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Protective Immunity against Disparate Tumors Is Mediated by a Nonpolymorphic MHC Class I Molecule

Eugene Y. Chiang and Iwona Stroynowski

Current peptide-based immunotherapies for treatment of model cancers target tumor Ags bound by the classical MHC class I (class Ia) molecules. The extensive polymorphism of class Ia loci greatly limits the effectiveness of these approaches. We demonstrate in this study that the murine nonpolymorphic, nonclassical MHC class I (class Ib) molecule Q9 (Qa-2) promotes potent immune responses against multiple syngeneic tumors. We have previously shown that ectopic expression of Q9 on the surface of class Ia-negative B78H1 melanoma led to efficient CTL-mediated rejection of this tumor. In this study, we report that surface-expressed Q9 on 3LLA9F1 Lewis lung carcinoma and RMA T cell lymphoma also induces potent antitumor CTL responses. Importantly, CTL harvested from animals surviving the initial challenge with Q9-positive 3LLA9F1, RMA, or B78H1 tumors recognized and killed their cognate tumors as well as the other cancer lines. Furthermore, immunization with Q9-expressing 3LLA9F1 or RMA tumor cells established immunological memory that enhanced protection against subsequent challenge with a weakly immunogenic, Q9-bearing melanoma variant. Collectively, the generation of cross-reactive CTL capable of eliminating multiple disparate Q9-expressing tumors suggests that this nonpolymorphic MHC class I molecule serves as a restriction element for a shared tumor Ag(s) common to lung carcinoma, T cell lymphoma, and melanoma. The Journal of Immunology, 2005, 174: 5367–5374.
and the nonresponsiveness of antitumor CTL against normal splenocytes and melanocytes in cytotoxicity assays. These results suggest that a nonpolymorphic class I molecule has the capacity to present tumor Ags shared by distinct lineages of cancer cells.

Materials and Methods

Mice

C57BL/6 or C57BL/6NCr (referred to as C57BL/6) mice were either bred and maintained in the microbiology animal colony at the University of Texas Southwestern Medical Center or purchased from The Jackson Laboratory or from the National Cancer Institute Animal Production Program. Adult mice (older than 8 wk) were used for all experiments.

Cell lines and gene transfection

Q9-expressing B6 mouse-derived B78H1 melanoma derivatives B78H1Q9TAP (clone H1Q9TAP.11) and GMQ9TAP (clone GMQ9TAP.13) as well as MHC class I-negative GMITAP have been previously described (14, 16). RMA T cell lymphoma (17), 3LLA9F1 Lewis lung carcinoma (18), and Hepa-1 hepatoma (19) tumor lines were transfected to express membrane-bound canonical Q9. The Q9-transfected RMA clone P5–5.6 (designated in this study as RMA-Q9) was generated as previously described (11). Hepa-1 and 3LLA9F1 transfectants expressing either membrane-bound Q9 or plasmid alone were generated as described for the derivation of B78H1 melanoma transfectants (16). Hepa-1 and 3LLA9F1 transfected to express Q9 are designated Hepa-Q9 (clone Hepa-Q9.1) and 3LL-Q9 (clone 3LLA9FIQ9.2), respectively. Culture medium consisted of 50% DMEM/50% RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals), 1 mM sodium pyruvate (Invitrogen Life Technologies), 0.1 mM nonessential amino acids (Invitrogen Life Technologies), and 10 U/ml penicillin/10 μg/ml streptomycin (Sigma-Aldrich). All tumor line transfectants were maintained in medium supplemented with 400 μg/ml G418. Melan-A2, an immortal differentiated melanocyte line derived from skin of B6 mice (20), was generously provided by Dr. V. J. Hearing (National Cancer Institute, National Institutes of Health, Bethesda, MD) and was cultured in RPMI 1640 supplemented with 10% FBS, 200 μM 12-O-tetradecanoyl phorbol acetate (Sigma-Aldrich), 50 μM 2-ME (Sigma-Aldrich), and penicillin/streptomycin. YAC-1 lymphoma and P815 mastocytoma cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 μg/ml streptomycin, 50 μM nonessential amino acids, 50 μg/ml amphotericin B (Invitrogen Life Technologies), 1 mM sodium pyruvate, 10 mM HEPES (Invitrogen Life Technologies), and penicillin/streptomycin. All cell lines were grown at 37°C and 5% CO2. To maintain Mycoplasma-free conditions, all cell lines were treated with 0.5 μg/ml Mycoplasma Removal Agent (ICN Biomedicals) and periodically cultured in medium supplemented with 10 μg/ml Ceﬂuroxim HCl (Mediatech).

Abs and flow cytometry

Anti-Qa-2 mAb 1-1-2 was purchased from BD Pharmingen. Anti-Qa-2 mAb MAb 24 (21), anti-Kb mAb Y3 (22), and anti-Fd mAb 28-14-8 (23) were purified from ascites or hybridomas as previously described (12). FITC-goat anti-mouse IgG (Cappell) was used as a secondary Ab. For flow cytometry analysis of class I MHC Ag cell surface expression levels, up to 1 × 106 cells was washed in staining buffer (PBS with 1% FBS and 0.1% sodium azide) and pelleted. A saturating amount of primary Ab was added and the cells were incubated with 100 μl of 1% SDS. Maximum release was determined by incubating target cells with 100 μl of 1% SDS.

Tumor challenge experiments

Tumor challenge experiments were performed as previously described (14, 15). Briefly, cells for injection were harvested from in vitro culture and the indicated dosage of live tumor cells were injected s.c. in a volume of 200 μl of HBSS into the right rear flank. Mice were monitored daily for tumor growth. Mice were considered tumor-bearing when the tumor was palpable and measured at least 3 × 3 mm. Tumors were continually monitored after tumor onset to observe if tumor regression occurred. Animals were sacrificed if tumor burden showed signs of being excessive to avoid pain and suffering for rechallenge experiments, mice that had completely rejected initial tumor challenge (tumor-free after day 100) were injected in the opposite flank with 1 × 106 live tumor cells. Number of tumor cells incubated for this study. Analysis of additional clones as well as bulk transfectants revealed that all Q9-expressing 3LLA9F1 and Hepa-1 cells exhibited dramatically reduced expression of surface class Ia (data not shown). Down-regulation of resident class I

In vitro cytotoxicity assays

Tumor-reactive CTL were generated as previously described (15). Briefly, splenocytes were harvested from mice that were tumor-free 100 days after initial tumor challenge and cultured at a concentration of 5 × 105 cells per well in 24-well plates with stimulator cells in culture medium supplemented with 10 U/ml IL-2. Stimulator cells were treated with 50 μg/ml mitomycin C, then plated with splenocytes at 2 × 105 cells/ml/well. Lymphokine-activated killer (LAK)3 cells were prepared by incubating splenocytes harvested from naive mice in the presence of 500 U/ml murine rIL-2 (16). CTL and LAK cell cultures were incubated in a humidified incubator at 37°C in 10% CO2 for 5–7 days. Killing assays were performed as previously described (15, 16), with 3-fold serial dilutions of viable effector cells incubated with 1000–5000 3H-labeled target cells in round-bottom 96-well plates at 37°C and 5% CO2 for 4 h. For in vitro Ab blocking of Qa-2, target cells were incubated with saturating amounts of mAb MAb46 during the labelling step. Data are expressed as the percent specific release, calculated as follows: ((experimental release − spontaneous release)/maximum release − spontaneous release) × 100. Maximum release was determined by incubating target cells with 100 μl of 1% SDS.

Results

Classical and nonclassical MHC class I expression on tumor lines

We have previously reported that Q9 expression is selectively silenced or severely depressed in a large number of tumor cell lines (13). We have also shown that restoration of Q9 expression in the B78H1 melanoma results in potent CTL-mediated antitumor immunity against Q9-bearing melanoma (14, 15). To investigate whether the ability of Q9 to act as a restriction element for anti-tumor CTL was specific to the class Ia-deficient melanoma system or if it is a more general phenomenon, several additional tumor lines were transfected with canonical Q9 cDNA. These tumor lines originated from distinct tissues/cells from H2b mice: SMA, a line derived by ethyl methane sulfonate mutagenesis of the RBL-5 Rauscher virus-induced T cell lymphoma of C57BL/6 origin (17); 3LLA9F1, a nonmetastatic line cloned from the 3LL Lewis lung carcinoma which spontaneously arose in a C57BL/6J mouse (18); and Hepa-1, a line derived from the BW7756 s.c. hepatoma that arose in a C57L/J mouse (19).

MHC class Ia molecules shared by the studied tumors, Kb and Dd, are expressed at varying levels on vector-transfected SMA, 3LLA9F1, and Hepa-1 tumor lines (Fig. 1). SMA and 3LLA9F1 express moderate to high levels of both Kb and Dd, while class Ia expression on Hepa-1 is low, with only Dd detectable on the cell surface. Q9 is detectable on SMA, albeit at ~10-fold reduced levels as compared with expression on normal T cells (Ref. 13 and data not shown); Q9 is not detectable on 3LLA9F1 or Hepa-1, consistent with our previous observations that Q9 expression is extinguished in most malignant cells. After transfection with Q9 cDNA, Q9 surface levels in SMA-Q9 transfecent were restored to levels observed on normal T cells (11). The 3LL-Q9 and Hepa-Q9 transfecents expressed similarly high levels of Q9, presumably exceeding the levels of Q9 on normal healthy epithelial cells of lung or liver (13).

Upon ectopic expression of Q9, class Ia expression in all three tumor lines was detectably reduced. Class Ia became undetectable on the surface of Q9-transfected 3LLA9F1 and Hepa-1 cells, while in the SMA cells presence of additional Q9 molecules was accompanied by moderate decreases of surface Kb and Dd. This observation was not limited to the 3LL-Q9 and Hepa-Q9 clones selected for this study. Analysis of additional clones as well as bulk transfectants revealed that all Q9-expressing 3LLA9F1 and Hepa-1 cells exhibited dramatically reduced expression of surface class Ia (data not shown). Down-regulation of resident class I

3 Abbreviation used in this paper: LAK, lymphokine-activated killer.
MHC upon transfection with exogenous class I MHC is commonly seen during engineering of class I-transfected derivatives of laboratory cell lines (I. Stroynowski, unpublished observations). We hypothesize that this effect may be caused by highly expressed ectopic class I H chains (usually transcribed from strong promoters such as the CMV promoter) competing successfully with resident class Ia for limiting supplies of endogenous β2-microglobulin or other class I chaperone proteins.

Syngeneic mice reject Q9-transfected lung carcinoma and T cell lymphoma

Earlier studies showed that C57BL/6 mice were protected against a s.c. challenge of live syngeneic B78H1 melanoma cells expressing Q9, and were capable of rejecting the Q9-positive melanoma derivative at tumor loads 10-fold higher than the minimal lethal dose required for 100% of mice to develop tumors after challenge with Q9-negative B78H1 variants (14, 15). To determine whether Q9 expression on different types of tumors can also mediate protective host immune responses, we challenged age-matched male C57BL/6 mice with live Q9-positive and Q9-negative 3LLA9F1, RMA, and Hepa-1 tumors by s.c. injection and monitored the animals for tumor growth. Using titrated tumor dosages ranging from $1 \times 10^4$ to $1 \times 10^6$ live tumor cells, the tumorigenicity of the Q9-negative tumors could be compared with that of Q9-transfected variants.

Mice were protected against 3LLA9F1 Lewis lung carcinoma and the Q9-transfected 3LL-Q9 variant to a similar degree, with complete protection against the lowest inoculum and increasing susceptibility to the formation of established tumors with increasing tumor loads (Fig. 2, A and D). 3LLA9F1 expresses class Ia molecules Kb and Db, but not Q9. The presence of Kb and Db on 3LLA9F1 contributes to its low metastatic potential (18, 24), and these class Ia molecules may mediate host immune responses independent of Q9. However, the 3LL-Q9 clone only expresses Q9. Thus, in this tumor derivative, Q9 is likely the class I that guides host antitumor immunity, and it does so to a level comparable to that elicited by the class Ia Kb and Db Ags. These conclusions were confirmed by additional experiments described in later sections of this study.

RMA T cell lymphoma, which expresses Kb,Db, and low level of Q9, was capable of establishing s.c. tumors when administered at the high dose of $1 \times 10^6$ cells (Fig. 2B). Using $1 \times 10^5$ cells, tumor outgrowth was observed in a few animals, but tumors regressed within 1 wk of tumor onset and all mice were tumor-free by day 25. The specific protective effect of Q9 was discernible when it was the dominant MHC class I Ag expressed on the cell surface. RMA-Q9 cells were unable to form detectable tumors, even at the highest, $1 \times 10^6$, tumor load (Fig. 2E). To demonstrate that the RMA-Q9 variant was in vivo competent, immunodeficient SCID Beige mice were challenged with $1 \times 10^5$ cells. Outgrowth of RMA-Q9 in these hosts, which have impaired T cell, B cell, and NK cell responses, confirmed the malignant potential of this variant (Fig. 2E). The ability of syngeneic C57BL/6 mice to be fully protected against the RMA-Q9 challenge (with tumors rejected in

FIGURE 1. MHC class I expression on tumor cell lines and transfectants. Flow cytometry analysis was performed on vector-transfected (left panels) and Q9-transfected tumor lines (right panels). Cells were stained with Q9-specific mAb 1-1-2 (filled histograms), anti-Kb mAb Y3 (thick solid line), or anti-Db mAb 28-14-8 (thin solid line). Background staining with secondary Ab FITC-GAM alone is shown with thin dotted line histograms.

FIGURE 2. Tumor take of Q9-transfected tumor cells in B6 mice. Animals were challenged with a s.c. injection in the right rear flank of live tumors cells at varying dosages (C, $1 \times 10^3$; Δ, $1 \times 10^2$; □, $1 \times 10^4$). Tumor growth was monitored for over 100 days and percent of mice with palpable tumor was recorded as Kaplan-Meier plots. The ability of vector-transfected 3LLA9F1 lung carcinoma (A), RMA T cell lymphoma (B), and Hepa-1 hepatoma (C) to establish tumors in B6 hosts was compared with that of Q9-transfected 3LL-Q9 (D), RMA-Q9 (E), and Hepa-Q9 (F), respectively. Ten mice were used for each group. Filled triangles (Œ) in E represent tumor take in SCID Beige animals (n = 5) to demonstrate in vivo competency of RMA-Q9 cells.
all mice) indicates that host responses evoked by Q9 may be more potent than those generated by K\(^\alpha\) and/or D\(^\alpha\) on parental RMA.

The Hepa-1 hepatoma, which only expresses low levels of D\(^\alpha\), is weakly tumorigenic in C57BL/6 mice, with tumor outgrowth only detectable with the highest initial tumor burden of 1 \(\times\) 10\(^6\) cells (Fig. 2C). Even at this high dose, tumors that do arise quickly regress. This phenotype may be due to the partial incompatibility between H2\(^\alpha\)-positive C57BL/6 hosts with the H2\(^\alpha\)-positive C57L/J-derived tumor (outside of the H2 locus) and the recognition of allelogenic/minor histocompatibility Ag-derived epitopes in the context of D\(^\alpha\). Alternatively, rejection may be mediated by presentation of hepatoma-specific tumor Ags by D\(^\alpha\), as suggested by studies of CTL involvement in the rejection of Hepa-1 in C57L/J mice (25). Intriguingly, Q9 expression on Hepa-1 (with concomitant down-regulation of D\(^\alpha\)) results in dramatically increased tumorigenicity, with nearly half of the mice developing tumors even when given the lowest challenge dose of 1 \(\times\) 10\(^4\) cells (Fig. 2F). Multiple mechanisms may contribute to enhanced tumor growth in the Q9-positive, class Ia-deficient hepatoma system. We favor a hypothesis that Hepa-1 tumor does not express Q9-restricted tumor antigenicity, with nearly half of the mice developing tumors even when given the lowest challenge dose of 1 \(\times\) 10\(^4\) cells (Fig. 2F).

Q9-restricted antitumor CTL exhibit cross-reactivity against multiple tumors

To assess whether Q9-restricted CTL were generated against -Q9 and RMA-Q9, in vitro cytotoxicity assays were performed using effector cells generated from animals that had rejected these tumors. In Fig. 3A, splenic CTL harvested from C57BL/6 survivors of 3LL-Q9 challenge were restimulated in vitro and tested for their ability to kill 3LL-Q9 cells and other targets. CTL derived in this manner not only killed 3LL-Q9 targets, but exhibited similar reactivity against the GMQ9TAP B78H1 melanoma derivative (GM-CSF transduced, Q9- and TAP-positive) and RMA-Q9 tumors. The cross-reactivity of these anti-3LL-Q9 CTL was not a general phenomenon, as Hepa-Q9 cells were not lysed. These cross-reactive antitumor CTL were nonetheless Q9-restricted, as Q9-negative GMTAP B78H1 melanoma (GM-CSF transduced, TAP-positive) and 3LLA9F1 were not killed. When GMQ9TAP cells were used as stimulators for CTL harvested from 3LL-Q9 survivor mice, a similar pattern of CTL recognition against the tumor targets was seen (Fig. 3D), providing further evidence that CTL generated against 3LL-Q9 in vivo display a shared specificity against Q9-bearing tumors.

Splenic CTL harvested from mice that had rejected RMA-Q9 challenge and restimulated with either 3LL-Q9 (Fig. 3B) or GMQ9TAP (Fig. 3E) exhibited similar cross-reactivity against 3LLA9F1, RMA, or B78H1 targets expressing Q9, as did CTL raised in mice that had rejected GMQ9TAP and were restimulated with either 3LL-Q9 (Fig. 3C) or GMQ9TAP (Fig. 3F). These results indicate that Lewis lung carcinoma, T cell lymphoma, and melanoma may share common tumor Ag(s) presented in the context of Q9, and that the Ag(s) is not expressed in hepatoma. The inability of Hepa-Q9 to be lysed was not due to expression of defective Q9, as Hepa-Q9 reacted with conformation-dependent anti-Q9 mAb (Fig. 1), was efficiently recognized by anti-Q9 allospecific CTL (data not shown), and inhibited NK cell cytotoxicity in in vitro killing assays (data not shown).

Cold target inhibition assays were performed to provide further evidence as to whether shared tumor Ags were presented (Fig. 4). The ability of CTL generated from GMQ9TAP challenged hosts to lyse \(^{51}\)Cr-labeled GMQ9TAP targets was reduced in the presence of unlabeled cold GMQ9TAP cells. Addition of cold Q9-positive tumor cells (RMA, RMA-Q9, and 3LL-Q9) inhibited lysis of GMQ9TAP targets to a similar degree. Addition of cold Q9-negative GMTAP, 3LLA9F1, and Hepa-1 cells had minimal impact on cytolysis of GMQ9TAP. Importantly, cold Hepa-Q9 did not inhibit killing of GMQ9TAP targets. Thus, tumor-reactive CTL appear to be restricted by Q9-presented epitope(s) derived from melanoma, Lewis lung carcinoma, and T cell lymphoma, but not hepatoma.

**FIGURE 3.** Cross-reactive antitumor CTL are generated in mice challenged with various Q9-expressing tumors. Effector CTL were obtained by harvesting splenocytes from mice that had rejected Q9-expressing 3LL-Q9 (A and D), RMA-Q9 (B and E), or B78H1 melanoma derivative GMQ9TAP (C and F) tumor challenge and restimulating them in vitro with mitomycin C-treated 3LL-Q9 (A–C) or GMQ9TAP (D–F) cells. CTL were then tested for their ability to lyse class I-negative B78H1 melanoma derivative GMTAP (C), Q9-transfected GMQ9TAP melanoma (D), Q9-negative 3LLA9F1 lung carcinoma (E), Q9-transfected 3LL-Q9 lung carcinoma (F), Q9-transfected RMA-Q9 T cell lymphoma (A), or Q9-transfected Hepa-Q9 hepatoma (B). Error bars are not shown for clarity; SE of triplicate measurements was <10% at each data point. Data shown are representative of five experiments.

**FIGURE 4.** Cold target inhibition assays. Splenocytes harvested from hosts challenged with GMQ9TAP cells were restimulated in vitro with GMQ9TAP to generate CTL. Effector were then incubated with \(^{51}\)Cr-labeled GMQ9TAP cells at an E:T ratio of 200:1 in the absence of cold inhibitor (D) or presence of increasing numbers of unlabeled cold targets (Q9-transfected or untransfected tumor cells as indicated on horizontal axis) added to give final percentages of 10% (E), 20% (F), and 40% (G) cold cells. Triplicate measurements were taken after 4 h of incubation and data is shown as mean ± SE. The experiment was performed twice with similar results.
The requirement for Q9 as a restriction element for these cross-reactive antitumor CTL is demonstrated by in vitro Ab blocking experiments, where the ability of CTL to kill GMQ9TAP, 3LL-Q9, and RMA-Q9 targets is abolished by preincubating target cells with anti-Q9 mAb (Fig. 5).

Cross-protection against melanoma is established in mice that have rejected disparate Q9-expressing tumors

The shared specificity of antitumor CTL implies that the animals that have rejected a challenge with one of these tumors may be immune to a subsequent challenge with a different tumor. We examined this postulate by testing the ability of the weakly immunogenic B78H1 variant, B78H1Q9TAP, to establish tumors in animals that have rejected 3LLA9F1, RMA or, as a control, Hepa-1. The B78H1Q9TAP melanoma variant has been previously shown to be eliminated in animals preimmunized with highly immunogenic GMQ9TAP melanoma in a CTL-dependent manner (15). In Fig. 6A, the seven mice that had survived a challenge with $1 \times 10^5$ live 3LL-Q9 tumor cells (Fig. 2D) received a s.c. injection of $1 \times 10^5$ live B78H1Q9TAP cells in the opposite flank. This dose leads to 100% mortality in naive C57BL/6 hosts. Remarkably, none of these 3LL-Q9 preimmunized animals developed detectable B78H1Q9TAP tumors, indicating that prior exposure to Q9-bearing Lewis lung carcinoma resulted in protective immunity against melanoma expressing Q9. In contrast, B78H1Q9TAP was able to establish tumors in Q9-negative 3LLA9F1-immunized animals with similar kinetics as observed in unimmunized naive C57BL/6 mice (Fig. 6A). These results indicate that protective host immunity against B78H1Q9TAP melanoma in 3LL-Q9-challenged mice is dependent on immunological memory established by Q9-restricted CTL. Similarly, mice that have been previously immunized with RMA (low Q9 expression) or RMA-Q9 (high Q9 expression) were able to reject subsequent challenge with B78H1Q9TAP (Fig. 6B). Thus, even though RMA and RMA-Q9 display differences in tumorigenicity (Fig. 2, B and E) due to differences in Q9 and/or class Ia MHC expression levels (Fig. 1), successful elimination of RMA or RMA-Q9 leads to the establishment of Q9-restricted immunological memory.

Mice that had been immunized with Q9-negative Hepa-1 were not protected against subsequent B78H1Q9TAP challenge (Fig. 6C). Although rejection of Hepa-1 is CTL-mediated in C57L/J mice (25), it is clear that any immunological memory established in response to Hepa-1 challenge in C57BL/6 animals is ineffective at eliminating D9-negative, Q9-positive B78H1Q9TAP cells. Indeed, even if Q9-restricted, anti-Hepa-1 CTL participated in the clearance of the initial challenge, they were directed against a tumor epitope that is not presented by melanoma cells. These results support in vitro cytotoxicity data showing that Q9-restricted antitumor CTL did not recognize Q9-expressing hepatoma targets.

Melanoma-reactive antitumor CTL are nonresponsive against syngeneic melanocytes or self lymphoblasts

In humans, peptide-based immunotherapies target tumor Ags presented by HLA class I to promote tumor-specific CTL responses. When effective CTL response are generated, autoimmune destruction of normal melanocytes and consequent vitiligo often results (26, 27). Similar autoimmune effects have also been observed in mouse models (28). We have not observed vitiligo in any of the animals that had rejected the melanoma challenge after prior immunization with any of the Q9-expressing tumor derivatives. To confirm the absence of autoimmunity using in vitro techniques, we tested whether CTL harvested from mice that had rejected GMQ9TAP or 3LL-Q9 tumors were reactive against a C57BL/6-derived melanocyte cell line, Melan-a2 (20). This immortalized differentiated line recapitulates many of the phenotypic properties of freshly isolated melanocytes, and therefore is used in lieu of primary melanocytes. Q9 is not serologically detectable on the surface of Melan-a2 but its expression is up-regulated upon stimulation with IFN-γ (Fig. 7A). When anti-GMQ9TAP (Fig. 7C) or

**FIGURE 6.** Rejection of Q9-expressing lung carcinoma or T cell lymphoma generates cross-protection against weakly immunogenic Q9-bearing melanoma. Animals that had remained tumor-free 100 days postchallenge received a s.c. injection of $1 \times 10^4$ live B78H1Q9TAP cells in the opposite flank, and tumor outgrowth of melanoma was monitored. A, Mice were initially challenged with 3LLA9F1 (open symbols, $n = 5$ for each tumor load) or 3LL-Q9 (filled symbols, $n = 10$ for $1 \times 10^4$, $n = 7$ for $1 \times 10^5$). B, Mice were initially challenged with RMA (open symbols) or RMA-Q9 (filled symbols), $n = 10$ for each tumor load. C, Mice were initially challenged with Hepa-1 (open symbols, $n = 5$ for each tumor load) or Hepa-Q9 (filled symbols, $n = 6$). Naive mice (diamonds, $n = 10$) were included to monitor normal tumor take of B78H1Q9TAP.

**FIGURE 5.** CTL effector function is inhibited by Q9-specific mAb. CTL generated by harvesting splenocytes from animals that had survived challenge with GMQ9TAP B78H1 melanoma derivative and in vitro restimulation with GMQ9TAP were used as effectors against target cells preincubated in the absence or presence of anti-Q9 mAb M46. Data shown are mean ± SE of triplicate measurements taken at an E:T ratio of 200:1. One representative experiment of four is shown.
anti-3LL-Q9 (data not shown) CTL were mixed with resting or IFN-γ-treated Melan-a2 cells, no lysis was observed. This indicates that Q9-restricted antitumor CTL do not recognize Ags presented on cells of the nonmalignant melanocytes. To test whether antitumor CTL would respond against other types of “self” cells, Con A-activated splenic T cell blasts were used as targets. Blast targets derived from C57BL/6 mice expressing high levels of Q9 on their surface (Fig. 7B) were not killed by anti-GMQ9TAP (Fig. 7C) or anti-3LL-Q9 (data not shown) CTL. This supports the notion that Q9 presents shared tumor-associated Ags aberrantly up-regulated in multiple tumors, rather than self Ags which could mark normal cells for autoimmune destruction.

**Discussion**

Membrane-bound Q9 is one of the most thoroughly characterized murine MHC class Ia proteins. It shares extensive amino acid sequence homology with class Ia H2-K, -D and -L alleles, comparable to the homologies of class Ia proteins with each other. The overall crystal structure of Q9 is also remarkably similar to the homologies of class Ia proteins with each other. The unique features of Q9-binding peptides needed for Q9 complex stabilization and anchoring via His/Arg interactions of peptide with the groove indicate that this class I MHC molecule evolved to accommodate a different set of ligands than those presented by class Ia. Furthermore, the tenuous contacts between the dominant self-peptide L19 with Q9 imply that well-fitting, high-affinity ligands will effectively displace L19 and other ill-fitting peptides. The competitive replacement may occur under conditions that lead to the generation/up-regulation of novel, Q9-tailored endogenous peptide. Such conditions may include cellular stress, pathogen infection, or neoplastic transformation. If this scenario is correct, it would result in elimination of Q9-bearing cells expressing stress or tumor-associated Q9-presented Ags.

Several lines of evidence indicate that Q9 is indeed involved in the elimination of malignantly transformed cells. Although Q9 is expressed in primary cultures and cell lines derived from different tissues, it is selectively shut off in a large number of established, in vivo-selected tumor lines (13). This observation suggests that Q9 renders tumors susceptible to immune surveillance and that tumors down-regulate this class Ib molecule, independently of other MHC class I, as an escape mechanism. Ectopic expression of Q9 in class Ia- and Ib-deficient B78H1 melanoma promoted rejection of this tumor by syngeneic CTL and led to induction of immunological memory (14, 15). The immunogenic properties of Q9-transfected B78H1 were dependent on the presence of functional TAP-generated Ag presentation pathway (15). This led us to propose that Q9 rendered B78H1 cells sensitive to CTL upon immune recognition of melanoma-expressed peptide Ags bound to this class Ib. Although many melanoma tumor Ags, such as tyrosinase, gp100, MART1, and tyrosinase-related proteins-1 and -2 (3, 26), are found uniquely in tumors of this lineage, others are shared among diverse cancers (3).

In this study, we tested whether Q9 complexes displayed on 3LLA9FI Lewis lung carcinoma, RMA T cell lymphoma, and Hepa-1 hepatoma served as restriction elements for antitumor CTL and if the effector CTL were cross-reactive. Although the individual actions of MHC class Ia molecules on immune recognition of these tumor systems have been well-characterized, the roles of the nonclassical class I in antitumor immunity have not been defined. For instance, in the Lewis lung carcinoma, Kk has been shown to present tumor-associated Ags MUT1 and MUT2 and induce CTL activity (29, 30). Because the class Ia molecules mediate host responses against these tumors, the impact of transfected Q9 Ag must be interpreted in the context of the overall class I expression pattern. Fortuitously, introduction of Q9 resulted in the loss of Kk and Dk surface expression, creating tumor models similar to the melanoma system (14) where Q9 effects could be more easily interpreted. Although we propose that Q9 is dominant in orchestrating the potent antitumor response against the cloned transfectants, we also acknowledge that host responses to these tumor variants may have been additionally altered due to the down-regulation of endogenous class I. These alterations may affect CTL, NK cell, and/or macrophage-mediated pathways.

Q9 on Lewis lung carcinoma and T cell lymphoma rendered the tumors susceptible to elimination by an adaptive immune response and led to establishment of protective immunity against subsequent challenges with syngeneic, disparate tumors. Mice that had been immunized against Lewis lung carcinoma or T cell lymphoma were capable of rejecting a weakly immunogenic, Q9-expressing B78H1 melanoma derivative. CTL raised in response to challenge with Q9-expressing 3LLA9FI lung carcinoma, RMA T cell lymphoma or GMQ9TAP B78H1 melanoma efficiently killed...
any of these tumors in a Q9-restricted fashion in cytotoxicity assays. Thus, CTL generated in the primary response establish a pool of memory cells that exhibit cross-reactivity against a number of different tumors. This favors the notion that Q9 presents a shared tumor Ag common to lung carcinoma, T cell lymphoma, and melanoma.

The idea of a shared tumor Ag is not novel. Many of the human tumor Ags that have been identified are shared among several different tumors (3). For example, a cancer/testis tumor Ag derived from GAGE-1, -2, -8 genes is commonly expressed in melanoma, sarcoma, non-small cell lung carcinoma, small cell lung carcinoma, seminoma, leukemias, lymphomas, head and neck tumors, bladder carcinoma, esophageal carcinoma, mammary carcinoma, colon carcinoma, and prostate carcinoma (31, 32). Cancer/testis Ags are typically expressed by a wide variety of tumors and result from the reactivation of genes that are normally expressed only by spermatocytes and/or spermatogonia of testis and perhaps placenta or ovary cells. Other widely expressed tumor Ags may be expressed on normal tissues, but at levels below the threshold required for T cell recognition. Although the identity of the Q9-presented tumor-associated Ag is currently unknown, it is not commonly expressed in normal healthy cells. The tumor-reactive CTL restricted by Q9-positive tumors did not recognize Q9-expressing normal splenocytes or melanocytes and did not induce vitiligo. The absence of autoimmune effects were in contrast to those observed in some human and mouse tumor systems where antitumor CTL provoked recognition of self normal cells (26–28).

Q9-expressing Hepa-1 cells were not rejected and were not recognized by cross-reactive antitumor CTL, indicating that this particular hematoma does not express the Q9-restricted shared Ag or other Q9-restricted epitopes that could lead to recognition and eradication of these tumor cells in a CTL-dependent fashion. Although Hepa-1 may have never expressed any Q9-binding tumor Ags, it is also possible that initial expression of any such immunogenic epitopes may have been extinguished during the progression of the tumor (33).

The increased capacity of Hepa-Q9 to establish tumors in the absence of Q9-restricted CTL raises questions about the potential importance of NK cells and other immune effectors in the elimination of different model tumors. We previously reported that NK cells were essential for rejection of large doses of Q9-positive melanoma cells, even in the presence of an active antitumor CTL response (14). In the absence of CTL, NK cells were sufficient for rejection of low doses of such melanoma challenge. NK cell-mediated rejection of Q9-bearing melanoma occurred despite the partially diminished capacity of bulk NK (LAK) cells to kill targets displaying Q9 (15, 16). In data not shown in this study, lung carcinoma and hepatoma were also found to be extremely sensitive to C57BL/6-derived LAK cells and transfection of Q9 into these cells rendered them ~30% less sensitive to killing in vitro. Although the partial protective effect of Q9 conferred upon target cells against NK cell-mediated cytolysis may be insufficient to change the outcome of tumor progression in the lung carcinoma model, we hypothesize that it may contribute to the observed outgrowth of Hepa-Q9. Interestingly, RMA-Q9 and RMA, which are inherently resistant to NK cells, grow poorly in vivo, suggesting that CTL responses are dominant in controlling their outgrowth. Thus, it is reasonable to surmise that in different tumor models, NK cells may contribute to the elimination of tumor cells to varying degrees, and that the early killing of tumor cells initiated by NK cells may be partially tempered by Q9 and/or other class I interactions with inhibitory NK cell receptors. The dynamic interplay between CTL, NK cells, and other effector cell populations mediated by Q9 in tumor immunity is of great interest to us and merits detailed investigation.

Our findings raise intriguing possibilities for the exploitation of nonpolymorphic class Ib molecules in immunotherapy against cancer. Identification of the shared tumor Ag(s) presented by Q9 would allow us to explore vaccine strategies in the mouse model. Application of this approach to the human system would require identification of human homologues or working substitutes for Qa-2. To date, no “true” orthologous relationships between mouse and human class I genes have been delineated (34, 35), and given the current knowledge of human class I products, it is unlikely that an exact homologue of the nonpolymorphic Q9 class I molecule will exist in humans. However, it is plausible that some of the properties of Qa-2 will be selectively associated with a specific HLA locus.

One candidate that merits evaluation is HLA-C. HLA-C polymorphism has been, until recently, considered to be negligible. At present, it is estimated that it constitutes only a fraction of that observed in HLA-A and -B loci, with ~40 known alleles (36). Because many HLA-C alleles may fall into the same dominant “supertype,” with overlapping peptide-binding motifs and reper- toires (37, 38), the functional polymorphism of HLA-C may be very limited indeed. Importantly, HLA-C-restricted, tumor-spe-
cific CTL have been generated against a number of melanoma-associated Ags that are shared among a large number of tumors (32, 39). Whether HLA-C may be considered as a candidate for “universal” vaccination strategies against conserved tumor Ags re-
mains to be determined.

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