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Lethality-based selection of recombinant genes in mammalian cells: Application to identifying tumor antigens

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Many biological processes result in either cell death or cessation of cell growth. However, plasmid- and retrovirus-based mammalian expression vectors in which it has been possible to construct representative cDNA libraries cannot be readily recovered from cells that are not actively dividing. This has limited the efficiency of selection of recombinant genes that mediate either lytic events or growth arrest. Examples include genes that encode the target antigens of cytotoxic T cells, genes that promote stem-cell differentiation and pro-apoptotic genes. We have successfully constructed representative cDNA libraries in a poxvirus-based vector that can be recovered from cells that have undergone lethality-based selection. This strategy has been applied to selection of a gene that encodes a cytotoxic T-cell target antigen common to several independently derived tumors.

Prospects for development of broadly effective cancer vaccines have been advanced by evidence that some non-mutated, normal gene products are recognized as tumor antigens by immune T cells[1–7]. Such gene products may serve as common target antigens in tumors of certain types arising in different individuals. Using a novel method for functional gene selection in cells that undergo a lytic event, we have identified an alternate germ-line–encoded gene for the ribosomal protein L3 that is systematically deregulated in multiple murine tumors and serves as a tumor-rejection antigen.

We have previously reported that cross-protective immunity is induced among three independently derived murine tumor cell lines[8]. These cell lines, BCA 22, BCA 34 and BCA 39, were derived by *in vitro* mutagenesis of independent subcultures of B/C.N, an immortalized, anchorage-dependent, contact-inhibited, non-tumorigenic cloned fibroblast cell line derived from a BALB/c embryo[9,10]. Immunization with any of these tumor cell lines, but not with B/C.N, protects against challenge with not only homologous tumor cells, but also against challenge with the heterologous tumors. Following immunization with any of the three tumor cell lines, $CD8^+$ cytotoxic T lymphocytes (CTLs) are induced that are cross-reactive *in vitro* with all three tumors, but not with the non-tumorigenic B/C.N cells from which the tumors were derived[8].

Selection of specific recombinants in a vaccinia-based vector

In order to progress from an immunological to a molecular definition of this shared tumor antigen, we developed a novel and efficient method for the identification of genes that encode CTL target epitopes. In this strategy, we constructed a representative tumor cDNA library in a modified vaccinia virus expression vector. A monolayer of antigen-negative cells is infected with this library such that on average a single viral plaque-forming unit (p.f.u.) infects each cell (multiplicity of infection (MOI) = 1). Following a 12-hour incubation to permit expression of recombinant genes, we added tumor-specific CTLs to the target-cell monolayer. Cells infected with a vaccinia virus recombinant that encodes the target antigen will process and present that antigen to tumor-specific CTLs and undergo specific lysis. As adherence is an energy-dependent process, cells that undergo a CTL-mediated lytic event are released from the monolayer and can be recovered in the floating population. We enriched virus collected from the floating cell population for recombinants that sensitize host cells to lysis. This selection process can be reiterated. The virus collected following one cycle of enrichment can be used as input for additional cycles of selection using fresh monolayers of target cells and fresh CTLs until the desired level of enrichment has been achieved. In a model experiment with CTLs specific for a known recombinant, we demonstrated that the specific recombinants could be enriched from an initial dilution of 0.001% to approximately 20% in six cycles of selection (Fig. 1*a*). At this level of enrichment, it is a simple matter to pick individual viral plaques for further characterization.

Construction of cDNA libraries in vaccinia virus

The ease of cloning and propagation in a variety of host cells has led to the widespread use of poxvirus vectors for expression of foreign proteins and as delivery vehicles for vaccine antigens[11]. The frequency of recombinants generated by homologous recombination, the most commonly used method to generate recombinant vaccinia virus, is of the order of 0.1% (ref. 12). This is sufficient to recover a recombinant of a specific DNA clone but far too low to permit construction of a representative cDNA library. Other methods generate recombinants at higher frequency but very low titer[13]. We have developed an alternative strategy to generate vaccinia virus recombinants at high frequency and good titer.

The rationale is that a high frequency of recombinants would be obtained if cells are transfected with defective vaccinia DNA that can be packaged into infectious particles only if it has undergone recombination. One way to accomplish this is to cut the vaccinia DNA in the middle of the thymidine kinase (*tk*) gene. As there is no homology between the two *tk* gene fragments, the two vaccinia arms cannot be linked by homologous recombination, except by bridging through the homologous *tk* sequences that flank the insert in the recombinant transfer plasmid. Because naked vaccinia DNA is not itself infectious, production of infectious particles requires transfection of cells also infected with a helper virus. As reported, fowlpox virus (FPV) does not replicate in mammalian cells but provides the necessary helper functions required for replication and packaging of mature wild-type or recombinant vaccinia virus particles[13,14].

A vaccinia vector, v7.5/*tk*, was constructed that incorporates the early/late 7.5k vaccinia promoter, as well as unique *Not*I and *Apa*I restriction sites downstream of the promoter[13] (Fig. 1*b*). Digestion with *Not*I and *Apa*I restriction endonucleases results in two large fragments approximately 80 and 100 kb, respectively. Each of the

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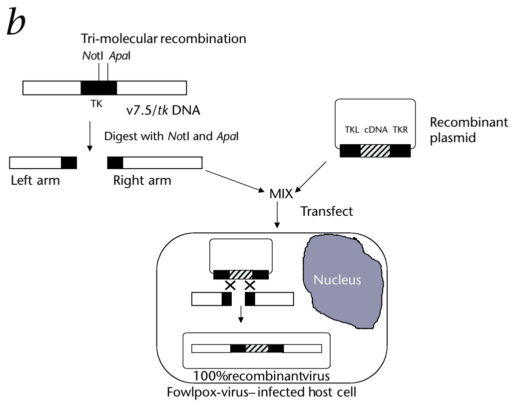
a

	Enrichment Cycle #	% VVova in Floating cells* Expt. 1	Expt. 2	Expt. 3
moi = 1	0	0.2	0.01	0.001
	1	2.1	0.3	nd
	2	4.7	1.1	nd
	3	9.1	4.9	nd
	4	14.3	17.9	1.4
	5	48.8	39.3	3.3
	6			18.6

b



c

DNA	Titer (p.f.u.x10^3) without BrdU	with BrdU	Percent recombinant
v7.5/*tk* untreated	2500	6	0.2
v7.5/*tk*NotI/ApaI-digested	0.2	0	0
v7.5/*tk*N/A+30-ng pELova/*tk*	68	74	100
v7.5/tkN/A+300-ng pELova/*tk*	21	29	100

d

Sample	Titer (p.f.u.x10^3) without BrdU	with BrdU
v7.5/*tk* DNA untreated	2200	0
v7.5/*tk* DNA *Not*I/*Apa*I+15ng cDNA plasmid library	92	92
v7.5/*tk* DNA *Not*I/*Apa*I+30ng cDNA plasmid library	77	75
v7.5/*tk* DNA *Not*I/*Apa*I+75ng cDNA plasmid library	26	27

Fig. 1 Selection of lethal recombinants and library construction in vaccinia virus by tri-molecular recombination. ***a***, Multiple cycles of enrichment for VVova. A vaccinia virus cocktail composed of wild-type v*NotI*/*tk* (ref. 13) spiked with the indicated concentrations of VVova (*tk*-) was subjected to CML selection. Percent VVova (titer with BrdU/titer without BrdU) × 100. ND, not determined. ***b***, Tri-molecular recombination. Genomic DNA from vaccinia strain v7.5/*tk* is digested with *Apa*I and *Not*I. Digested vaccinia 'arms' are mixed with recombinant vaccinia transfer plasmid and transfected into FPV-infected BSC1 cells[13]. Virus is collected 72 h post transfection. ***c***, 0.5 µg digested vaccinia 'arms' were mixed with either 30 ng or 300 ng of pELova/*tk* recombinant transfer plasmid (ref. 13) for transfection into FPV helper virus infected confluent monolayers of approximately 5 × 10^5 BSC1 cells. One sample was transfected with 600 ng uncut genomic v7.5/*tk* DNA. 72 h later, cells were collected and packaged virus was extracted. The frequency of recombinant virus was determined by plaque assay on *tk*-143 B cells in the presence and absence of BrdU. ***d***, Vaccinia tumor cDNA library. The titer and frequency of vaccinia recombinants was determined following transfection with the indicated amount of DNA from a plasmid tumor cDNA library.

digested viral arms includes a non-overlapping fragment of the *tk* gene for bridging by a transfer plasmid. Almost all infectious vaccinia virus produced in FPV-infected cells triply transfected with the two vaccinia arms and a specific transfer plasmid are recombinant for a DNA insert as determined by BrdU resistance (Fig. 1*c*). Individual clones were confirmed to be recombinant by PCR analysis using primers that flank the insertion site in the *tk* gene (data not shown). We have termed this strategy for selection of a high frequency of viral recombinants tri-molecular recombination.

Selection of virus encoding tumor rejection antigen

We constructed a cDNA expression library in a vaccinia transfer plasmid from poly A$^+$ RNA of BCA 39 tumor cells. Titration of the relative amount of plasmid DNA used for tri-molecular recombination again demonstrated that although the recombination frequency remains close to 100% at all concentrations, excess plasmid DNA results in lower viral titers (Fig. 1*d*). Analysis of individual vaccinia clones demonstrated that cDNA inserts were of diverse size, with a predominant distribution between 300 and 2,000 bp, which is indistinguishable from the size range of inserts observed in the plasmid cDNA library employed as an intermediate to construct the vaccinia library (data not shown). To construct a high-titer library, 39 tri-molecular recombination reactions were carried out at the optimal plasmid concentration as described in Fig. 1*d*. The titer of the library of pooled recombinants was 5 × 10^6 p.f.u. To identify the tumor antigen, we infected a monolayer of 5 × 10^5 antigen-negative B/C.N cells with an equal number of recombinant p.f.u. of the tumor library (MOI = 1). CTLs specific for BCA 34 tumors cells were added to the target-cell monolayer at an effector-to-target ratio that gives approximately 50% lysis of tumor cells in a standard ^{51}Cr-release assay. We used CTLs specific for the heterologous BCA 34 tumor cells to select recombinants from the BCA 39 library in order to focus selection on antigens common to at least these two tumors. Following four cycles of selection with tumor-specific CTLs, individual recombinant viral plaques were expanded and used to infect separate cultures of non-tumorigenic, antigen-negative

a

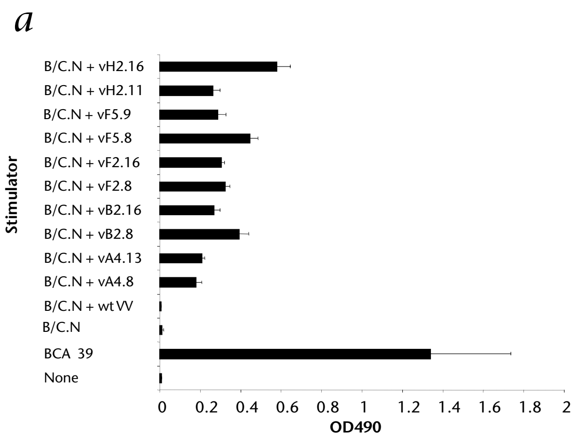


Fig. 2 CML-selected recombinant vaccinia cDNA clones stimulate tumor-specific CTLs. ***a***, B/C.N cells infected with CML-selected vaccinia clones were assayed for the ability to stimulate tumor-specific CTLs to secrete IFN-γ. Cytokine secretion was measured by ELISA and is represented as OD490. An OD490 of 1.4 and 0.65 are approximately equal to 4 ng/ml and 1 ng/ml of IFN-γ, respectively. ***b***, CML-selected clones sensitize host cells to lysis by tumor-specific CTLs. Monolayers of B/C.N in wells of a 6-well plate were infected with the indicated vaccinia virus clones at MOI = 1. After 14 h of infection the cells were collected and labeled with ^{51}Cr along with the indicated control targets. Target cells were incubated with tumor-specific CTLs at the indicated effector:target ratio for 4 h at 37 °C and the percent specific lysis was determined. This experiment was repeated 3 times with similar results.

b

Target	Effector: Target 10:1	2:1
BCA 34	68.4	54.8
BCA 39	36.6	23.4
B/C.N	0.2	0.3
B/C.N + vF5.8	47.5	34.6
B/C.N + vH2.16	67.8	56.2
B/C.N + vaccinia vector	0	0.2

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a **Normal L3**

Amino acid position	45 46 47 48 49 50 51 52 53 54 55 56
Sequence	A F L G Y K A G M T H I
Nucleotide	GCC TTT CTG GGT TAC AAG GCT GGC ATG ACC CAC ATC

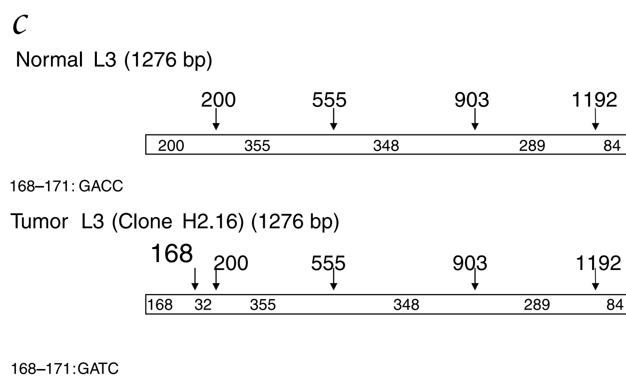
Tumor L3 (Clone H2.16)

Amino acid position	45 46 47 48 49 50 51 52 53 54 55 56
Sequence	A F L G Y K A G M I H I
Nucleotide	--- --- --- --- --- --- --- --- --- -T- --- ---

b

Target	Effector: Target 10:1 (% specific lysis)	Effector: Target 2:1 (% specific lysis)
BCA 34	62.4	32.1
BCA 39	49.7	23.6
B/C.N	3.3	0.2
B/C.N + L3 peptide 48–56 (I54) (1 μM)	46.0	16.1
B/C.N + L3 peptide 48–56 (I54) (10 nM)	35.2	9.3
B/C.N + L3 peptide 48–56 (T54) (100 μM)	2.0	0
B/C.N + L3 peptide 45–54 (I54) (100 μM)	0	0

c



d

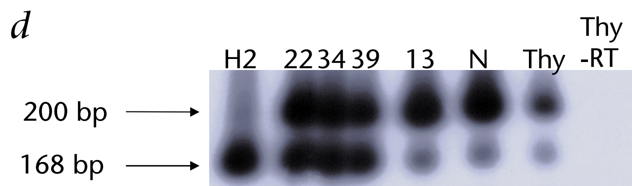


Fig. 3 The tumor antigen is encoded by a ribosomal protein *L3* gene. ***a***, Sequence of tumor (clone H2.16) and normal ribosomal protein L3 genes from aa45 to aa56. The amino acid (in single letter code) and nucleotide sequence of cDNA clone rpL3 are shown. ***b***, B/C.N cells were pulsed with peptides at the indicated concentrations and use as target cells in a ^{51}Cr-release assay with BCA 34 tumor-specific CTLs (as in Fig. 2*b*). ***c***, *Sau*3AI map of the published normal ribosomal protein L3 and the tumor L3(C170T) clone (H2.16). Shown are the *Sau*3AI restriction map for the *L3* gene predominantly expressed in normal cells (top) and for the H2.16 clone, *L3*(C170T)-encoded cDNA, overexpressed in tumors (bottom). ***d***, *L3*-specific RT-PCR products from each indicated sample were generated using a ^{32}P-labeled 5′ PCR primer and an unlabeled 3′ primer. No PCR product was observed when RNA for each sample was used as PCR template without RT, indicating that none of the samples was contaminated with genomic DNA. The PCR products were gel-purified, digested with *Sau*3AI and resolved on a 3% agarose gel. No PCR product was observed in a control PCR sample that had no template added to it. This result was reproduced 3 times.

B/C.N cells. Each culture of infected cells was assayed for the ability to stimulate tumor-specific CTLs to secrete interferon (IFN)-γ (Fig. 2*a*), or for sensitization to lysis by tumor-specific CTLs (Fig. 2*b*). We isolated ten viral clones, all of which conferred upon B/C.N the ability to stimulate a line of tumor-specific CTLs to secrete IFN-γ. Neither uninfected B/C.N nor B/C.N infected with empty vector induced significant IFN-γ secretion by tumor-specific T cells. All ten clones contained a similar size cDNA insert of approximately 1,300 bp. Sequence analysis confirmed that these all represented the identical full-length cDNA.

Deregulated *L3* gene is the tumor target antigen

A homology search of the cloned sequence revealed that except for a single nucleotide substitution at position 170, this cDNA is identical to the murine ribosomal protein *L3* gene[15] (GenBank Accession no. Y00225). The single C170T nucleotide substitution results in a threonine-to-isoleucine substitution at aa54 of the translation product. The tumor gene encoding ribosomal protein L3 was designated *L3*(C170T). In all, 6/6 CTL lines that were generated by immunization with BCA 34 demonstrated specificity for the antigen encoded by this gene product.

It seemed likely that the altered amino acid (Ile 54) in L3(C170T) might be part of a tumor-specific peptide that binds to class I major histocompatibility complex (MHC) for presentation to tumor-specific CTLs. This hypothesis was supported by the demonstration that a vaccinia virus recombinant for a gene fragment encoding the first 63 amino acids of the *L3*(C170T) tumor gene sensitizes B/C.N to lysis by tumor-specific CTLs (data not shown). Two peptide epitopes encoded within this region were identified that are predicted to bind with relatively high affinity to the class I MHC molecule H2-K^d using a computer algorithm (Fig. 3*a*)[16]. These two peptides, $L3_{45-54}$(I54) and $L3_{48-56}$(I54) were synthesized and tested for the ability to sensitize B/C.N cells to lysis by tumor-specific CTLs. As shown in Fig. 3*b*, peptide $L3_{48-56}$(I54) sensitizes B/C.N to lysis, whereas $L3_{45-54}$(I54), and the normal L3 gene, $L3_{48-56}$(T54), do not. It was determined that 10 nM $L3_{48-56}$(I54) is sufficient to sensitize targets to lysis by CTLs, whereas 100 μM $L3_{48-56}$(T54) or $L3_{45-54}$(I54) does not. These results demonstrate that peptide $L3_{48-56}$(I54) is a target epitope recognized by tumor-specific CTLs.

There are two ways to account for the origin of the new *L3* RNA in tumor cells. Either the *L3*(C170T) gene expressed in these tumors is a somatic mutant of the wild-type gene or there are multiple germ-line genes of *L3*, at least one of which is deregulated during the process of tumor transformation and gives rise to an immunogenic product. We considered the first hypothesis unlikely because the cross-reactive BCA 39, 34 and 22 tumors were each independently derived. It would be remarkable if the same mutant epitope was randomly generated in all three tumors. In support of the alternative hypothesis of differential gene regulation, Southern blots of different restriction digests of genomic DNA from BCA 39 and B/C.N indicated that there are multiple *L3* genes in the murine genome. This is consistent with previous reports of multiple copies of *L3* in genomes of both the rat and the cow[17,18]. It remained to be demonstrated that different *L3* genes in the germ line are subject to differential regulation in tumors and normal cells.

The published *L3* nucleotide sequence from position 168 to 171 is GACC. The sequence of *L3*(C170T) in this same region is GATC

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a

Percent Specific Lysis

Immunogen

Target	vH2.16 E:T 40:1	vH2.16 10:1	v7.5/tk 40:1	v7.5/tk 10:1
BCA 34	33.6	12.9	5.7	4.0
BCA 39	22.1	9.0	5.3	3.1
B/C.N+L3 48-56(I54)	48.2	20.2	3.9	1.5
B/C.N+L3 48-56(T54)	6.4	1.4	1.8	2.9
B/C.N	7.1	5.7	6.1	2.8
YAC	1.2	2.5	0	1.8

b

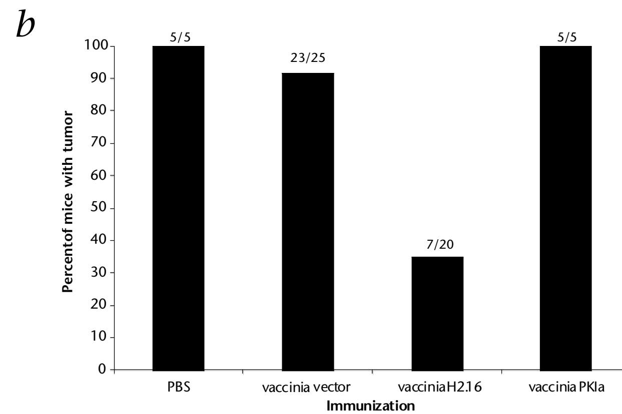

Fig. 4 A shared murine tumor antigen is immunogenic and immunoprotective. ***a***, Mice were immunized with either vaccinia recombinant for the tumor *L3* gene (vH2.16) or with empty vector (v7.5/*tk*). Splenocytes were collected, restimulated and tested in a ^{51}Cr-release assay. ***b***, Female BALB/cByJ mice were immunized twice as indicated. The mice were challenged by subcutaneous injection of 200,000 viable BCA 34 tumor cells into the abdominal wall 14 d following the secondary immunization. Results are tabulated as number of mice with tumor/total number of mice in each group. Mice were killed when their tumors grew large enough (> 100 mm^2) to result in skin breakage with attendant risk of infection. Animals that rejected tumor have remained tumor-free for more than 6 mo. Data are from 3 independent experiments.

(Fig. 3*c*). This new palindrome is the recognition sequence for a number of restriction endonucleases, including *Sau*3AI. A *Sau*3AI digest of normal L3 cDNA is expected to generate fragments of 200, 355, 348, 289 and 84 bp, whereas a *Sau*3AI digest of the H2.16 clone of L3(C170T) cDNA would generate a 168-bp fragment in place of the 200-bp fragment (Fig. 3*c*). This difference in the *Sau*3AI digestion products was used to confirm that the three immunologically cross-reactive tumor cell lines BCA 39, 34 and 22 express at least two different *L3* genes. L3 reverse transcriptase (RT)-PCR products from cell lines and from thymus RNA were synthesized using a ^{32}P-labeled 5′ *L3* sequence-specific primer and unlabeled 3′ *L3* sequence-specific primer. PCR products were digested with *Sau*3AI and analyzed on an agarose gel. The predicted 168-bp fragment is generated from the cloned H2.16 cDNA recombinant, whereas both the 200- and 168-bp fragments are generated from cDNA derived from the three immunologically cross-reactive tumors BCA 39, 34 and 22, indicating approximately equivalent expression of the two different L3 genes (Fig. 3*d*). In contrast, normal cells, thymus and the unrelated BCB13 tumor express predominantly the 200-bp fragment with low but clearly detectable levels of the *L3*(C170T) tumor gene derived 168-bp fragment. Quantitative analysis indicated that the ratio of 200- to 168-bp fragments in the BCA tumors is 2:1, whereas the ratio of the same fragments detected in B/C.N, BCB13, and thymus is approximately 18:1. We also observed equivalent low levels of expression of this immunogenic *L3*(C170T) tumor gene relative to the normal *L3* gene when RNA from kidney, heart and skeletal muscle was analyzed. Expression of the *L3*(C170T) gene product in normal tissues indicates that it is encoded in the germ line and is not a product of somatic mutation in tumor cells. Evidently, in some tumors, gene deregulation associated with the transformation process results in upregulated expression of this germ line gene by a factor of nine and renders the corresponding gene product immunogenic.

If broadly effective vaccines are to be developed based on expression of shared tumor antigens, then it is critical to demonstrate that such antigens can be immunoprotective. So far, most shared antigens have been identified in human tumors. Clinical trials employing these antigens for immunotherapy are still at an early stage. In mice, where immunotherapeutic strategies could be more thoroughly investigated, very few shared tumor antigens have been identified. It was therefore important to determine whether immunization with L3(C170T) recombinant vaccinia virus would induce tumor-specific CTLs that are immunoprotective against tumor challenge[19]. Immunization of BALB/c mice with vaccinia virus recombinant for the *L3*(C170T)-encoded cDNA (H2.16) induces CTLs that are able to lyse targets sensitized with the specific $L3_{48-56}$ (I54) peptide epitope. Importantly, they also lyse both BCA 34 and 39 tumor cells, but not normal B/C.N (Fig. 4*a*). This confirms that the target peptide is appropriately processed and presented in tumor cells. To test the immunoprotective potential of this antigen in a prophylactic model, we immunized mice with vaccinia virus recombinant for *L3*(C170T) before challenge with viable BCA 34 tumor cells. Immunization with L3(C170T) results in significant protection against tumor challenge (Fig. 4*b*). In contrast, mice immunized with empty viral vector or a control vaccinia recombinant were unable to reject this tumor challenge. Importantly, immunization with *L3*(C170T) recombinant vaccinia virus is also immunoprotective against challenge with viable BCA 22 tumor cells (data not shown). This is a formal demonstration that L3(C170T) is a shared tumor rejection antigen.

It is particularly intriguing that the immunogenic *L3* gene is also expressed, albeit at a nine-fold reduced level, in normal thymus. This level of expression is evidently not sufficient to tolerize all T cells with functional avidity for the nine-fold higher level of *L3*(C170T) expressed in some tumors. Nevertheless, the observation that although B/C.N and BCB13 express low levels of the tumor *L3* gene they are not susceptible to lysis by tumor-specific CTLs suggests that higher affinity T cells have been tolerized. These observations emphasize that tolerance to a self-protein is not absolute but must be defined in relation to quantitative levels of expression[20,21].

Lethality-based selection

Our strategy for selection of recombinant genes that encode target antigens of cytotoxic T cells has several advantages over expression cloning methods that have been previously applied for this purpose. Viral infection is a far more efficient means than

Methods

Generation of recombinant viruses by tri-molecular homologous recombination. Viral genomic DNA of v7.5/*tk* (ref. 13) (0.5 μg/sample) was digested with *Apa*I and *Not*I restriction endonucleases. Digested vaccinia DNA was transfected alone, or mixed with the indicated amount of transfer plasmid pELova/*tk* (ref. 13), or plasmid purified from the BCA 39 cDNA library. The DNA was transfected using lipofectamine into confluent monolayers of approximately 5×10^5 BSC1 cells, which had been infected 2 h previously with FPV HP1 at MOI = 1. One sample was transfected with 500 ng untreated genomic v7.5/*tk* DNA. 72 h later the cells were collected, and the virus extracted by 3 cycles of freeze/thaw. Recombinant virus in crude stocks was titered by plaque assay on *tk*-143 B cells in the presence and absence of 125 mg/ml BrdU.

Construction of cDNA library. Total RNA was isolated from BCA 39 tumor cells and used to construct a dT-primed cDNA library in plasmid p7.5/*tk* (ref. 13) following standard procedures.

Generation of CTLs. BALB/cByJ mice (Jackson Laboratories, Bar Harbor, Maine) were immunized twice with 2×10^6 irradiated (6,500 cGy) BCA 34 cells. One week following the second immunization splenocytes were collected, and stimulated *in vitro* with irradiated, mitomycin C-treated BCA 34 cells. Viable T cells were purified weekly using Lympholyte-M and restimulated with irradiated (5,000 cGy) BALB/c spleen cells and irradiated, mitomycin C-treated BCA 34 cells.

CML selection for VVova. A specific vaccinia recombinant, VVova, encoding the well-characterized ovalbumin 257–264 peptide (SIINFEKL) was diluted with non-recombinant virus so that it initially constituted either 0.2%, 0.01% or 0.001% of total viral p.f.u. Adherent monolayers of MC57G cells (H-2^b) were infected with each viral mix at MOI = 1. Following a 12-h infection, target cells were incubated with ovalbumin peptide-specific CTLs. The monolayer was then gently washed, and both floating cells and the remaining adherent cells were separately collected. Virus extracted from each cell population was titered for the frequency of recombinant (BrdU-resistant) viral p.f.u. In the presence of BrdU only the *tk*- recombinant VVova give rise to p.f.u. on *tk*- 143B host cells. Following the fourth cycle of selection, more rapid enrichment was obtained if the MOI was reduced from MOI = 1 to 0.1. Virus extracted from floating cells was then used as input to another enrichment cycle with fresh adherent MC57G cells and ovalbumin peptide-specific CTLs.

CML selection with tumor-specific CTLs. A confluent monolayer of B/C.N in a well of a 12-well plate was infected with vaccinia BCA 39 cDNA library at MOI = 1. At 12 h post-infection, the monolayers were washed 3 times with media, and 2.5×10^6 CTLs were added to the wells in a 250-μl volume. The T cells and targets were incubated at 37 °C for 4 h. Following the incubation, the supernatant of floating cells was collected, and the monolayer gently washed 3 times with media (250 μl). Virus was released from cells in the pooled supernatant by freeze/thaw. Titers were determined by plaque assay on BSC1 cells. The selected virus population (floating cells in cultures that received specific T cells) was subjected to 3 additional enrichment cycles.

Identification of tumor antigen recombinant clones. Virus from the fourth enrichment cycle was divided into 40 pools of 5 p.f.u. each. Each pool was amplified on BSC1 cells in a 96-well plate and used to infect monolayers of B/C.N in a 96-well plate. As a control, a monolayer of B/C.N was infected with v*Not*I/*tk* (ref. 13). At 5-h post-infection, 2×10^4 washed CTLs were added to each well. The cells were incubated at 37 °C for 18 h. The cells were then pelleted by centrifugation, supernatant was collected and assayed for IFN-γ by ELISA. Individual clones were picked from 5 positive pools and assayed as above.

Analysis of *L3* mRNA expression. Total RNA (2 μg) was converted to cDNA using an oligo-dT primer. cDNA was used as the template for PCR amplification with *L3* sequence-specific primers. Before the PCR reaction the upstream primer was end-labeled using ^{32}P and T4 kinase. These PCR products contained the region of L3 between nucleotide position 3 and 1252. The PCR products were gel purified, digested with *Sau*3AI, and resolved on a 3% agarose/ethidium bromide gel.

Immunization with L3(C170T). Adult female BALB/cByJ mice (n = 2 per group) were immunized by subcutaneous injection of 5×10^6 p.f.u. of vH2.16, or v7.5/*tk* (empty vector). 7 d following the immunization, splenocytes were collected and cultured with 1 μM peptide $L3_{48-56}$(I54). After 7 d, the viable T cells were purified using Lympholyte-M, and stimulated with 1 μM peptide and irradiated (5,000 cGy) BALB/c spleen cells. The T cells were tested in a ^{51}Cr-release assay 5 d following this second restimulation. Mice were maintained in compliance with federal and state law, and NIH policy.

Tumor challenge. Adult female BALB/cByJ mice were immunized by subcutaneous injection of 10×10^6 p.f.u. of vH2.16, vPKIa (non-specific control recombinant of PKI-α; ref. 22), v7.5/*tk* or PBS. A secondary boost with the same immunogen was given 21 d later. Mice were challenged by subcutaneous injection of 2×10^5 BCA 34 tumor cells 14 d following the last immunization.

DNA transfection for introducing recombinant genes into antigen presenting cells (APCs). Moreover, because of the broad poxvirus host range, it is possible to employ readily available autologous APCs such as fibroblasts or Epstein–Barr-virus-transformed B cells. This obviates the need for prior identification and cloning of MHC restriction elements for expression in the few available highly transfectable cell lines. Autologous APCs used in this method naturally express all the relevant MHC restriction elements that might be required for T-cell recognition. Finally, the strategy described is based on selection rather than labor-intensive screening. Selection is intrinsically more efficient than screening as it is not necessary to assay numerous negative clones or pools. By employing specific CTLs to select recombinant virus from multiple cultures of 500,000 cells each infected with an equal number of viral recombinants, a library with a titer of several million can be readily surveyed.

A limitation of the selection strategy described here is that it is dependent on lytic activity. However, as will be described elsewhere, even without the advantage of selection, the sensitivity of epitope detection using the poxvirus expression system is such that this method has been successfully employed to screen for target antigens with a polyclonal population of human tumor-infiltrating lymphocytes that are induced to secrete lymphokines by autologous tumor cells but have low cytolytic activity (E.S.S. *et al.*, manuscript in preparation). The ability to identify target specificities in a polyclonal population has the significant advantage that it avoids the loss of specificities associated with relatively low T-cell cloning efficiencies. Specific T cells can be more readily isolated and their MHC-restriction specificities determined once the target antigens are known.

Construction of representative cDNA libraries in a poxvirus vector creates the opportunity for lethality-based selection strategies that cannot be efficiently applied using alternative mammalian expression vectors. Selection strategies such as that described here for identification of the target molecules recognized by cytotoxic T cells are enabled because fully packaged and infectious poxvirus can be recovered—even from cells that have undergone lytic or apoptotic death. As examples of further applications of this technology, we have developed additional strategies employing this vector system to express and select human antibodies in mammalian cells and for functional selection of genes that regulate terminal differentiation of stem cells.

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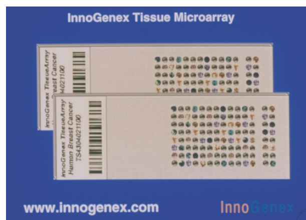

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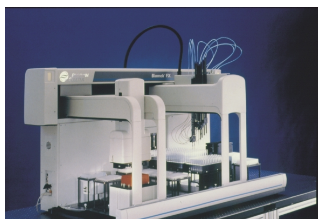
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