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Identification of a K^b-restricted CTL Epitope of β-Galactosidase: Potential Use in Development of Immunization Protocols for ''Self'' Antigens

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The use of recombinant and synthetic vaccines in the treatment of cancer has recently been explored using model tumor associated antigens (TAA), many of which do not model the immunological state of affairs in which the TAA is expressed by normal tissues. One potentially useful model Ag is β-galactosidase (β-gal). Because the activity of this enzyme is so easily detectable, this gene has been inserted into a large number of recombinant viruses and tumors useful to the cancer vaccinologist. In addition, numerous transgenic mouse colonies that have tissue-specific expression of β-gal have been developed, enabling the modeling of tolerance to ''self'' Ags. Since most of these mice have an H-2^b background, we generated cytotoxic T lymphocytes (CTL) capable of recognizing β-gal-expressing tumor cells of C57BL\6 origin and have determined that their restriction element is the K^b molecule. Using an allele-specific epitope forecast to generate a panel of candidate peptides, we have determined that the K^b-restricted sequence is DAPIYTNV and corresponds to amino acids 96–103 of the intact β-gal molecule. A recombinant vaccinia virus (rVV-ES β-gal_{96-103}) was constructed that encoded the peptide epitope preceded by an endoplasmic reticulum insertion signal sequence. Tumor cells infected with this rVV were recognized by the original CTL that had been used to identify the epitope. Furthermore, splenocytes of mice immunized with a rVV encoding the full-length β-gal molecule and restimulated with the DAPIYTNV peptide specifically recognized tumor cells expressing β-gal. The identification of this immunogenic β-gal sequence enables the modeling of immunization strategies in animal models of malignant disease in which the target antigen is a ''self'' protein.

Studies of the induction of cellular immune responses have focused on the use of defined protein antigens. The availability of genes that encode antigens and the identification of specific MHC class I and II restricted peptide epitopes have contributed greatly to our understanding of T-cell-mediated immune responses. Furthermore, the generation of transgenic and knock-out mice has enabled the modeling of immune responses *in vivo*. Recently, we and others have focused on generating immune responses against tumor antigens using a variety of recombinant and synthetic vaccines (1–5).

To facilitate the study of the efficacy and mechanisms of anti-tumor responses we developed a tumor model consisting of an adenocarcinoma stably transfected with the *Escherichia coli* LacZ gene. An advantage of using such a model tumor antigen is the availability of a broad spectrum of reagents. The LacZ gene has been sequenced (6) and cloned into a wide range of recombinant DNA vectors, the purified protein as well as polyclonal and monoclonal antisera and ELISA kits for quantitation are readily available, and the X-gal staining reagent facilitates the determination of qualitative and quantitative expression of the protein. Numerous cell lines stably transfected with the LacZ gene have been generated, as well as recombinant and synthetic vaccine vectors that encode β-galactosidase (β-gal). Furthermore, the immunogenic H-$2L^d$-restricted β-gal peptide TPHPARIGL has been identified. Our group has employed recombinant fowlpox virus, vaccinia virus, modified vaccinia Ankara, adenovirus, and influenza

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virus as well as helium-driven ballistic device ("gene gun")-mediated recombinant DNA transfer for successful vaccination of mice against the β-gal L^d epitope. (1–3, 7, 8). Additionally, we have identified adjuvants and immunization strategies to further increase the magnitude of the immune response against tumors expressing β-gal (9–12).

Most of the shared antigens that have been identified on human tumor cells are nonmutated proteins expressed by normal tissue as well, e.g., MAGE-1 (13), gp100 (14), MART-1/Melan-A (15), tyrosinase (16), and HER-2/*neu* (17, 18). Since β-gal is not expressed in mice, our model does not account for possible tolerization or active suppression of tumor antigen-specific cytotoxic T lymphocytes (CTL). However, the widespread use of β-gal as a reporter gene in developmental studies has led to the generation of transgenic mouse strains engineered to express β-gal in normal cells, thereby making it a "self protein." Most of these mice are bred on a C57BL/6 background, presenting the need to identify an immunogenic H-2^b-restricted epitope of β-gal to take advantage of developed vaccination strategies. Furthermore, this peptide would enable the modeling of our current clinical efforts in peptide-based vaccinations of melanoma patients in a self-antigen setting. In this report, we present the identification of such a peptide and the implications for new models of cancer immunotherapy.

MATERIAL AND METHODS

Animals and Cell Lines

Female C57BL/6 (H-2^b) mice, 6–10 weeks old, obtained from Frederick Cancer Research Center (Frederick, MD) and maintained in a barrier facility, were used for all experiments. The CT26 colon carcinoma (H-2^b) and the β-gal transfectant C25 have been described previously (1–3), as have the EL-4 thymoma (H-2^b) and the β-gal transfectant E22 (1, 7). CT26, EL-4, and MC-38, a C57BL/6-derived colon carcinoma (19), were maintained in CM (RPMI 1640 with 10% heat inactivated FBS (Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 50 μg/ml gentamicin sulfate (NIH Media Center)). C25 and E22 were maintained in CM + 400 μg/ml of bioactive G418. BSC-1 cells (ATCC; American Type Culture Collection, Rockville, MD) were maintained in EMEM with 10% heat inactivated FBS (Biofluids), 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 50 μg/ml gentamicin sulfate (NIH Media Center).

Recombinant Vaccinia Viruses

All recombinant vaccinia viruses (rVV) used in this study were generated by insertion of the foreign genes into the rVV thymidine kinase (TK) gene by homologous recombination, resulting in the generation of TK-negative progeny as previously described. Purified virus was prepared using a modification of the method described by Earl and Moss (20). Briefly, 175-cm^2 flasks of HeLa cells were infected at a multiplicity of infection (m.o.i.) of 5–10 PFU and incubated at 37°C for 72 h. Infected cells were harvested and centrifuged at 1000*g* for 10 min. Cells were resuspended in 10 mM Tris (pH 9.0) and lysed by 30 strokes of a Dounce homogenizer. Nuclei and cell debris were partially removed by centrifugation for 5 min at 1000*g*. The crude lysate was then loaded onto a cushion of 36% sucrose in 10 mM Tris and centrifuged at 30,000*g* for 40 min. The pellet was resuspended in 3 ml of PBS and stored at −80°C. Titering of the virus was performed on BSC-1 cells as described. The rVV synthetic early/late (S E/L) β-gal employs a completely synthetic early/late promoter, driving high-level expression of β-gal (21). The rVV V69 encodes the influenza nuclear protein (NP) driven by the strong late promoter p11 (22).

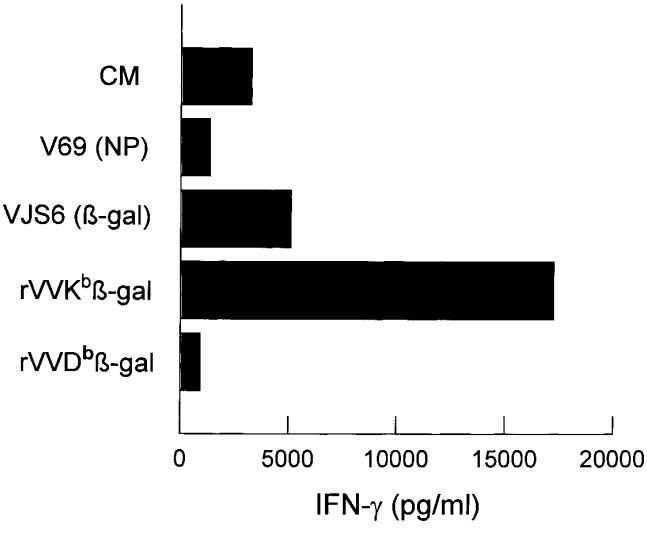

FIG. 1. β-Gal-specific CTL are H-$2K^b$ restricted. BSC-1 cells were infected with rVV encoding NP, β-gal, H-$2D^b$ and β-gal, or H-$2K^b$ and β-gal. CTL were added at a 1:1 E:T ratio and supernatants were taken after 24 h. IFN-γ release was measured by ELISA.

Both viruses have been shown to generate *in vivo* specific immune responses against the heterologous protein. The rVVK^b and rVVD^b, both expressing β-gal, were provided by Drs. J. Bennink and J. Yewdell, NIAID, NIH, Bethesda, MD. The rVVs encoding the β-gal 96–103 and 876–884 peptides were constructed using the adenovirus E3/19K signal peptide sequence fused to the β-gal minigene sequences and inserted downstream from an early VV promoter in VV expression plasmid pKT1430. This plasmid vector facilitates the insertion of foreign genes into the viral ribonucleotide reductase large subunit locus. Infection of target cells with rVV was performed for 2 h at an m.o.i. of 10 in CM. Cells were washed once in CM before use.

Peptides

The synthetic peptide SIINFEKL, spanning amino acids 257–264 of ovalbumin (OVA), ASNENMETM, spanning amino acids 366–374 of NP, and DAPIYTNV, spanning amino acids 96–103 of β-gal, were synthesized by Peptide Technologies (Washington, DC) to a purity greater than 99% as determined by HPLC and amino acid analysis. The OVA- and β-gal-derived peptides are H-2K^b restricted; the NP peptide is restricted by H-2D^b.

Generation of a β-Galactosidase-Reactive T-Cell Line

The generation of the β-gal-reactive cell line has been described elsewhere. Briefly, mice were immunized iv with 1×10^9 rAd2CMVβ-gal, and spleen cells were harvested at Day 21 and cultured with K78 hepatoma (H-2^b) infected with rAdCMVβ-gal in CM + 30 I.U./ml rIL-2 (Chiron Corporation, Emeryville, CA). After two weekly restimulations, subsequent restimulations were performed using E22. The line was >98% CD8$^+$ by FACS (not shown).

Evaluation of CTL Responses

Mice were immunized by iv injection with the specified rVV. Spleens were harvested on Day 21,

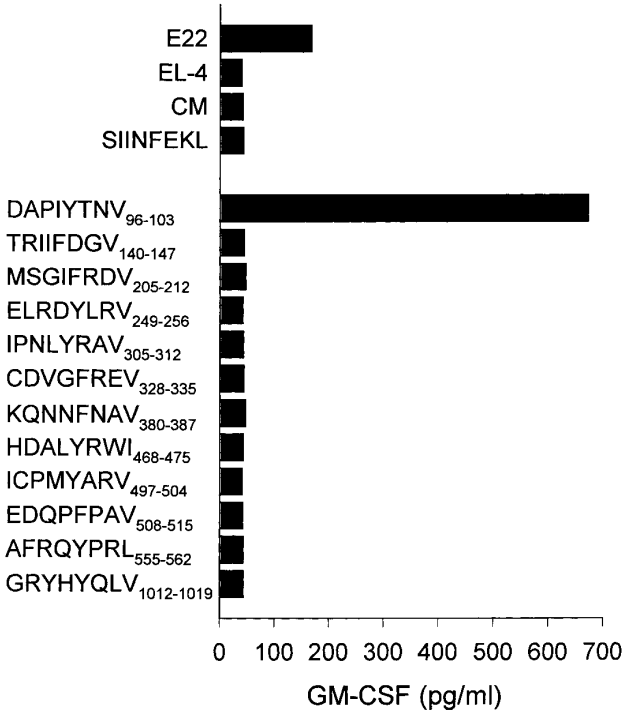

FIG. 2. β-Gal-specific CTL recognize the β-gal peptide 96–103 DAPIYTNV. CTL were added at a 1:1 E:T ratio to EL-4 cells pulsed with each of 12 peptides containing the optimal K^b-binding motif. Supernatants were taken after 24 h and GM-CSF release was measured by ELISA.

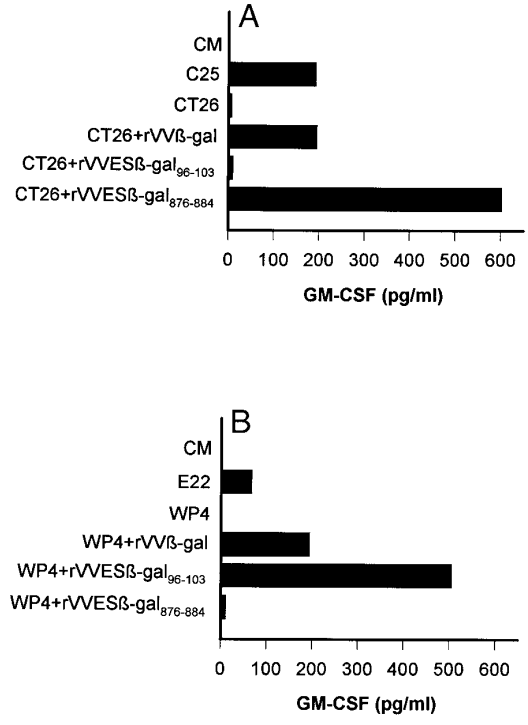

FIG. 3. Target cells infected with rVV encoding minimal determinant eptiopes of β-gal are recognized by CTL in a MHC-I restricted fashion. Target cells CT26 (H-2^d) and WP4 (H-2^b) were infected at an m.o.i. of 10 with rVV encoding minigenes for the L^d-restricted β-gal epitope TPHPARIGL, the K^b-restricted β-gal epitope DAPIYTNV, or the full-length β-gal molecule. The targets were incubated for 3 h in a 96-well plate (10^5 cells/well), and β-gal-specific H-2^d-restricted CTL (A) or H-2^b-restricted CTL (B) were added at an E:T ratio of 1:1. Supernatants were taken after 24 h and GM-CSF release was measured by ELISA.

separated into single-cell suspension, and cultured in 75-cm^2 flasks at 3 spleen equivalents/flask in 30 ml CM. Peptide was added to a final concentration of 10 μg/ml. After 6 days of culture, cells were harvested, counted, and used in a cytokine release assay.

Cytokine Release Assay

To determine cytokine release, 4×10^5 effector cells were added to 4×10^4 target cells/well in round-bottom 96-well plates and incubated overnight in CM. Supernatants were collected and tested using the mIFN-γ ELISA kit (Endogen, Cambridge, MA) or the mGM-CSF ELISA kit (R&D systems, Minneapolis, MN) according to the manufacturer's protocol.

RESULTS

The H-2^b β-Gal Response Is K^b-Restricted

To identify the restriction element presenting the dominant MHC class I β-gal epitope in C57BL/6 mice, green monkey kidney cells (BSC-1) were infected with rVV encoding β-gal and either K^b or D^b to allow presentation of β-gal-derived peptides on either K^b or D^b MHC-I molecules. BSC-1 cells infected with VJS6, which encodes β-gal but no MHC-I molecules, or V69, encoding influenza NP, did not induce significant IFN-γ release. Specific IFN-γ release by the β-gal-specific CTL line was observed only against cells infected with the virus encoding K^b and β-gal, indicating the anti-β-gal response was K^b-restricted (Fig. 1).

A Unique Peptide Is Recognized by β-Gal-Specific CTL

Since β-gal is a foreign antigen, and tolerance against high binders for MHC class I was therefore not expected, all 12 peptides present in the β-gal molecule that corresponded to the optimal binding motif for K^b (XXXX{F,Y}XX{L,M,I,V}) (23) were identified and synthesized. Pulsing of these peptides onto EL-4 cells (H-2^b) resulted in specific cytokine release by CTL to only one peptide, spanning amino acids 96–103 that has the sequence DAPIYTNV. EL-4 cells pulsed with OVA control peptide were not recognized, but the β-gal-expressing cell line E22 evoked GM-CSF release as well (Fig. 2). Similar results were obtained using an IFN-γ release assay (data not shown). Target cells infected with rVV encoding the DAPIYTNV peptide epitope, but not the H-2^d-restricted TPHPARIGL β-gal peptide epitope, were also recognized by β-gal-specific CTL, further confirming the identity of DAPIYTNV as an immunogenic epitope in the β-gal molecule (Fig. 3).

Specific CTL Can Be Generated Using the DAPIYTNV Peptide

To examine whether the DAPIYTNV peptide was able to restimulate primed splenocytes *in vitro*, splenocytes from naive mice or mice immunized on Day 0 with 1×10^7 rVV encoding S E/L β-gal were harvested on Day 21 and incubated with 10 μg/ml of indicated peptide in CM for 6 days. Cells were used in a GM-CSF release assay (Fig. 4). Only splenocytes from animals that had been immunized with S E/L *in vivo* and restimulated with DAPIYTNV *in vitro* released GM-CSF to E22 or peptide-pulsed EL-4 cells. This release was specific, since unpulsed and OVA peptide-pulsed EL-4 cells were not recognized.

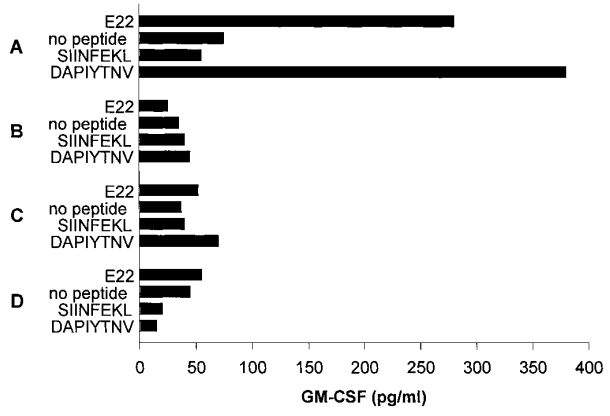

FIG. 4. DAPIYTNV peptide induces specific CTL in secondary splenocyte cultures. Splenocytes from naive mice (C and D) or from mice immunized with rVV encoding β-gal (A and B) were cultured for 6 days in the presence of 10 μg/ml of DAPIYTNV β-gal$_{96-103}$ peptide (A and C) or SIINFEKL OVA$_{257-264}$ peptide (B and D). These cells were washed and tested in a 10:1 E:T ratio against E22, EL-4 pulsed with DAPIYTNV peptide, EL-4 pulsed with SIINFEKL peptide, or EL-4 alone. Supernatants were taken after 24 h and GM-CSF release was measured by ELISA.

DISCUSSION

The identification of specific tumor-associated antigens (TAA) in human and murine systems has greatly accelerated the development of recombinant and synthetic cancer vaccines (24–26). The recognition of the self-protein nature of TAA has sparked

interest in studying murine models of anti-self immune responses. We have previously reported studies employing β-gal as a nonself antigen on tumor cells, showing the efficacy of *in vivo* vaccination approaches, leading to curative, long-lasting anti-tumor immunity (reviewed in (8)). These efforts effectively model the situation in virally and possibly chemically induced human cancers, where viral or mutated proteins are expressed exclusively on tumor cells and may be recognized as nonself targets to the immune system (e.g., cervical cancer (HPV), Burkitt's lymphoma (EBV), hepatocellular carcinoma (HBV)).

Recently, many TAA have been identified as nonmutated self proteins, expressed in normal tissues. We are currently undertaking peptide-based vaccinations in a self-antigen setting. Peptides provide a relatively low-cost, stable, well-defined, proven effective immunogen that can easily be made under GMP conditions, but experience with peptide vaccination against self proteins is limited (27). Transgenic mice expressing a defined self antigen could provide a powerful tool for developing effective peptide and other immunization strategies. A variety of such β-gal transgenic mouse strains has been generated, modeling whole-body or tissue-specific protein expression (for a selection of strains; Table 1). Mice expressing β-gal throughout the body and bearing a β-gal-transduced tumor represent a model for cancers expressing a self antigen such as HER-2/*neu*, present on a large variety of normal cell types, but overexpressed on a population of breast, ovarian, and stomach cancers (17, 18). Tissue-specific expression mimics the presence of TAA that are expressed selectively, such as MAGE-1 (testis) and the melanocyte differentiation antigens gp100, MART-1, tyrosinase, and TRP-1 (26, 28). The identification of an immunogenic peptide epitope from β-galactosidase enables the use of these transgenic mice to explore vaccination strategies against self antigens.

Preliminary experiments show that *in vitro* restimulation with DAPIYTNV peptide of splenocytes from TgR ROSA mice (expressing β-gal in virtually all tissues in the body) vaccinated with rVV encoding β-gal does not lead to induction of β-gal-specific CTL, whereas β-gal-negative control mice readily gener-

TABLE 1

β-Gal Transgenic H-2^b Mouse Models

Name	Promoter driving LacZ	Expression pattern
TgR ROSA	Unidentified endogenous	Whole body (30)
LacZ Syx	Syx	Testis (31)
LacZ Mt1	Metallothionein	Liver (32)
Hpx 700	Human hemopexin	Brain, liver (33)
hNF-L	Human neurofilament light gene	Central/peripheral neurons (34)
TnI LAcZ 1/29	Quail fast troponin I	Skeletal muscle (35)
Tie	Human/mouse tie	Vascular endothelium (36)
KPOLac 34	Human keratin 18	Various tissues (not thymus) (37)
TMLacZ	Endogenous thrombomodulin	Vascular endothelium, dermis (38)
hNF-L2	Human neurofilament light gene	Central/peripheral neurons (39)
-1627 COL β-gal	Mouse collagen α_1(I)	Liver/tendon (40)
GFAP-LacZ	Glial fibrillary acidic protein	Astrocytes (41)
Myo-D LacZ	Human myoD	Skeletal muscle (42)
Des1-nLACZ	Desmin	Skeletal muscle (43)
TH3.6LAC	Mouse tyrosine hydroxylase	Brain, some also adrenal gland (44)
CalsperminLacZ	Rat calspermin	Testis, after Day 22 pp (45)
RIβLacZ	Mouse RIβ subunit of cAMP-dep. PK	Brain (no other organs tested) (46)
β2nZ 2	β_2-microglobulin	Skin, central neurons, thymus (47)
hβ1integrin	Human β1 integrin	Varying throughout embryonic/fetal development (48)
H1TLacZ	Rat histone H1T	Testis (49)
RARE-LacZ	Retinoic acid response element	Ductus arteriosus (prenatal) (50)
STF-1 LacZ	Homeobox factor STF-1	Pancreas/duodenum (51)
MER βgal	Mouse estrogen receptor	Most organs (52)
K5LacZ	Human epidermal keratin	Embryonal/adult epidermis (53)
17-LacZ	Mouse pro-α1(I) collagen	Osteoblast (54)
xx-LacZ	Mouse pro-α1(I) collagen	Skin, some osteoblast/odontoblast, som tendon/fascia (55)
hAAT-β1LacZ	Human α_1-antitrypsin	Liver (56)
LacZ-βLCR	Human β-globin	Erythrocyte (57)

ate vigorous anti-β-gal CTL responses (R.S. Chamberlain, Surgery Branch, pers. comm.). This suggests deletion or tolerization of β-gal-specific T cells in the transgenic mice, probably due to constitutive expression of β-gal in the thymus throughout development. Possibly, tissue-specific antigen expression will prevent deletion or strong tolerization of β-gal-specific T cells and therefore allow generation of specific CTL. If so, this suggests that immunization against tumor-associated self proteins is feasible. If not, immunization protocols will have to be developed to break tolerance against specific self proteins, taking advantage of recent insights in enhancement of immunization through addition of cytokines or provision of costimulation (9–12).

It is unclear whether successful immunization to a self protein will lead to destruction of normal tissues. It is conceivable that a successful anti-tumor immune response directed against a normal self protein will also destroy all other cells bearing that antigen, which is acceptable in the case of the epithelial cells of nonessential tissues or organs such as melanocytes, prostate, and breast. In melanoma patients, vitiligo is known to correlate with favorable prognosis in patients undergoing IL-2 treatment (29). It will be interesting to explore whether this phenomenon can be modeled in mice expressing β-gal in an organ-specific fashion. Can β-gal-positive tumor cells be destroyed without affecting other β-gal-expressing tissues, or will autoimmunity be a relatively prevalent side effect of successful self-antigen-directed cancer immunotherapy?

In conclusion, β-gal transgenic mice, together with the wide array of β-gal-based reagents, provide a highly useful model to gain insight into the immune response against self antigens on tumor cells. The identification of the immunogenic β-gal-derived peptide provides an opportunity to define peptide vaccination strategies leading to therapeutic anti-tumor immune responses.

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