicitation with approximately 0.2 μg of VSV protein per well. In contrast, *bm3* CTL reached 50% of maximal activity at a nearly 10-fold higher dose of around 2.0 μg per well. Even at the maximum dose used in these experiments (40 μg), the activity of *bm3* anti-VSV CTL was somewhat less than the activity of B6 CTL when assayed as a function of E:T ratios (see below). That the defect in the *bm3* mutant can be at least partially overcome at high antigen doses suggests that $H\text{-}2K^{bm3}$ is capable of functioning as a restricting element for anti-VSV CTL, provided that the dominant specificities of anti-VSV CTL are not altered by the higher antigen dose.

Specificity of anti-VSV CTL elicited in *bm3* mice. The predominant specificity of anti-VSV CTL from $H\text{-}2^{b}$ mice is for surface antigens derived from the VSV nucleocapsid protein (18) in the context of $H\text{-}2K^{b}$ rather than $H\text{-}2D^{b}$ (8). We tested whether the *bm3* CTL elicited at high doses of VSV displayed the same specificity or whether the increased antigen dose elicits CTL with a different specificity. Primed spleen cells from B6 or *bm3* mice were stimulated in vitro with UV-inactivated VSV (40 μg) and then tested for activity against MC57G targets infected with vaccinia virus recombinants that express either the VSV N protein or the VSV G protein (Fig. 3). The specificities of both B6 and *bm3* CTL for viral antigens was similar. Both CTL populations lysed cells infected with the N protein vaccinia virus recombinant to an extent slightly less than VSV-infected cells, whereas little activity was observed with the G protein recombinant.

Geiger et al. (8) have shown that the majority of $H\text{-}2^{b}$ anti-VSV CTL are restricted by $H\text{-}2K^{b}$ rather than by $H\text{-}2D^{b}$. To determine whether the anti-VSV CTL response generated in *bm3* mice involves *H-2K*-restricted CTL, the

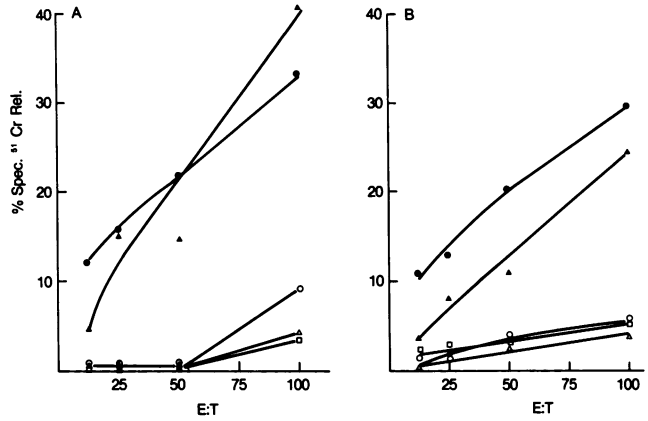

FIG. 3. Lysis of targets infected with vaccinia virus recombinants by B6 or *bm3* secondary anti-VSV CTL. Spleen cells from B6 (A) or *bm3* (B) mice primed with VSV at least 4 weeks earlier were secondarily stimulated in vitro with 40 μg of UV-inactivated VSV per 8×10^6 cells for 6 days. The secondary anti-VSV CTL were tested against ^{51}Cr-labeled MC57G cells infected with VSV (●), vaccinia recombinant that expresses N protein (▲), vaccinia recombinant that expresses G protein (△), wild-type vaccinia virus (○), or uninfected MC57G cells (□). The percent spontaneous release for uninfected cells was 8.9%. The percent spontaneous release for infected cells was: VSV, 13.5%; wild-type vaccinia, 10.5%; vaccinia (N protein), 12.4%; and vaccinia (G protein), 11.5%. Data shown are means of triplicate determinations, with the standard deviation not exceeding 11.0% of the mean. Spec., Specific; Rel., release.

lytic activity of *bm3* CTL was tested against VSV-infected conA blasts from recombinant mouse strains which differ at their H-2K and H-2D loci. The data for targets from HTI mice (K^b, D^d) are shown in Table 1. CTL from *bm3* mice showed lytic activity against VSV-infected HTI targets, but this activity was significantly less than that of B6 or HTI effectors. These results are similar to those obtained with B6 targets (e.g., see Fig. 2 and 3). The results in Table 1 are representative of three separate experiments. Lytic activity of B6 and *bm3* CTL against HTG targets (K^d, D^b) was less consistently demonstrated. In some experiments, H-2D^b-restricted killing of HTG targets was detected, and in those experiments, B6 and *bm3* CTL showed an equivalent low level of killing (data not shown).

TABLE 1. Killing of recombinant HTI (*b*, *d*) conA spleen cell blasts by anti-VSV effector cells

Effectors[a]	% Specific ^{51}Cr release by HTI target cells[b]			
	Infected		Uninfected	
	100:1[c]	50:1	100:1	50:1
bm3	26.8	17.9	8.0	2.3
B6	39.9	36.1	4.0	3.2
HTI (*b*, *d*)	40.6	33.2	4.4	3.2
HTG (*d*, *b*)	−2.6	−1.3	1.7	−0.7

[a] Mice were primed in vivo with 1.0×10^6 PFU of VSV at least 4 weeks earlier. Spleen cells were stimulated in vitro with 40 μg of UV-inactivated VSV per 8×10^6 cells for 6 days. Cells were harvested and tested for lytic activity.

[b] HTI conA spleen cell blasts were infected with VSV (multiplicity of infection, 30) or mock infected and then were ^{51}Cr labeled and incubated with effector cells at the indicated E:T ratio for 4 h. The percent spontaneous release levels for infected and uninfected cells were 24.6 and 23.2% respectively. Data shown are means of triplicate determinations, with the standard deviation not exceeding 10.7% of the mean.

[c] E:T ratios.

TABLE 2. Lytic activity of anti-VSV effector cells against various targets

Effector[a]	VSV-infected target cells (LU/culture)[b]				
	bm3	B6	EL-4	MC57G	P815
bm3	6.08	15.84	22.80	22.96	≤0.02
B6	6.56	57.52	60.24	176.08	≤0.02
BALB/c	NT[c]	NT	0.02	≤0.02	26.48

[a] Mice were primed in vivo with 1.0×10^6 PFU of VSV at least 4 weeks earlier. Spleen cells were harvested, cleared of erythrocytes, and stimulated in vitro with 40 μg of UV-inactivated VSV per 8×10^6 cells for 6 days. Cells were harvested and tested for cytolytic activity.

[b] B6 or *bm3* conA spleen cell blasts and EL-4, MC57G, or P815 cells were infected with VSV (multiplicity of infection, 30), ^{51}Cr labeled, and incubated with effector cells as described in Materials and Methods (1 lytic unit [LU] gives 40% specific release). The percent spontaneous release levels of B6, *bm3*, EL-4, MC57G, and P815 cells in the absence of effector cells were 21.1, 22.7, 9.2, 13.9, and 9.1%, respectively. LU were calculated from the mean of triplicate determinations, with the standard deviation never exceeding 11.8% of the mean.

[c] NT, Not tested.

Activity of *bm3* cells as targets for anti-VSV CTL. A decreased ability of infected *bm3* cells to be recognized as targets for anti-VSV CTL could also contribute to the low activity of *bm3* anti-VSV CTL shown in Fig. 2. To test this point, the ability of various cell types to be lysed by B6 and *bm3* CTL was measured (Table 2). Spleen cells from B6 or *bm3* mice primed at least 4 weeks earlier were cultured for 6 days with 40 μg of UV-inactivated virus per well. Titration of E:T ratios similar to those in Fig. 1 was analyzed by the method of Cerrotini and Brunner (2) with 1 lytic unit representing 40% specific release. There is less lytic activity against infected *bm3* targets than against B6 targets, regardless of the source of CTL (Table 2). For instance, B6 effectors lyse infected B6 conA blasts nearly 10-fold better than *bm3* conA blasts. Likewise, *bm3* effectors show more activity against B6 targets than against *bm3* targets. This suggests that the low CTL response in the *bm3* mice also includes a decreased ability of the *bm3* target cell to be recognized and lysed by anti-VSV CTL. Two *H-2*^{b} tumor cell lines (EL-4 and MC57G) were more efficient targets than conA blasts with both *bm3* and B6 CTL. Similar to the data presented in Fig. 2, B6 effectors killed both tumor cell lines more efficiently than did *bm3* effectors. However, at the high virus dose used to elicit the CTL effectors in this experiment, the difference in activity between B6 and *bm3* CTL is more pronounced when a range of E:T ratios is analyzed, as represented by the lytic unit calculation, compared with the single E:T ratio shown in Fig. 2. These CTL were shown to be *H-2* restricted in that P815 cells (*H-2*^{d}) were not killed by either B6 or *bm3* CTL.

The results presented in Table 2 indicate that *bm3* targets are recognized poorly by both B6 and *bm3* anti-VSV CTL. To provide further support for this conclusion, the lysis of B6 ^{51}Cr-labeled cells was competitively inhibited with unlabeled B6 or *bm3* cells in a cold-target inhibition assay. Secondary B6 (Fig. 4) or *bm3* (Fig. 5) CTL were tested for their ability to lyse a constant number of labeled B6 target cells in the presence of various numbers of unlabeled B6 or *bm3* cells. The data presented in Fig. 4 show that VSV-infected B6 cells effectively inhibited the lysis of VSV-infected B6 targets when B6 CTL were tested. In contrast, inhibition by virus-infected *bm3* cells was comparable to the nonspecific inhibition by uninfected B6 and *bm3* cells. Similarly, the lysis of labeled virus-infected targets by *bm3* anti-VSV CTL was inhibited by B6 but not by *bm3* unlabeled

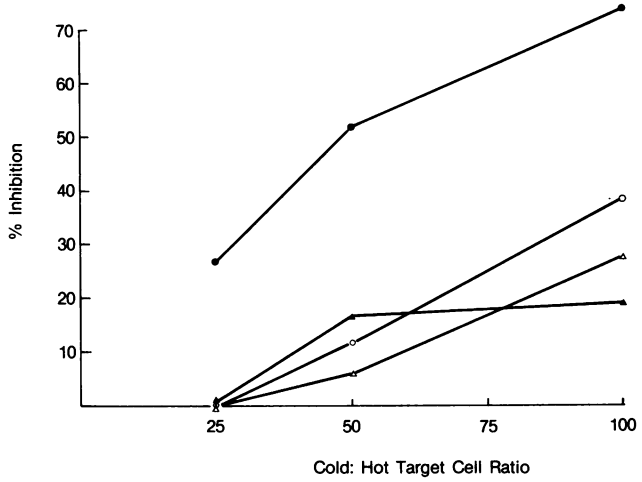

FIG. 4. Competitive inhibition of B6 anti-VSV CTL lysis of VSV-infected B6 conA blasts. B6 spleen cells, primed at least 4 weeks earlier in vivo with 1.0×10^6 PFU of VSV, were incubated in culture for 6 days with 40 μg of UV-inactivated VSV per 8×10^6 cells. Cells were harvested and incubated with either VSV-infected B6 (●), uninfected B6 (○), VSV-infected *bm3* (▲), or uninfected *bm3* (△) unlabeled conA blast inhibitor cells for 30 min at 4°C. ^{51}Cr-labeled VSV-infected B6 conA blasts were added (E:T, 6.25:1), and after 4 h of incubation at 37°C, lytic activity was determined from the amount of radioactivity released into the supernatant. The percent specific ^{51}Cr release in the absence of inhibitor cells was 23.8%. The points represent the mean of triplicate determinations, with the standard deviation never exceeding 13.1% of the mean.

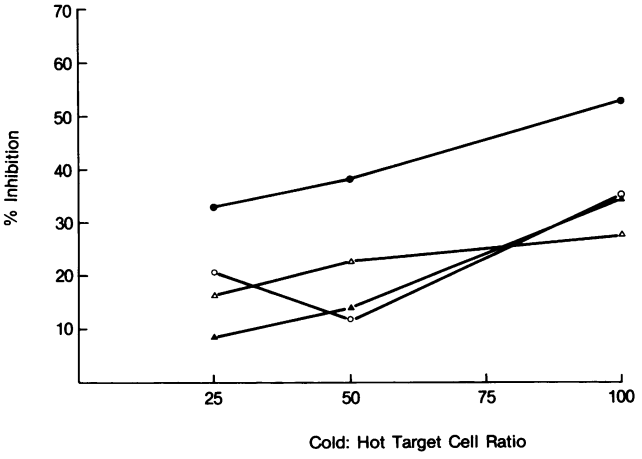

FIG. 5. Competitive inhibition of *bm3* anti-VSV CTL lysis of VSV-infected B6 conA blasts. *bm3* spleen cells, primed at least 4 weeks earlier in vivo with 1.0×10^6 PFU of VSV, were incubated in culture for 6 days with 40 μg of UV-inactivated VSV per 8×10^6 cells. Effector cells were harvested and incubated with either VSV-infected B6 (●), uninfected B6 (○), VSV-infected *bm3* (▲), or uninfected *bm3* (△) unlabeled conA blast inhibitor cells for 30 min at 4°C. ^{51}Cr-labeled VSV-infected B6 conA blasts were added (E:T ratio, 100:1), and after 4 h of incubation at 37°C, the lytic activity was determined from the amount of radioactivity released into the supernatant. The percent specific ^{51}Cr release in the absence of inhibitor cells was 37.7%. The points represent the means of triplicate determinations, with the standard deviation never exceeding 8.3% of the mean.

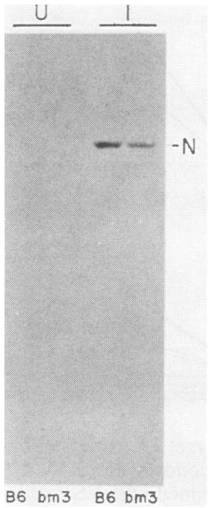

FIG. 6. *bm3* or B6 conA spleen cell blasts were either infected with VSV (I) or uninfected (U). Cells were labeled with [^{35}S]methionine from 4 to 5 h postinfection, lysed with detergent, and immunoprecipitated with a monoclonal antibody specific for the VSV N protein. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, a fluorograph of which is shown.

VSV-infected cells (Fig. 5). These data suggest that the failure of *bm3* targets to be lysed by anti-VSV CTL is caused by a defect in the recognition of the target cell by the CTL rather than by the ability of the target cell to be lysed.

Expression of viral and *H-2K* proteins in B6 and *bm3* target cells. The expression of viral proteins and *H-2K* in conA blasts from B6 and *bm3* mice was examined to determine whether defective expression of these proteins could account for the poor activity of *bm3* cells as targets for anti-VSV CTL. VSV-infected and uninfected conA spleen cell blasts from B6 and *bm3* mice were labeled with [^{35}S]methionine from 4 to 5 h postinfection. The labeled N protein was immunoprecipitated with a monoclonal antibody against N protein and was analyzed by SDS gel electrophoresis and fluorography (Fig. 6). The levels of expression of N protein in B6 and *bm3* cells were similar, although the *bm3* cells synthesized slightly less N protein than did B6 cells. It is unlikely that this minor difference in N protein synthesis could account for the difference in CTL reactivity. Studies with cell lines that express N protein from recombinant vectors have shown that lytic activity of anti-VSV CTL is only weakly dependent on the amount of N protein produced, even if the levels of N protein are too low to be detected by immunoprecipitation experiments such as those presented in Fig. 6 (18).

The expression of *H-2K* in B6 and *bm3* spleen cell blasts was examined by surface iodination with ^{125}I followed by immunoprecipitation (Fig. 7). The VSV G protein, which is the major viral surface protein, was also immunoprecipitated from these extracts, as a further test of whether *bm3* cells are defective in replication of VSV. It is not possible to analyze the surface expression of N protein by this technique, because labeling of even a small fraction of the large internal pool of N protein obscures the very low level of N protein antigens expressed on the cell surface (18, 27). The data presented in Fig. 7 show that similar amounts of labeled G protein are precipitated from both infected B6 and *bm3* cells, providing further support for the conclusion that *bm3* cells are not defective in expression of VSV proteins. Uninfected

bm3 cells express less surface-labeled *H-2K* than do uninfected B6 cells. However, when B6 or *bm3* cells are infected with VSV, the expression of *H-2K* on the cell surface is similar in both B6 and *bm3* cells. A band comigrating with G protein is seen when *H-2K* is immunoprecipitated from infected cells. Since G protein is the major band seen when infected cells are surface labeled, this could represent a nonspecific interaction with the *H-2K* antigen-antibody complex. It is conceivable that minor differences in the level of viral or *H-2K* antigens contribute to the low activity of *bm3* targets; however, the data in Fig. 6 and 7 strongly suggest that an inability of *bm3* cells to express viral proteins or a deficiency in expression of *H-2K* cannot explain the low response of anti-VSV CTL to *bm3* targets.

DISCUSSION

Mutations in $H\text{-}2K^b$ have a variable effect on the CTL response, depending on the antigen being recognized. The two amino acid substitutions that make up the *bm3* mutation result in the phenotype of a low CTL response to Sendai virus (4, 5) and the minor histocompatibility antigens H-3.1 and H-4.2 (26). In contrast, $H\text{-}2K^b$ CTL specific for Moloney leukemia virus (22, 23), vaccinia virus (10, 29), and simian virus 40 (17) are able to efficiently recognize viral antigens in association with $H\text{-}2K^{bm3}$. An overall conclusion of these studies is that the determinants recognized on the $H\text{-}2K^b$ molecule are dependent on the foreign antigen studied. In this study, we found that the *bm3* mutation produces a low response in the anti-VSV CTL system, as originally described by Clark et al. (4). The basis for this effect was characterized by (i) comparing the amount of virus required to stimulate B6 or *bm3* CTL in vitro, (ii) determining the differences in the levels of recognition of virus-infected B6 and *bm3* target cells, (iii) performing competitive inhibition studies, and (iv) comparing the amount of surface *H-2K* and viral antigens expressed on VSV-infected B6 and *bm3* target cells.

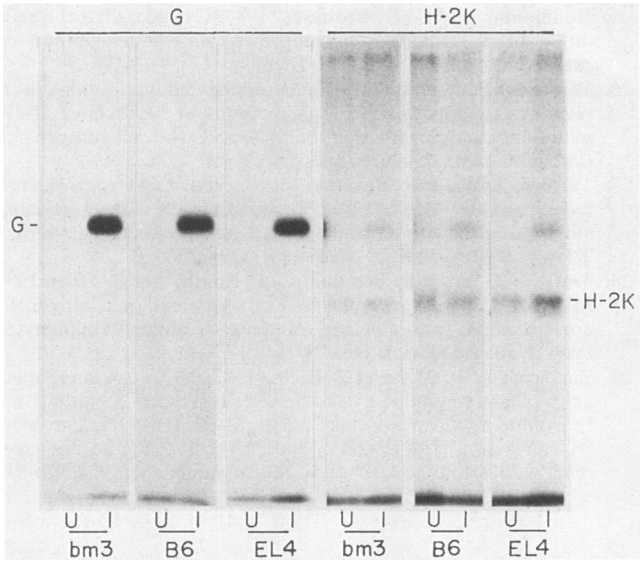

FIG. 7. *bm3*, B6, or EL-4 cells, either infected with VSV (I) or uninfected (U), were surface labeled with ^{125}I by the lactoperoxidase method, lysed with detergent, and immunoprecipitated with a monoclonal antibody specific for either VSV G protein (left panel) or *H-2K* (right panel). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, a fluorograph of which is shown.

The low activity of *bm3* anti-VSV CTL against autologous VSV-infected *bm3* cells (e.g., as shown in Fig. 1) consists of an inability of infected *bm3* cells to be recognized as targets as well as a defect in the elicitation of *bm3* anti-VSV CTL. The defect at the target level is shown by the observation that both *bm3* and B6 CTL effectors express a low level of lytic activity against *bm3* targets. In addition, in cold-target inhibition experiments, unlabeled infected *bm3* cells are less efficient competitors than B6 cells, regardless of whether *bm3* or B6 CTL are used as effectors.

The *bm3* mutation also results in a deficiency at the level of elicitation of memory anti-VSV CTL. This is shown by the low level of killing expressed in the *bm3* population, which is always lower than that seen in the B6 spleen cell population regardless of whether $H\text{-}2^{bm3}$ or $H\text{-}2^b$ targets are used. In addition, *bm3* CTL are not able to respond to low doses of antigen that are sufficient to elicit B6 CTL (Fig. 2). The defect at the target cell level and the deficiency at the level of activation of memory CTL show that the *bm3* mutation results in an alteration in functional determinants required by the VSV CTL system. However, no evidence was obtained for acquisition of new antigenic specificities as a result of the *bm3* mutation. For example, the higher activity of *bm3* anti-VSV CTL on B6 versus *bm3* targets (Table 2) and the competitive inhibition experiments (Fig. 5) indicate that anti-VSV CTL that are uniquely restricted by $H\text{-}2K^{bm3}$ rather than by $H\text{-}2K^b$ are not a significant part of the CTL population. Likewise, the *bm3* mutation does not alter recognition of the N protein as the predominant antigen for anti-VSV CTL (Fig. 3). Other explanations for the lytic activity of *bm3* effectors, such as induction of natural killer activity, are ruled out by controls which show that these effectors are VSV specific (no lysis of uninfected or vaccinia virus-infected cells), are MHC restricted (Tables 1 and 2), and show surface markers typical of CTL (see Materials and Methods).

Two hypotheses have been considered to explain the low responsiveness of MHC mutants to a given antigen, such as the one used here. (i) First, the *bm3* mutation could reduce the avidity of the *H-2K* molecule for either the viral antigens or for the T-cell receptor. This decreased avidity would reduce the probability of forming the functional complex of T-cell receptor, viral antigen, and *H-2K*. The decrease in the formation of these functional complexes would be seen at the elicitation stage as a lower number of memory *bm3* CTL activated and at the effector stage as a decreased ability of VSV-infected *bm3* cells to act as targets or as competitive inhibitors. (ii) On the other hand, the low *bm3* anti-VSV CTL response could be caused by a mechanism similar to the clonal deletion hypothesis set forth by Schwartz and Klein (11, 20) for T cells restricted by class II MHC antigens. In this case, the combination of $H\text{-}2K^{bm3}$ and VSV antigens results in determinants which cross-react with self-determinants shared between *bm3* and B6. The cross-reactivity of self-determinants and determinants produced by $H\text{-}2K^{bm3}$ and viral antigen would result in a blind spot in the T-cell repertoire of B6 and *bm3* mice representing those clones recognizing VSV antigens in the context of $H\text{-}2K^{bm3}$. Because the defect arises in both the B6 and *bm3* populations at the time of education of the T-cell repertoire, neither B6 nor *bm3* CTL are able to recognize VSV-infected *bm3* cells. The work presented here would favor the idea that the *bm3* mutation changes the avidity of association of *H-2* with viral antigens over the clonal deletion mechanism. The evidence

for this is the observation that the defect at the level of elicitation can be at least partially overcome at higher doses of antigen (Fig. 2). Currently, work is in progress to compare the association of viral and *H-2K* proteins incorporated into liposomes and the effect of the association of these proteins of the ability of liposomes to elicit secondary anti-VSV CTL in vitro.

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