

# Immunization with an Antigen Identified by Cytokine Tumor Vaccine-assisted SEREX (CAS) Suppressed Growth of the Rat 9L Glioma *in Vivo*<sup>1</sup>

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## ABSTRACT

We have reported previously that s.c. immunization of rats with IL-4 transduced 9L gliosarcoma cells (9L-IL-4) induced a potent antitumor immunity against intracranial, parental 9L tumors. Subcutaneous implantation of 9L-IL-4 influenced the systemic humoral response, which was demonstrated by Th2-type isotype-switching and the induction of cellular immune responses, which played a critical role in the rejection of tumors. Serological analyses of recombinant cDNA expression libraries (SEREX), has recently emerged as a powerful method for serological identification of tumor-associated antigens (TAAs) and/or tumor rejection antigens (TRAs). Because IL-4 is known to activate B cells and to promote humoral responses, and inasmuch as induction of humoral responses by central nervous system tumors has been reported to be minimal, we investigated whether the induction of a potent humoral immune response against 9L TAAs or TRAs in rats immunized s.c. with 9L-IL-4 could be demonstrated. Screening of  $5 \times 10^5$  independent clones of 9L-expression cDNA library for the presence of reactive antibodies in the serum from a 9L-IL-4 immunized rat led to the identification of three different TAAs. One 9L TAA (clone 29) was demonstrated to be calyculin, a member of the S-100 family of calcium-binding proteins. The second 9L TAA (clone 37) was demonstrated to be the rat homologue of the J6B7 mouse immunomodulatory molecule. The third TAA (clones 158 and 171) was determined to be the rat homologue of the mouse Id-associated protein 1 (MIDA1), a DNA-binding, protein-associated protein. Northern blotting demonstrated that message for calyculin was overexpressed in 9L cells. Message encoding MIDA1 was highly expressed in parental 9L cells and thymus and, to a lesser degree, in testis, suggesting that MIDA1 was comparable with the cancer/testis category of TAAs. Sera obtained from animals bearing 9L-IL-4 were found to have a higher a frequency and titer of antibodies to these antigens when compared with sera obtained from rats bearing sham-transduced 9L (9L-neo) cells. To determine whether immunization with these TAAs induced antitumor immunity, animals were immunized by intradermal injection with expression plasmids encoding calyculin or MIDA1. Subsequent challenge of rats with parental 9L resulted in significant suppression of tumor growth in animals immunized with MIDA1, but not with calyculin. These results indicate that MIDA1 is an effective 9L TRA and will be useful for the investigation of specific antitumor immunity in this glioma model. Furthermore, these results suggest that this approach, termed “cytokine-assisted SEREX (CAS),” may serve as an effective strategy for identification of TRAs for in animal-glioma models of cytokine gene therapy, and potentially in humans undergoing cytokine gene therapy protocols as well.

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## INTRODUCTION

Although the CNS<sup>5</sup> has been considered to be immunologically privileged (1–4), specific cellular immune responses against antigens present in the CNS, such as myelin basic protein, have been induced by systemic immunization of hosts in models of autoimmune disease (5, 6). We have reported that s.c. implantation of 9L gliosarcoma cells expressing the murine *IL-4* gene (9L-IL4) induced effective protection and some degree of therapeutic immunity against parental 9L tumors implanted intracranially (7). The antitumor immunity induced by 9L-IL4 appears to be mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T cells (8). Although clinical trials of *IL-4* gene-transduced tumor vaccines for patients with malignant gliomas have been launched (9), one can easily recognize concerns that vaccination with whole tumor cells could potentially result in the development of a deleterious CNS autoimmune encephalitis, as demonstrated by studies in animal models (10). However, allergic encephalitis has not been observed in human clinical trials of whole-cell brain tumor vaccines, with the exception of one case of mild, transient encephalopathy (11–13). Nevertheless, the use of specific TRAs is favored for safer and more effective immunotherapy for brain neoplasms. The potential for therapeutic use of glioma-specific TRAs is clearly supported by studies using DCs pulsed with tumor-specific antigens. For instance, we have demonstrated that therapeutic antitumor immunity to intracranial C3 tumors can be generated after systemic administration of tumor-specific, E7 human papillomavirus peptide-pulsed DCs (14). However, the clinical value of this approach in patients with malignant intracranial neoplasms will require additional preclinical development in models using native glioma-associated TRAs and, ultimately, the identification of appropriate human glioma TRAs.

SEREX has recently emerged as a powerful methodology for identifying human tumor antigens eliciting humoral immune responses (15, 16). To date, more than 600 TAAs have been serologically identified using the SEREX method, of which nearly one third are novel (16, 17). Categories of TAAs identified include differentiation antigens such as tyrosinase, amplified/overexpressed proteins such as galectin 9, mutated antigens such as p53, and C-T antigens such as MAGE (reviewed in Ref. 17). These data suggest that SEREX will, in fact, be useful for defining antigens similar to those originally identified by cloning target cell-eluted peptides recognized by CTLs, such as MAGE and tyrosinase (18, 19). Furthermore, a novel C-T TRA, known as NY-ESO-1 and initially defined by SEREX (20), has been demonstrated to be an antigen recognized in the context of both HLA class I and II by specific CTLs derived from patients with melanoma (21, 22). Thus, serological screening of cDNA libraries prepared from human cancers can be used to identify antigens eliciting a cellular immune response, including CTLs, circumventing the

<sup>5</sup>The abbreviations used are: CNS, central nervous system; IL-4 interleukin 4; TRA, tumor rejection antigen; SEREX, serological analysis of recombinant cDNA expression libraries; DC, dendritic cell; C-T, cancer-testis; MIDA1, mouse Id-associated protein 1; CAS, cytokine-assisted SEREX; F344 rats, Fischer 344 rats; PBS/Tw, PBS supplemented with 0.5% Tween 20; OD, optical density; ORF, open reading frame; HLH, helix-loop-helix; i.d., intradermal.

need for established cultured autologous cell lines and stable CTL clones.

Intracranial tumors are generally poorly immunogenic. For example, antibodies against p53 can be detected readily in sera from patients with tumors having abnormalities in p53 (23–25). In contrast, sera from patients with malignant gliomas rarely contain detectable level of anti-p53 antibodies (26) despite the fact that mutation and/or over-expression of p53 in malignant gliomas is as frequent as in other types of tumors (27). The poor immunogenicity of gliomas could be attributable to a lack of access of glioma-derived antigens to the periphery or access of afferent immune elements to the CNS, resulting in a limited response. Alternatively, it could be attributable to the elaboration of immunosuppressive substances such as transforming growth factor  $\beta$  from gliomas (28). Either possibility suggests a need to enhance the immune response to gliomas and/or a need for the development of new strategies for identification of glioma-associated TRAs.

SEREX technology provides an important new means of tumor antigen identification. However, it is applicable in a limited number of patients, because antibodies against most antigens can only be detected in 10–30% of patients bearing a tumor expressing the respective antigen, and it is without a correlation to any obvious clinical parameter (16). Therefore, we have sought to develop a means of improving the potential for identification of glioma-derived TAAs and/or TRAs by SEREX. To that end, we have altered the process by using cytokine gene-transduced tumor cells to enhance an immune response. Initially, we have used tumor cells genetically engineered to produce IL-4, as this approach has been reported to induce potent protective (29, 30) and therapeutic immunity (31, 32) in various animal models. IL-4 has pleiotropic effects on multiple lineages of immune cells, including T cells (33, 34), myeloid cells such as eosinophils (35), natural killer cells (36), natural killer/T cells (37), DCs (38, 39), and B cells (40). In the context of SEREX, IL-4 can be viewed as an attractive cytokine for use because it has multiple modulatory effects that enhance humoral responses. These effects include inducing differentiation of (T helper) Th precursors into Th2 cells, which drive the proliferation, differentiation, and isotype class switching of antigen-binding B cells (41). IL-4 also up-regulates expression of class II, CD40, B7.1, and B7.2 (42), all of which promote an enhanced capacity for antigen presentation to T cells. There is also evidence for IL-4-driven enhancement of differentiation and immunoglobulin production by B cells (43). Therefore, we hypothesized that IL-4, when expressed peritumorally, would enhance antitumor humoral responses and, therefore, enhance the potential for identification of TAAs by SEREX.

In this report, we describe the use of CAS for identification of glioma TAAs using the rat 9L gliosarcoma. In this model, we observed enhanced antibody responses to three 9L TAAs, including calcyclin, an S100 family calcium-binding protein; the rat homologue of the J6B7 immunosuppressive factor; and the rat homologue of MIDA1, a DNA binding protein-associated protein. Further, we determined that MIDA1 serves as a TRA for 9L using naked DNA immunization with a cDNA encoding this molecule. These data clearly suggest that the use of CAS can provide an efficient modification of SEREX with improved potential for identifying TRAs.

## MATERIALS AND METHODS

**Animals and Cell Lines.** Rat 9L gliosarcoma cells derived from F344 rats were maintained in RPMI 1640 supplemented with 5% fetal bovine serum, 100 units/ml penicillin G, and 100 units/ml of streptomycin sulfate. 9L cells transduced to express murine IL-4 (9L-IL4) and sham transduced 9L (9L-neo) cell lines were produced as described previously (7). Briefly, parental 9L cells

were infected with a retroviral vector DFG-mIL4-neo carrying cDNAs for murine IL-4 and *neoR* or were infected with MFG-neo carrying only *neoR*. Transduced 9L cells were subsequently selected with 1 mg/ml of G418 to generate 9L-IL4 and 9L-neo. Selected cultures of cells were expanded, maintained in culture, and periodically determined to be free of *Mycoplasma* contamination using a *Mycoplasma* detection kit (Boehringer Mannheim, Indianapolis, IN). IL-4 production by 9L-IL4 cells was measured by ELISA using anti-IL-4 antibody obtained from PharMingen (San Diego, CA) and was determined to be  $\sim 100$  ng/ $10^6$  cells/48 h. Male F344 rats (Harlan Sprague Dawley, Indianapolis, IN) were housed and handled in accordance with the guidelines for animal care established by the University of Pittsburgh Animal Care and Use Committee.

**Immunization with s.c. Injection of Gene-modified Tumor Cells.** Tumor immunizations were performed s.c. by injecting 0.5 ml of HBSS containing exponentially growing  $2 \times 10^6$  9L-IL4 in the right flank of syngeneic F344 rats. 9L-neo cells and 9L-IL4 cells used as irradiated tumor vaccines were exposed to 5000 rads using a Gammacell 1000 Elite cell irradiator (MDS Nordion, Kanata, Ontario, Canada) before injection.

**Detection of Serum Isotype Switching.** For hyperimmunization, animals were injected s.c. with  $2 \times 10^6$  irradiated or nonirradiated 9L-IL4, irradiated 9L-neo, or HBSS on days 0, 4, 7, 11, 14, and 17. Animals receiving a single immunization were given a s.c. injection of  $2 \times 10^6$  irradiated 9L-IL4 or 9L-neo cells on day 0. Animals given cells intracranially received  $1 \times 10^4$  9L cells on day 0. For all groups, animals were sacrificed on day 24, and sera were obtained. The concentrations of IgG1, IgG2a, IgG2b, and IgM isotypes in pools of normal and immune sera were determined by ELISA. Enhanced protein-binding ELISA plates (Nunc, Rochester, NY) were coated with 2  $\mu$ g/ml mouse antirat IgG1, IgG2a, IgG2b, or IgM (PharMingen, San Diego, CA) diluted in 0.1 M NaHCO<sub>3</sub> (pH 8.2) for 1 h at 37°C. Plates were washed three times with PBS/Tw. The plates were blocked by incubating with blocking buffer which was PBS supplemented with 10% FBS and washed three times with PBS/Tw. The plates were then incubated with the pools of normal and immune sera for 1 h at room temperature. After washing three times with PBS/Tw, plates were incubated with 2  $\mu$ g/ml biotinylated mouse antirat light chain and mouse antirat immunoglobulin heavy chain in blocking buffer for 1 h at room temperature. Plates were then washed six times with PBS/Tw, incubated with avidin-peroxidase in blocking buffer for 30 min at room temperature, and washed six times with PBS/Tw. The ELISA was developed by incubating the plates with substrate buffer containing 0.015% v/w 3-ethylbenzthiazoline-6-sulfonic acid (Sigma, St. Louis, MO) in 0.05 M citric acid (pH 4.35) and 1:1100 diluted 30% H<sub>2</sub>O<sub>2</sub>. The color reaction was stopped by adding 1% SDS and the absorbance (OD) 405 nm was read using the Bio-Rad microplate reader (Hercules, CA). The relative isotype concentration was calculated by using the formula as follows:

$$\frac{\text{OD of isotype immune sera}}{\text{OD isotype normal sera}} \times 100$$

**RNA Extraction, cDNA Library, and Immunoscreening of the cDNA Library.** Total RNA was extracted from cultured 9L line by the guanidium isothiocyanate/phenol/chloroform method, and mRNA was purified using the PolyAtract kit (Promega, Madison, WI). A cDNA library was prepared commercially in the  $\lambda$ Triplex vector system as a custom cDNA library by Clontech (Palo Alto, CA). The amplified cDNA library was screened with sera obtained from animals hyperimmunized with nonirradiated 9L-IL4 cells. The sera were diluted 1:10, preabsorbed with lysate of a *Escherichia coli* strain XL-1 to remove antibodies reacting with *E. coli* components. XL-1 infected with recombinant phage vectors were plated onto Luria-Bertani agar plates. Expression of recombinant proteins was induced with isopropyl  $\beta$ -D-thiogalactoside. Plates were incubated at 37°C until plaques were visible and then blotted onto nitrocellulose membranes. The membranes were then blocked with 5% skim milk in Tris-buffered saline and incubated with 1:400 dilution of the absorbed serum (final dilution of serum 1:400) overnight at room temperature. After washing, the filters were incubated with 1:10,000 diluted alkaline phosphatase-conjugated goat antirat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and reactive phage plaques were visualized by incubating with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium. Phagemid clones reactive against pre-immune sera were subsequently

eliminated during the secondary screening, and tumor specific positive clones were subcloned to monoclonality.

**Isotype Analysis of Serum Antibodies Recognizing Positive Clones.** Slight modifications were made on the above protocol for defining the serum IgG isotypes reactive to positive clones. Briefly, positive clones were mixed with control negative clones at the ratio of approximately 1:5 and cultured on agar plates with a bacterial lawn, and then blotted onto nitrocellulose. After incubation with serum from 9L-IL4 immunized rats, blots were incubated with alkaline phosphatase-conjugated goat antirat immunoglobulin isotype specific (anti IgG1, IgG<sub>2a</sub>, IgG<sub>2b</sub>, and IgM) secondary antibodies (PharMingen) to analyze the serum isotypes recognizing positive clones.

**Sequence Analyses of the Reactive Clones.** The reactive phage clones were excised and converted to pTriplEx plasmid forms according to the manufacturer's instructions. Inserted cDNAs were sequenced by the University of Pittsburgh DNA Sequencing Facility using an ABI PRISM automated sequencer (Perkin-Elmer, Norwalk, CT). DNA and predicted amino acid sequences were compared with sequences in GenBank using the BLAST program.

**Northern Blot Analyses.** To evaluate the relative level of expression of message for each of the three cloned genes, Northern blot analyses were performed with total RNA extracted from 9L cells, MADB106 cells, and normal tissues of F344 rats. The integrity of the RNAs was checked by electrophoresis in 1% agarose gel containing 5% formaldehyde and 20 mM 3-(*N*-morpholino)propanesulfonic acid. Gels containing 10  $\mu$ g total RNA per lane were blotted onto nylon membranes. After prehybridization, the membranes were incubated with the specific <sup>32</sup>P-labeled MIDA1 or calcyclin PCR of cDNA templates cloned in pTriplEx plasmid with the specific primers: CTCGGGAAGCGCCATTGTGTTGGT and ATACGACTCACTATAG-GGCGAATTGGCC (LD-Insert Screening Amplimer Sets; Clontech), overnight at 55°C in Church buffer (44). The membranes were then washed with 2 $\times$  SSC and 0.5% SDS at 55°C for 45 min. followed by another wash with 0.5 $\times$  SSC-0.1% SDS at 55°C for 45 min. Autoradiography was conducted at -80°C for up to 5 days using Kodak, Biomax MR; (Rochester, NY) films and intensifying screens. After exposure, the filters were stripped with Denhardt's solution (45) and rehybridized with a probe for 18S-rRNA to prove RNA integrity and consistency in RNA loading.

**Immunization with s.c. Injection of Naked Plasmid DNA and following Tumor Challenge.** A cloned cDNA fragment of calcyclin in the pTriplEx plasmid was excised by *Bst*XI-*Xba*I digestion and ligated into the expression plasmid vector pCDNA3.1(+) (Invitrogen, Carlsbad, CA). Because the stop codon was missing in the ORF of the cDNA fragment for the MIDA1 homologue, an artificial stop codon was introduced into the expression plasmid of this gene. A PCR fragment was designed with primers CCAGATGG-GAAGTCATTGCT and GCTCTAGATCACTGCTCTTCTGTT so that a *Dra*I site of rat MIDA1 was included, and the reverse primer introduced a stop codon followed by a *Xba*I recognition site. The *Bst*XI-*Dra*I fragment, from the original cloning vector, and the *Dra*I-*Xba*I-digested fragment of the PCR fragment were cloned into the pCDNA3.1(+) by ligation to form one ORF for rat MIDA1. A plasmid pCDNA3.1/CAT encoding chloramphenicol acetyl transferase was used as an unrelated control gene vector. Cloned genes were sequenced and 10 mg of plasmid for each clone were prepared using the Endo-Free Giga-prep system (Qiagen, Chatsworth, CA), and the endotoxin level was confirmed to be <100 U/mg DNA. F344 rats were immunized intradermally at the base of the tail with 0.5 ml of PBS containing 0.5 mg of purified expression plasmid using a 26-gauge needle (day 0). Immunization was repeated on days 10 and 20 in the same manner. Animals were then challenged s.c. in the right flank on day 27 with  $1 \times 10^5$  parental 9L tumor cells suspended in 0.1 ml of HBSS. Tumor size was calculated using calipers by multiplying the measured value of the long axis of the tumor by the measured value of the greatest perpendicular axis.

**Statistical Analyses.** Growth of SQ tumors and values for OD readings were evaluated for statistical significance using Student's *t* test for two samples with unequal variances. Statistical significance was assigned at the level of *P* < 0.05.

## RESULTS

**9L-IL4 Induces Th2 Isotype Switching *in Vivo*.** IL-4 is known to preferentially promote Th2 type responses and to promote the isotype

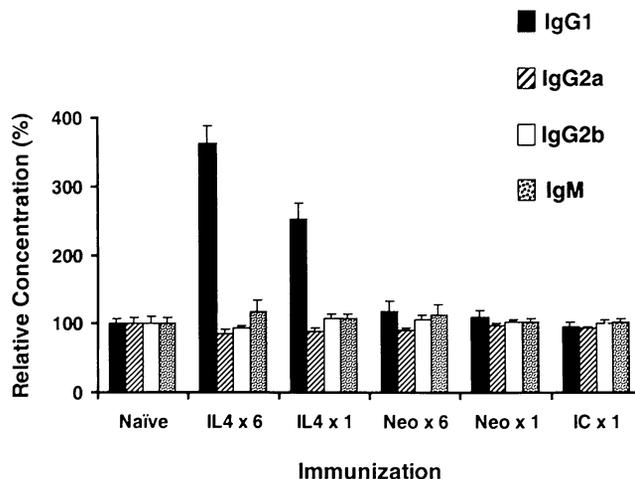


Fig. 1. Serum isotype switching induced by immunization with 9L-IL4. The concentration of IgG1, IgG2a, IgG2b, and IgM isotypes in nonimmune and immune sera was determined by ELISA assay. Data representing relative concentrations of each isotype in sera from rats after one ( $IL4 \times 1$ ) or six ( $IL4 \times 6$ ) s.c. injections of 9L-IL4 tumor cells, in sera from rats after one ( $neo \times 1$ ) or six ( $neo \times 6$ ) s.c. injections of 9L-neo tumor cells, in sera from rats given one ( $IC \times 1$ ) intracranial injection of 9L-neo, and serum from a nonimmunized rat. Relative concentration (%) was determined by dividing the absorbance (OD) of colorimetric determinations by ELISA of isotype concentrations by the OD of colorimetric determinations in normal sera  $\times 100$ .

class switching in humoral responses leading to increased levels of IgG1 (41, 46). To determine whether immunization of rats with 9L-IL4 influenced systemic humoral responses, we investigated whether differences in serum immunoglobulin isotype concentrations could be detected by ELISA among rats injected with 9L-IL4 cells or 9L-neo cells *versus* normal, control rats. As shown in Fig. 1, a significant increase in IgG1, but not in other isotypes, was detected in sera obtained from rats given a single injection of 9L-IL-4 ( $IL4 \times 1$ ;  $P = 0.0084$ ) or hyper-immunized with 9L-IL4 ( $IL4 \times 6$ ;  $P = 0.0036$ ) compared with serum obtained from a naive rat. In contrast, immunization one or six times with 9L-neo did not induce a demonstrable difference in the concentration of IgG1 or other isotypes in sera. Similarly, intracranial injection with 9L-neo, which simulated naturally occurring intracranial gliomas, did not result in a demonstrable change in serum isotype concentrations. Data shown represents one of three independent experiments yielding similar results; and values and error bars are means and standard deviations of triplicates. Strikingly, none of these conditions of immunization resulted in any demonstrable change in the concentration of the other isotypes tested, including IgG2a, IgG2b, and IgM. These results strongly suggest a potent, *in vivo* influence of IL-4 production by 9L on the antitumor humoral response, particularly in terms of production of IgG1.

**Isolation of Antigens Recognized by Serum Response following 9L-IL4 Immunization.** To determine whether immunization of rats with 9L-IL4 induced a humoral response to 9L associated antigens, we carried out SEREX using sera obtained from rats hyperimmunized with 9L-IL-4. A cDNA library was constructed from parental 9L cell line and was expressed in *E. coli* using a  $\lambda$ TriplEx phage system. Screening of at least  $5 \times 10^5$  recombinant clones from the parental 9L cDNA library with serum (1:400) from a 9L-IL4 hyperimmunized F344 rat led to the identification of four clones expressing antigens against which the animal had specific IgG antibodies. These were designated as clones 29, 37, 158, and 171. These four positive clones of the phage library were converted to the form of pTriplEx plasmids, and the nucleotide sequences of the cDNA inserts were determined. Sequence analyses revealed that the four clones encoded three distinct genes. Clone 29 was a 1.6-kb transcript, which was determined to be 100% homologous to the entire ORF of rat calcyclin, an S100 family

(S100A6) calcium-binding protein (47, 48). Clone 37 was determined to be encoded in a 2.7-kb transcript that represented a 98% homologous, partial sequence of mouse J6B7, an immunosuppressive molecule that is secreted by a T cell hybridoma and which suppresses mixed lymphocyte reactions (49). Clones 158 and 171 encoded identical 1852-bp fragments, which were 95% homologous to the mouse Id-associated protein (MIDA1) at the nucleic acid level. Clone 158 was also determined to be 98% homologous to MIDA1 at the predicted amino acid level (50). The sequences of clones 37 and 158 have been deposited in the European Molecular Biology Laboratory database under the accession numbers AF118854 and AF118853, respectively.

**Enhanced Detection of Tumor Antigens Using Sera from 9L-IL4 Immunized Rats.** To confirm that immunization of rats with 9L-IL4 versus 9L-neo resulted in a higher frequency of induction of 9L antigen-specific immunoglobulin, we compared immunoreactivity against calcyclin, and MIDA1 and J6B7 in sera from rats immunized 1× or 6× with irradiated 9L-neo or 9L-IL4. Immunoreactivity was also addressed in sera obtained from animals harboring intracranial 9L-neo tumors, simulating the situation of glioma patients' without vaccine therapy. Table 1 demonstrates the seroreactivity at 1:400 dilution of sera for all groups. Antibodies against calcyclin were detected in 5 of 5 animals with the 9L-IL4 × 6 immunization protocol, in 3 of 5 animals with the 9L-IL4 × 1 immunization protocol, in 3 of 5 animals in the 9L-neo × 6 immunization protocol, and only 1 of 5 animals with the 9L-neo × 1 immunization protocol. Antibodies recognizing J6B7 were detected in 4 of 5 animals with the 9L-IL4 × 6 immunization protocol, in 2 of 5 animals with the 9L-IL4 × 1 immunization protocol, in 2 of 5 animals with the 9L-neo × 6 immunization protocol, and in only 1 of 5 animals with the 9L-neo × 1 immunization protocol. Similarly, antibodies recognizing MIDA1 were detected in 5 of 5 animals with the 9L-IL4 × 6 immunization protocol, in 5 of 5 animals with the 9L-IL4 × 1 immunization protocol, in 2 of 5 animals with the 9L-neo × 6 immunization protocol, and in 0 of 5 animals with the 9L-neo × 1 immunization protocol. These data clearly indicate that immunization with 9L-IL4 × 6 conferred a higher frequency of induction of specific reactivity over immunization with 9L-neo × 6, and immunization with 9L-IL4 × 1 enhanced the induction of specific reactivity over immunization with 9L-neo × 1. None of sera obtained from animals with control HBSS or intracranial 9L injections contained detectable levels of antibodies against these clones by this method.

These data led us to determine the actual titer of antibody response in sera obtained from immunized animals. Because the reactivity against MIDA1 at 1:400 dilution was clearly higher in MIDA1 than with other antigens, sera from animals immunized with either 9L-IL4 or 9L-neo were titered against a phage clone coding for MIDA1 (clone 158). In addition to the experiment demonstrated in Table 2, we examined the titer of sero-response against MIDA1. A total of eight animals in each group was immunized with 9L-IL4 or 9L-neo six times, sera from these animals were serially diluted, and reactivity against MIDA1 was examined using phage blots. Representative positive signals on the blots are demonstrated in Fig. 2. Among a total of eight animals immunized with 9L-IL4, three animals demonstrated serum response against MIDA1 with titers of 1:400,000 or higher,

Table 2 Titer of anti-MIDA1 antibody in immunized animals

In a separate experiment, MIDA1 clone (clone 158) was tested for reactivity against 10-fold serially diluted sera (1:400 to 1:40,000) from animals hyperimmunized with 9L-IL4 or 9L-neo using the same plaque assay. Assays are scored positive only if MIDA1 clone is clearly distinguishable from negative control phages.

	Titer <sup>a</sup>
Animal(s) with 9L-IL4 vaccine	
3/8	1:40,000+
4/8	1:4,000
1/8	1:400
Animal(s) with 9L-Neo vaccine	
2/8	1:4,000
3/8	1:400
3/8	1:400-

<sup>a</sup> 1:40,000+ indicates that the titer is 1:40,000 or higher. 1:400- indicates that the titer is lower than 1:400.

four demonstrated 1:40,000, and one animal had a titer of 1:400. All animals were positive for MIDA1 at least at 1:400. In contrast, two of eight animals immunized with 9L-neo demonstrated titer of 1:4,000, three had 1:400, but the other three animals were negative for anti-MIDA1 response even at the lowest titer tested (1:400). These data suggest that immunization with IL-4 transfected 9L tumor enhances the detectability against MIDA1 antigen over to the condition immunized with non-cytokine-transfected 9L tumors.

**Analyses of Immunoglobulin Isotypes Recognizing the Cloned Antigens.** Inasmuch as we have generated data indicating that rats injected with 9L-IL-4 had increased levels of IgG1 isotype immunoglobulin in their sera (Fig. 1), we also analyzed the humoral response induced by immunization with 9L-IL4 to determine whether there was a corresponding production of antigen-specific IgG1. To that end, we analyzed the isotypes of antibodies recognizing calcyclin, J6B7, or MIDA1 expressed in *E. coli* using antirat IgG isotype-specific antibodies. As shown in Table 3, calcyclin was recognized by both immunoglobulins of both IgG1 and IgG2b isotypes. The J6B7 homologue was found to be recognized mainly by IgG2b and, to a somewhat lesser extent, by IgG1. Reactivity for the rat homologue of MIDA1 was greater in the sera assayed in comparison with reactivity for calcyclin or for the J6B7 homologue. In addition, IgG1 was found to be the predominant isotype with reactivity for the MIDA1 homologue. However, there was some reactivity for this molecule among IgG2b antibodies. These data support the conclusion that immunization with 9L-IL4 resulted in an enhanced humoral response against all three antigens and promoted production of reactive immunoglobulin of an IgG1, as opposed to an IgG2a, isotype against MIDA1.

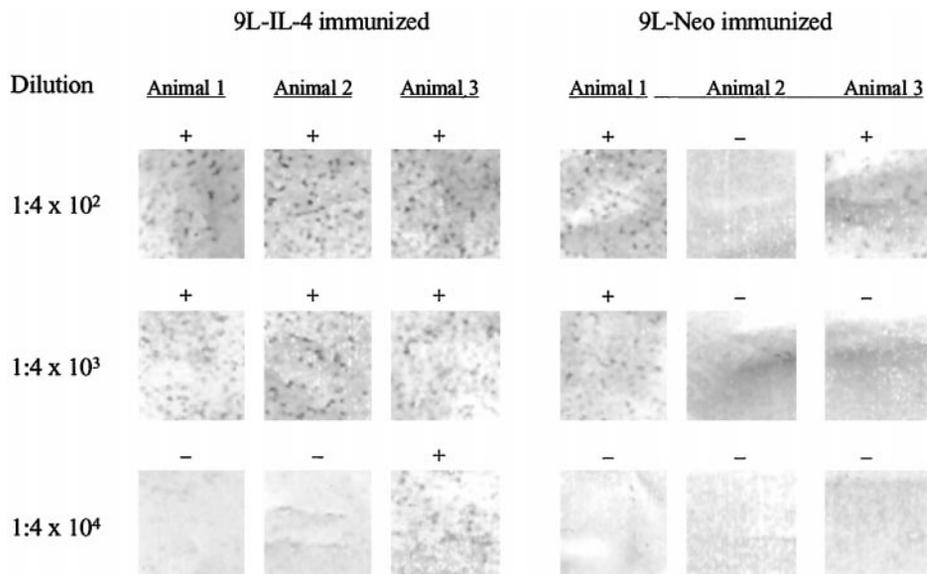
**Expression of Calcyclin, J6B7, and MIDA1 in Tumor Cells and Normal Tissues.** Screening of human tumor libraries by SEREX technology has resulted in the demonstration of serological reactivity against >600 antigens, and these have been divided among several categories, including mutational antigens, amplified/overexpressed antigens, and C-T antigens, because of their expression in tumor cells and testis (17). To assess both the level of expression of message and the normal tissue distribution of calcyclin, rat J6B7, and MIDA1, we carried out Northern blot analyses on RNA isolated from 9L tumor cells, brain, thymus, heart, lung, liver, spleen, muscle, and testis. As illustrated in Fig. 3, a high level of message for calcyclin was detected in 9L; and a lesser level of message was detected in lung. However,

Table 1 Sera from 9L-IL4 immunized animals confer higher sensitivity for detection of antigens

Phages for positive clones were plated on bacterial lawns, and blots were incubated with sera from animals following various immunization conditions for detection of reactive antibodies. Numbers represent animals' sera with positive reactivity/total animals tested at a dilution of 1:400, showing frequency of seroreactivity against three antigens.

Clones	9L-IL4 × 6	9L-IL4 × 1	9L-Neo × 6	9L-Neo × 1	9L-IC	HBSS
Calcyclin	5/5	3/5	3/5	1/5	0/5	0/5
J6B7 homologue	4/5	2/5	2/5	1/5	0/5	0/5
MIDA1 homologue	5/5	5/5	2/5	0/5	0/5	0/5

Fig. 2. Seroreactivity against MIDA1 clone in animals immunized six times with 9L-IL4 or 9L-neo. Serum dilutions are indicated. A  $\lambda$ Triplex phage fraction with no reactivity against 9L-rat sera was mixed with the MIDA1 clone and serves as internal negative control; these are visible as a background to the positive clones. Assays are scored positive only if test clones are clearly distinguishable from control phages. A total eight animals/group were analyzed (Table 2), and representative blots were demonstrated here. Anti-MIDA1 seroreactivity can be observed up to 1:400,000 in some of animals immunized with 9L-IL4, and positive reactivity was found in all 9L-IL4 immunized animals at least at 1:400. On the other hand, reactivity was weaker or absent in animals immunized with 9L-neo.



it could not be detected by Northern blotting in brain, heart, liver, spleen, muscle, or testis. These data support the conclusion that calcyclin is comparable with the amplified/overexpressed category of antigens. Northern blotting for MIDA1 resulted in the observation that message for this protein was readily detected in RNA from 9L. Message for MIDA1 was also detected in thymus and testis, however, it was not detected in brain, heart, lung, liver, spleen, or muscle. These data suggest that MIDA1 is comparable with the C-T category of antigens. Message for J6B7 was not detected in either 9L nor any of the normal tissues analyzed by Northern blotting (data not shown).

**DNA Vaccination with an MIDA1-encoding Construct Induced Significant Inhibition of Growth of Parental 9L Tumors.** To investigate the potential for use of calcyclin or MIDA1 as TRAs, we immunized rats with cDNA constructs encoding these proteins using

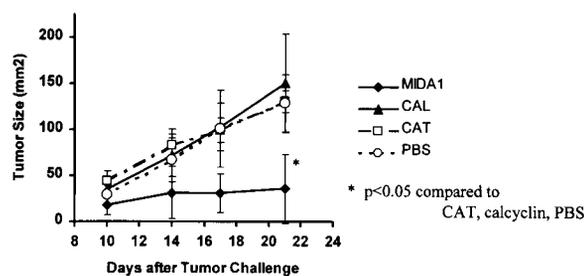


Fig. 4. Immunization of rats with a cDNA encoding MIDA1 resulted in the reduced tumorigenesis of parental 9L tumor cells. Rats were immunized (3 $\times$ ) by i.d. injection of 0.5 mg of plasmid encoding MIDA1, calcyclin, or CAT, or with PBS, on days 0, 10, and 20. On day 27, rats were given  $1 \times 10^5$  9L cells s.c. Tumor volumes were determined serially on the days indicated. \*,  $P < 0.05$  compared with CAT, calcyclin, and PBS.

Table 3 IgG isotypes recognizing genes clones by CAS<sup>a</sup>

Genes	IgG	IgG1	IgG2a	IgG2b
Calcyclin	+	+	-	+
J6B7 homologue	+	±	-	+
MIDA1 homologue	++	++	-	±

<sup>a</sup> -, negative; ±, weakly positive; +, positive; ++, strongly positive on blots.

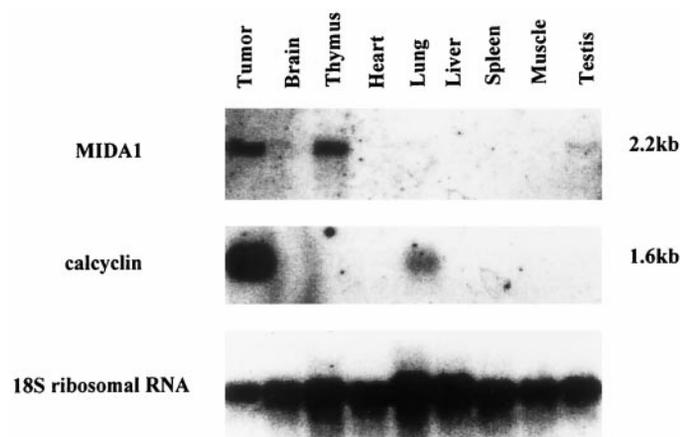


Fig. 3. Northern blot analyses of MIDA1 and calcyclin. Expression of MIDA1 mRNA was detected in 9L, in thymus, and in testis, but not from any other organ tested, including brain, heart, liver, spleen, and muscle. Message for calcyclin was detected as an intense signal in 9L and, to a lesser, degree, in lung; but not from any other organ tested. A probe for 18S rRNA was used as an internal control.

naked DNA delivered intradermally (3 $\times$ ) and then evaluated the effects of this immunization on s.c. 9L tumor growth. Expression of the transgenes *in vivo* was assessed by the demonstrating antibodies in sera reactive to calcyclin or MIDA1 by Western blotting of these proteins expressed in *E. coli* (data not shown). As illustrated in Fig. 4, immunization of rats with a cDNA encoding MIDA1 homologue resulted in the significantly reduced growth of parental 9L tumors in comparison with those immunized with a cDNA encoding calcyclin or a control plasmid vector encoding an unrelated gene chloramphenicol acetyl transferase (CAT). In addressing this issue, we have attempted both i.d. delivery of plasmid; delivery of MIDA1 encoded in an adenoviral vector; as well as gene gun delivery for immunization. In two experiments using i.d. immunization with plasmids encoding MIDA1, growth of parental 9L was inhibited. In two experiments involving immunization with adenoviral vector encoding MIDA1, growth of parental 9L was inhibited (data not shown). However, in two experiments using gene gun delivery, we were unable to detect antitumor effects of MIDA1 immunization (data not shown). Cumulatively, our results strongly support the conclusion that CAS is an effective means of identifying TAAs, which may be candidate TRAs for gliomas, and that MIDA1 represents a rat 9L TRA.

## DISCUSSION

In this report, we present data indicating that peripheral immunization of syngeneic rats with IL-4-transduced 9L gliosarcoma cells

resulted in an enhanced immune response to antigens associated with parental 9L tumor cells. To identify 9L TAAs recognized after immunization with 9L-IL4, we used sera from 9L-IL4 hyperimmunized rats for serological screening of a 9L cDNA library. As our screening, CAS, was based on the use of an expression library constructed from parental 9L cells, the possibility of identifying antigens uniquely associated with transfection of IL-4 was eliminated. In this fashion, we demonstrated an increased serological reactivity against three 9L TAAs, including calcyclin and the rat homologues of J6B7 (50) and MIDA1 (49). Although we have clearly been able to establish potent serological reactivity to these three proteins, additional factors must be considered for determining whether they will serve as potential TRAs, and, more importantly, whether these preclinical findings may ultimately direct avenues of research for human glioma TRAs.

Calcyclin, S-100A6, is known to be expressed by chemically induced rat mammary carcinoma cells (47) and by various human tumor cells (51, 52), and it is also known to serve as a progression marker for human melanomas (48). However, despite extensive screening of a melanoma cDNA library with sera from melanoma patients, human calcyclin has not been identified by SEREX to date (17). Our contrasting data using 9L could be attributable to a higher population of S-phase cells, in which calcyclin can be up-regulated in animal tumor models (52). It is also possible that future analyses may reveal IL-4 enhances the potential for recognition of calcyclin in melanoma, as was the case with 9L; but to date, such analyses have not been reported. However, an additional factor suggests some concerns for the suitability of calcyclin for use as a TRA. That is, we demonstrated a high level of expression of message for calcyclin in normal lung tissue, suggesting a potential for deleterious effects of hyperimmunization against calcyclin. However, we have carried out experiments immunizing rats with constructs encoding calcyclin, and we have not seen any impact of calcyclin immunization on normal tissues upon necropsy (data not shown).

The second 9L TAA identified by CAS was the rat homologue of the mouse J6B7 immunosuppressive molecule. Similar to mouse J6B7, the rat homologue identified here was predicted to be a secreted protein, rather than an intracellular or cell-surface protein. This suggested that this J6B7 may not be the most attractive candidate for evaluation as a TRA. Therefore, we have not yet pursued experiments examining J6B7 as a potential TRA.

The third TAA identified by CAS was the rat homologue of MIDA1, which is a 621-amino acid/M<sub>r</sub>74,000 protein that can associate with Id, a HLH protein (50, 53). On the basis of its predicted amino acid sequence, MIDA1 consists of a Zuotin (a Z-DNA-binding protein in yeast) homology region and tryptophan-mediated repeats similar to those in the c-Myb oncoprotein. MIDA1 associates with the HLH region of Id via a conserved region adjacent to a eukaryotic DnaJ conserved motif within the Zuotin region, although it does not have any canonical HLH motif (53). MIDA1 has now also been shown to bind Z-DNA, and the binding of MIDA1 to Id1 stimulated sequence-specific DNA-binding activity, although it inhibited the Z-DNA-binding activity (53). In functional analyses, the addition of antisense oligonucleotides of MIDA1 to an erythroleukemia line inhibited growth without interfering with erythroid differentiation, indicating that it regulates cell growth (50).

Northern blot analyses to determine message levels for MIDA1 in RNA derived from tumor and normal tissues indicated its selective expression in tumor, testis, and thymus. This distribution was similar to the pattern of expression of a number of known TRAs, *i.e.*, so-called C-T antigens (54, 55). Given the similarity of MIDA1 to TRAs defined using traditional SEREX (17) and using tumor-specific T cell clones (18), we investigated whether direct immunization against MIDA1 was capable of inducing an anti-9L tumor response.

As shown in Fig. 4, an inhibition of tumor growth was observed in animals immunized with plasmid encoding MIDA1. These data clearly support the hypothesis that MIDA1 is a TRA for 9L and suggests the potential for use of MIDA1 for inducing protective immune responses. However, high-level expression of MIDA1 in thymus raises a concern of autoimmune destruction of thymus, which would induce central tolerance, if MIDA1 is used as a vaccine. Nevertheless, in our vaccination experiments, we did not see any effect on thymus upon necropsy (data not shown). Therefore, the immunogenicity of 9L-derived MIDA1 may be attributable to mutations of MIDA1 in gliomas. We are currently analyzing the sequence of normal organ-derived MIDA1. We are also trying to isolate T cells reactive against MIDA1 from the splenocytes of animals immunized with MIDA1. Human gliomas are currently being examined for the expression of this gene in our laboratories.

Our data suggest that using CAS may have resulted in an enhanced capacity to identify glioma TAAs with characteristics similar to TAAs identified by traditional SEREX (17). However, the quantitative and qualitative differences between humoral immune responses elicited by IL4-secreting rat 9L glioma vaccines *versus* by irradiated 9L-neo vaccines, or by irradiated 9L glioma cells modified to secrete other cytokines, have not been fully characterized. This potentially enhanced capacity could be the result of several factors. For instance, IL-4 could increase the potential for antigen detection through an enhancement of B cell activation and isotype switching. In fact, our data on the relative concentration of different isotypes of immunoglobulin and titer of antibody response against MIDA1 in sera from parental 9L-neo *versus* 9L-IL4 hyperimmunized rats supports this hypothesis. In these experiments, there was clear evidence for induction of Th2 type isotype switching as the concentration of IgG1 was significantly up-regulated in 9L-IL4 immunized rats. More importantly, antibodies reactive with MIDA1 and the other tumor antigens in our detection system were predominantly isotypes of IgG. Therefore, positive clones selected in this screening would likely express proteins to which CD4<sup>+</sup> T cell responses had been elicited, as the presence of reactive IgG implies T cell help for class switching (56). Although a number of studies have supported a role for CD8<sup>+</sup> cells in mediating antitumor activity to CNS tumors (14, 57), CD4<sup>+</sup> T helper responses have been shown to play an important role for the induction of CD8<sup>+</sup> CTLs against MHC class II negative tumor cells (58). Furthermore, we have reported that CD4<sup>+</sup> T cells are essential for expression of immunity induced by immunization with 9L-IL4 (8). In that vein, CAS using IL-4 may provide a powerful method to identify important epitopes for induction of effective antitumor responses. Cumulatively, the models we have developed and the data we have presented provide compelling support for the continued use of CAS for the search of human tumor rejection antigens such as gliomas with IL-4 transduced tumor vaccine.

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## Immunization with an Antigen Identified by Cytokine Tumor Vaccine-assisted SEREX (CAS) Suppressed Growth of the Rat 9L Glioma *in Vivo*

Hideho Okada, Jason Attanucci, Katinka M. Giezeman-Smits, et al.

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