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CTL Response and Protection Against P815 Tumor Challenge in Mice Immunized with DNA Expressing the Tumor-Specific Antigen P815A

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ABSTRACT

A DNA immunization approach was used to induce an immune response against the tumor-specific antigen P815A in DBA/2 mice. The *P1A* gene, which encodes the P815A antigen, was modified by the addition of a short sequence coding for a tag epitope recognized by the monoclonal antibody AU1, and cloned into the eukaryotic expression vector pBKCMV, resulting in plasmid pBKCMV-P1A. L1210 cells stably transfected with pBKCMV-P1A expressed P1A mRNA and were lysed by the syngeneic P815A-specific cytotoxic clone CTL-P1:5, thus confirming that the tag-modified P1A protein underwent correct processing and presentation. A single intramuscular injection of 100 μg of pBKCMV-P1A induced the expression of P1A mRNA for at least 4 months. Eighty percent of DBA/2 mice injected three times with 100 μg of pBKCMV-P1A generated cytotoxic T lymphocytes (CTL) that lysed P815 tumor cells, whereas mock-inoculated animals failed to show any cytotoxicity. Moreover, experiments designed to evaluate the protection of pBKCMV-P1A-immunized mice against a lethal challenge with P815 tumor cells showed that 6 of 10 immunized mice rejected the tumor, and 2 mice showed prolonged survival compared to control animals.

OVERVIEW SUMMARY

The murine *P1A* gene, which codes for the tumor-specific antigen P815A, was cloned into a eukaryotic expression vector and employed to evaluate the feasibility of a DNA immunization approach in a physiological model that closely resembles human cancer. The DNA immunization procedure resulted in cytotoxic T lymphocyte generation and protection against a lethal challenge with tumor cells expressing the relevant antigen.

INTRODUCTION

In the past few years, many human tumor antigens have been identified and cloned molecularly. Some of these new antigens, whose prototype is represented by the MAGE family, are considered tumor-specific antigens (TSA), because they are produced from genes not expressed in normal tissues, with the exception of testis. A second group of antigens consists of differentiation antigens, which are present both on tumors and on normal cells having the same histological origin; finally, the third group of antigens is represented by point mutants of normal cellular proteins, and can be considered specific for individual tumors (Boon and Van der Bruggen, 1996; Robbins and Kawakami, 1996). The discovery of a growing number of tumor antigens raises the possibility that they could be used as targets for active immunotherapy.

In contrast to the numerous TSA identified in humans, only a few have been described in animal models (Jaffee and Pardoll, 1996). In fact, with the exception of antigens derived by mutation of proto-oncogenes, the only identified murine tumor antigen originating from a normal gene is P815A, which represents a major tumor rejection antigen of the mastocytoma cell line P815, a methylcholanthrene-induced tumor (Van den Eynde *et al.*, 1991). The P815A antigen is recognized by specific cytotoxic T lymphocyte (CTL) clones in the context of the major histocompatibility complex (MHC) class I molecule L^d, and has been mapped to amino acids 35–43 of the P1A protein (Lethé *et al.*, 1992). The *P1A* gene, like human genes belong-

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ing to the MAGE, GAGE, and BAGE families, is silent in normal tissues, with the exception of testis and placenta. However, notwithstanding *P1A* gene expression in these organs, CTL generation against P815A antigen does not induce autoimmune side effects (Uyttenhove *et al.*, 1997). Although it was originally reported that expression of the *P1A* gene was confined to mastocytomas (Van den Eynde *et al.*, 1991), a wider expression of the gene has recently been found among different tumor cell lineages (Ramarathinam *et al.*, 1995). These observations, on the whole, indicate that P815A may be considered a murine counterpart of human TSA expressed by melanomas and other tumors, and therefore might represent a useful model for the development of tumor vaccination strategies in humans.

Injection of "naked DNA" plasmid vectors (also referred to as DNA immunization) either into skeletal muscle or intradermally has been shown to be effective in eliciting a strong immune response against a variety of infectious agents (Hassett and Whitton, 1996; McDonnell and Askari, 1996; Ulmer *et al.*, 1996). Upon injection, the plasmid DNA is taken up by myocytes and professional antigen-presenting cells, and induces the expression of vector-encoded antigens that are able to prime both MHC class I-restricted CTL as well as MHC class II-restricted helper T cells (Corr *et al.*, 1996). In fact, it has been reported that *in vivo* delivery of plasmid DNA encoding specific antigens induced humoral and cellular immune responses to influenza (Donnelly *et al.*, 1995), hepatitis B (Michel *et al.*, 1995; Kuhöber *et al.*, 1996), and human immunodeficiency viruses (Wang *et al.*, 1995), to parasites such as the malaria circumsporozoite (Sedegah *et al.*, 1994), and to mycobacterium tuberculosis (Huygen *et al.*, 1996; Tascon *et al.*, 1996).

However, only a few studies have examined whether immunization of mice with plasmid DNA encoding tumor antigens could elicit an immune response capable of destroying the tumor cells (Spooner *et al.*, 1995). Moreover, although the antigens used thus far for vaccination, such as β-galactosidase (Irvine *et al.*, 1996), SV40 large tumor antigen (Bright *et al.*, 1996), and human carcinoembryonic antigen (Conry *et al.*, 1995), represent useful tumor antigen models, they cannot be considered physiological tumor antigens because they are not normal mouse gene products.

To test the DNA vaccination approach with TSA comparable to those detected in humans, we injected mice intramuscularly with a plasmid coding for the P1A protein. The present report demonstrates that this immunization procedure was able to induce the generation of tumor-specific CTL, and resulted in protection against a challenge with a tumor cell line expressing the relevant TSA.

MATERIALS AND METHODS

Mice and cell lines

Five to six-week-old female DBA/2 mice were purchased from Charles River Laboratories (Calco, Como, Italy). The P815 mastocytoma and L1210 lymphoma cell lines were originally obtained from DBA/2 mice that had been treated with methylcholanthrene. P1 is a clonal line isolated from a permanent cell line derived from the transplantable P815-X2 subline (Uyttenhove *et al.*, 1980); the P1 line was used throughout the study and hereafter is referred to as P815. Cell line IR5P1A$^-$ is a P815A-loss variant isolated from a P815 tumor that grew in a DBA/2 mouse that had been challenged with P815 cells after immunization with the irradiated cell line. The murine fibrosarcoma cell line WEHI 164 was used as a tumor necrosis factor (TNF) cytotoxicity-sensitive target. HLtat cells (a derivative of human HeLa cells; see Schwartz *et al.*, 1990) and T6 cells (a murine fibroblast cell line; D. Saggioro *et al.*, manuscript in preparation) were used in transient transfection assays. The P815A-specific CTL clone CTL-P1:5 was a gift from Dr. B. Van den Eynde (Ludwig Institute for Cancer Research, Brussels) and was cultured as previously reported (Van den Eynde *et al.*, 1991). All tumor cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Paisley, UK) supplemented with 2 m*M* L-glutamine, 10 m*M* HEPES [4-(2-hydroxy-ethyl)-1-piperazine ethanesulfonic acid], 20 μM 2-mercaptoethanol, 150 U/ml streptomycin, 200 U/ml penicillin, and 5% heat-inactivated fetal calf serum (FCS) (PAA Laboratories, Linz, Austria).

Plasmid construction

The *P1A* open reading frame was amplified by the polymerase chain reaction (PCR) from the complete *P1A* cDNA (kindly provided by Dr. B. Van den Eynde) using the following primers: 5′-GGTACCCTTTGTGCCATGTC-3′ (P1, sense) and 5′-TTATATATATCGATATGTATCAGGTGAGAAGCCATCCGGG-3′ (P2AU, antisense). The 5′ terminus of P2AU coded for a 6-amino-acid tag epitope (DTYRYI) recognized by the monoclonal antibody (mAb) AU1 (BAbCO, Richmond, CA), followed by a stop codon. PCR amplification was performed using Vent DNA polymerase (New England BioLabs, Hitchin, UK), 0.5 μM each primer and 240 ng of template, and consisted of 20 rounds of amplification, each of 40 sec at 95°C, 40 sec at 63°C, and 1 min at 72°C. The 708-bp PCR product was purified from an acrylamide gel, phosphorylated using T4 polynucleotide kinase (New England BioLabs) and cloned into the filled-in *Bam* HI site of pBluescript KS$^-$ (Stratagene, La Jolla, CA); the insert was then transferred to the eukaryotic expression vector pBKCMV (Stratagene) as a *Hind* III–*Sac* I fragment. This plasmid was digested with *Nhe* I and *Spe* I and religated to remove sequences between the human cytomegalovirus (CMV) promoter and the *P1A* open reading frame (ORF), resulting in pBKCMV-P1A. The complete *P1A* sequence of pBKCMV-P1A was verified by sequence analysis (fMol, Promega, Madison, WI). Plasmid DNA was purified using Qiagen columns (QIAGEN GmbH, Hilden, Germany) following the supplier's protocol.

Generation and selection of L1210-CMV-P1A clones

L1210 cells (5×10^6) were transfected in 0.5 ml of DMEM-10% FCS with 20 μg of pBKCMV-P1A or with the pBKCMV vector lacking the insert by electroporation using a Gene Pulser System (Bio Rad Laboratories, Richmond, CA) set at 300 V, 960 μF in 0.4 cm-gap cuvettes. Selection of transfected cells in mass cultures was carried out in complete DMEM supplemented with 1 mg/ml Geneticin (G-418) (Boehringer Mannheim GmbH, Mannheim, Germany). After 14 days, the cells were seeded in 96-microwell plates at 10 cells per well in

100 μl of DMEM-10% FCS; 8 days later, the resulting L1210-CMV-P1A and L1210-CMV sublines were incubated with CTL-P1:5 cells in DMEM-5% FCS for 4 hr and the supernatants were analyzed in TNF cytotoxicity assay using WEHI 164 cells. After 20 hr, cell viability was measured by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO). The L1210-CMV-P1A sublines that induced the highest levels of TNF were cloned by limiting dilution in 96-microwell plates at 0.3 cell per well. Some of the resulting clones were analyzed for P1A expression by reverse transcription (RT)-PCR, as described below, and tested in ^{51}Cr-release assays with CTL-P1:5 cells as effectors.

DNA immunization protocol and analysis of P1A mRNA expression

Female DBA/2 mice were anesthetized by ethyl ether inhalation; a 1.0-cm incision was made on the posterior thigh and the quadriceps muscle was exposed. The pBKCMV-P1A plasmid (100 μg in 100 μl of saline solution) was injected to a depth of about 2 mm using a 1-ml syringe and a 27-gauge needle. The entire quadriceps muscles were removed at days 1, 7, 15, 30, 60, or 135 after the inoculation and immediately frozen in liquid N_2. Total RNA was extracted using the RNAfast isolation system (Molecular System, San Diego, CA), treated with DNase I (Boehringer Mannheim), and 2-μg portions were reverse-transcribed in a 20-μl final volume by extension with oligo(dT)$_{12-18}$, using SuperScript II (M-MuLV) reverse transcriptase (GIBCO-BRL). cDNA corresponding to 500 ng of RNA was used for PCR amplification in a total volume of 50 μl, containing 200 μ*M* of each dNTP, 2 m*M* $MgCl_2$, 1.25 units of *Taq* polymerase (Promega Corporation, Madison, WI), and 0.5 μ*M* sense P1 primer and antisense P2 primer (5′-CTAAGGTGAGAAGCCATCCGGG-3′) in PCR buffer. Amplification was performed for 35 cycles at 95°C for 40 sec, 63°C for 40 sec, and 72°C for 1 min. A 30-μl aliquot of each PCR reaction was analyzed in a 1.2% agarose gel and visualized by ethidium bromide staining. RNA from P815 cells was processed as above and utilized as a positive control for the expected 690-bp P1A amplification product; RNA extracted from muscles of DBA/2 mice 1 day after injection with pBKCMV lacking insert was used as a negative control. PCR amplification of samples not subjected to reverse transcription was also carried out to verify the absence of contaminating DNA. Amplification of β-actin cDNA was performed to check integrity of the RNA samples.

Mixed lymphocyte tumor cell culture

Female DBA/2 mice were injected three times at 10-day intervals with 100 μg of pBKCMV-P1A as described above. Mice inoculated three times subcutaneously (s.c.) with 5×10^6 irradiated P815 cells or intramuscularly (i.m.) with the pBKCMV vector lacking insert served as positive and negative controls, respectively. Ten days after the last inoculation of plasmid DNA, the animals' spleens were removed and 2.5×10^7 splenocytes were restimulated *in vitro* in a mixed lymphocyte tumor cell culture (MLTC) with 5×10^5 irradiated P815 cells in 15 ml of DMEM-10% FCS. Cultures were maintained in 25-cm^2 tissue culture flasks (Falcon, Becton Dickinson, Lincoln Park, NJ) at 37°C and 5% CO_2. After 5 days of incubation, MLTC cells were tested for their lytic activity in a ^{51}Cr-release assay.

Chromium release assay

Cytolytic activity was measured as described elsewhere (Rosato *et al.*, 1994). Briefly, ^{51}Cr-labeled target cells were incubated with effector cells at various effector/target ratios in 96-well microplates. After incubation for 4 hr at 37°C supernatants were harvested and radioactivity was counted in a microplate scintillation counter (Top-Count, Packard Instruments Company, Meriden, CT). For peptide-pulsing, 10^6/ml ^{51}Cr-labeled IR5P1A$^-$ cells were incubated with a peptide corresponding to amino acids 35–43 of the P1A protein (10 μg/ml) for 30 min at 37°C, and then washed three times before use.

Tumor protection assay

Mice vaccinated three times with pBKCMV-P1A plasmid DNA as described above were challenged s.c. with 1×10^6 P815 cells 3 weeks after the last DNA immunization, and then followed for 120 days after tumor inoculation. Mice injected with pBKCMV and noninoculated animals served as negative controls.

RESULTS

Functional analysis of plasmid pBKCMV-P1A

To evaluate P1A expression, we modified the gene by tagging it with a sequence coding for the AU1 epitope and cloned it into a eukaryotic expression vector driven by the human CMV promoter, resulting in plasmid pBKCMV-P1A. The expression of P1A protein from pBKCMV-P1A was verified by anti-AU1 immunoblotting of lysates prepared from transiently transfected human (HLtat) and murine (T6) cells. Results revealed that pBKCMV-P1A expressed the expected 38-kD P1A product, which appeared to be phosphorylated on the basis of *in vivo* [^{32}P]orthophosphate-labeling experiments (data not shown). The intracellular distribution of the P1A protein was studied by immunofluorescence analysis of transiently transfected HLtat and T6 cells using anti-AU1 mAb and anti-mouse Ig-FITC. We observed that the P1A protein had a mixed distribution in HLtat cells, accumulating in the cytoplasm of some cells and in the nucleus of others, and appeared to be excluded from nucleoli; T6 cells showed an almost exclusive cytoplasmic accumulation of the protein (data not shown). The observed apparent size, phosphorylation, and subcellular distribution of P1A produced from pBKCMV-P1A were consistent with those of natural protein expressed in P815 cells (Amar-Costesec *et al.*, 1994).

To assess whether the AU1 tag-modified P1A protein underwent correct processing and presentation of the natural antigenic peptide, we transfected syngeneic L1210 cells, which do not express endogenous P1A, with pBKCMV-P1A. P1A-expressing clones were selected by their ability to induce a strong release of TNF by the P815A-specific CTL clone CTL-P1:5. Six of these positive clones were randomly chosen for further analysis. As shown in Fig. 1A, all six clones expressed P1A mRNA, as demonstrated by RT-PCR analysis, and were lysed by CTL.P1:5 when tested in a short-term ^{51}Cr release assay, al-

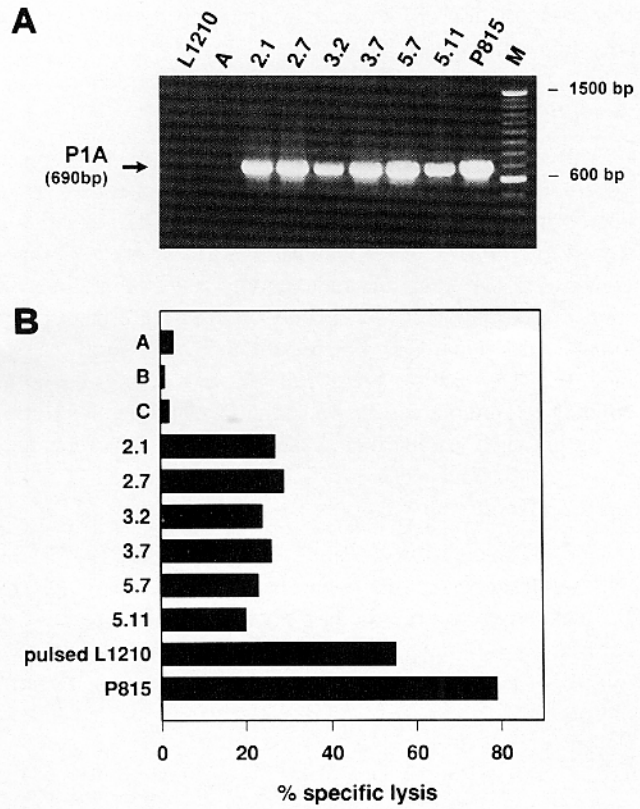

FIG. 1. Generation of *P1A*-expressing L1210 clones. A. P1A mRNA expression. RNA was extracted from untransfected L1210 cells (lane 1), from a mock-transfected clone A, (lane 2), and from six L1210-CMV-P1A clones (2.1, 2.7, 3.2, 3.7, 5.7, 5.11, lanes 3–8), reverse-transcribed, and the resulting cDNA was amplified by PCR using P1A-specific primers (RT-PCR). Products were visualized by ethidium bromide staining after electrophoresis in a 1.2% agarose gel. Lane 9, Positive control (P815 cells); lane 10, 100-bp ladder DNA size markers (M). B. Lytic activity of CTL-P1:5 toward L1210-CMV-P1A clones. Three ^{51}Cr-labeled, mock-transfected clones (A,B,C) and six L1210-CMV-P1A clones (see above) were incubated with CTL-P1:5 at an effector/target ratio of 10 in a 4-hr ^{51}Cr-release assay. L1210 cells pulsed with a peptide corresponding to amino acids 35–43 of the P1A protein and P815 cells were tested as positive controls.

beit to a lower extent compared to L1210 cells pulsed with a peptide corresponding to amino acids 35–43 of the P1A protein (Fig. 1B). L1210 cells transfected with vector lacking the insert did not express P1A mRNA and were not lysed by CTL-P1:5 (Fig. 1A,B). These observations demonstrated that pBKCMV-P1A was able to drive the expression of P1A protein, and that the presence of the AU1 tag did not alter the normal processing and presentation of the P815A antigen.

To study whether the injection of pBKCMV-P1A into skeletal muscle would result in efficient and prolonged expression of the *P1A* gene, 6 DBA/2 mice received a single inoculation of 100 μg of pBKCMV-P1A into the quadriceps muscle. RT-PCR analysis was then performed on mRNA extracted from the injected muscles after different periods of time. Results revealed a positive signal for more than 4 months after DNA injection (Fig. 2, days 1–135); no signal was detected in muscles from control mice injected with pBKCMV vector lacking the insert (Fig. 2, lane C). Therefore, a single i.m. injection of pBKCMV-P1A was sufficient to induce long-lasting expression of the *P1A* gene at the inoculation site.

CTL generation and in vivo *protection against P815 tumor cell challenge in mice immunized with pBKCMV-P1A*

To evaluate whether *P1A* naked DNA immunization was able to elicit a tumor-specific CTL response, DBA/2 mice were injected i.m. three times with 100 μg of pBKCMV-P1A at 10-day intervals; mice inoculated three times with the same amount of pBKCMV vector lacking the *P1A* gene were used as negative controls. Ten days after the last inoculation, splenocytes from injected mice were stimulated in MLTC with irradiated P815 tumor cells; 5 days later, the cytotoxic activity was evaluated using P815 cells as targets. As shown in Fig. 3, CTL activity against P815 cells was observed in 12 out of 15 pBKCMV-P1A-injected mice; the 3 remaining mice did not show significant cytotoxicity, because their values differed by less than 3 SD from the mean value of pBKCMV-injected control mice. It should be noted that in 5 of the 12 positive mice, the lytic activity was very high and comparable to that of MLTC prepared using spleen cells of mice that were immunized three times with 5×10^6 irradiated P815 tumor cells.

To ensure that the lytic activity observed in the MLTC was specific for the P815A antigen, an antigen-loss variant of the original P815 cell line was isolated from a tumor that grew in a DBA/2 mouse that had been immunized with irradiated P815 cells and then challenged with P815 cells. This cell line, designated IR5P1A$^-$, did not express P1A mRNA as assessed by RT-PCR, and was not lysed by CTL-P1:5. However, *in vitro* loading of the cell line with the antigenic peptide corresponding to amino acids 35–43 of the P1A protein completely restored susceptibility of the cells to lysis by CTL-P1:5 (data not shown). Figure 4 shows that MLTC prepared using splenocytes from pBKCMV-P1A-injected mice did not disclose any appreciable reactivity against IR5P1A$^-$ cells. However, cytotoxic activity of the MLTC was fully restored upon pulsing the IR5P1A$^-$ cells with the antigenic peptide, thus verifying that the cytotoxic activity elicited by the *in vivo* DNA immunization approach was highly specific and directed against the antigen expressed by the DNA vaccine.

Experiments were then performed to evaluate whether immunization with pBKCMV-P1A would protect mice against a lethal challenge with P815 tumor cells. Ten DBA/2 mice were injected three times with 100 μg of pBKCMV-P1A at 10-day intervals; 10 mice inoculated with pBKCMV vector lacking the *P1A* gene and 10 nontreated mice served as negative controls. Three weeks after the final DNA inoculation, the mice were injected s.c. with 10^6 P815 tumor cells. As shown in Fig. 5, six of ten mice that had been vaccinated with pBKCMV-P1A were able to reject the tumor. Two mice showed a prolonged survival, whereas the remaining 2 died in a period comparable to that of the control animals. Two of the 10 control animals injected with pBKCMV and 2 control mice that did not receive any treatment spontaneously regressed the neoplasia; this finding is not surprising, because spontaneous regression of P815 tumor cell challenge in untreated mice has also been reported by others (Brichard *et al.*, 1995; Levraud *et al.*, 1996).

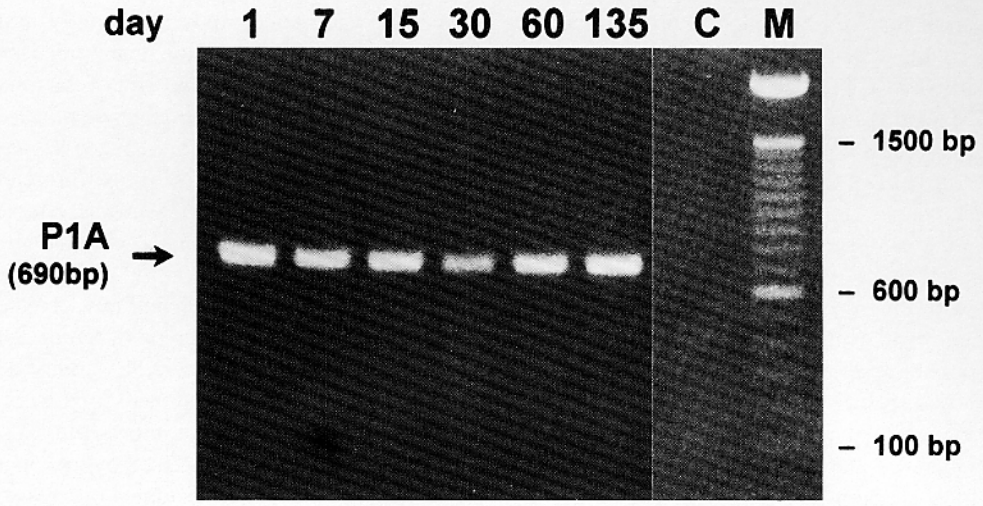

FIG. 2. Kinetics of *in vivo* expression of P1A mRNA. Total RNA was isolated at different timepoints from muscles injected with pBKCMV-P1A, reverse-transcribed, and the resulting cDNA was amplified by PCR using P1A-specific primers. Products were visualized by ethidium bromide staining after electrophoresis in a 1.2% agarose gel. Lanes 1–6, mice injected with pBKCMV-P1A; lane 7, mock-injected mouse (negative control, C); lane 8, 100-bp ladder DNA size markers (M).

DISCUSSION

Significant results have already been achieved in the field of gene therapy of cancer in animal models by the use of a variety of viral vectors expressing experimental antigens such as β-galactosidase from *Escherichia coli*, chicken ovalbumin, and nucleoprotein from vesicular stomatitis virus (for review, see Restifo, 1996); however, the heterologous nature of these models and strong immunogenicity of the antigens employed make it difficult to extrapolate results of these studies to human neoplasias. We chose to use the murine tumor antigen P815A to develop a mouse model that would more closely resemble the situation in human cancers. In fact, the gene coding for the P815A antigen shares many characteristics with already identified human tumor antigens (Jaffee and Pardoll, 1996).

Studies of TSA in cancer cells have often been hampered by difficulties in obtaining appropriate mAb or antisera. Few reports have described the generation of mAb recognizing human TSA, such as MAGE-1 (Schultz-Thater *et al.*, 1994), MAGE-3 (Kocher *et al.*, 1995), and MAGE-4 (Shichijo *et al.*, 1995). To provide a general means to evaluate TSA expression and to trace the protein product, we modified the *P1A* gene by the addition of a sequence coding for the AU1 tag epitope, recognized by a specific mAb. Introduction of the tag epitope facilitated the characterization of the P1A protein produced, whose apparent molecular weight, intracellular distribution, and post-translational modifications matched those described for the natural protein (Amar-Costesec *et al.*, 1994). In addition, process-

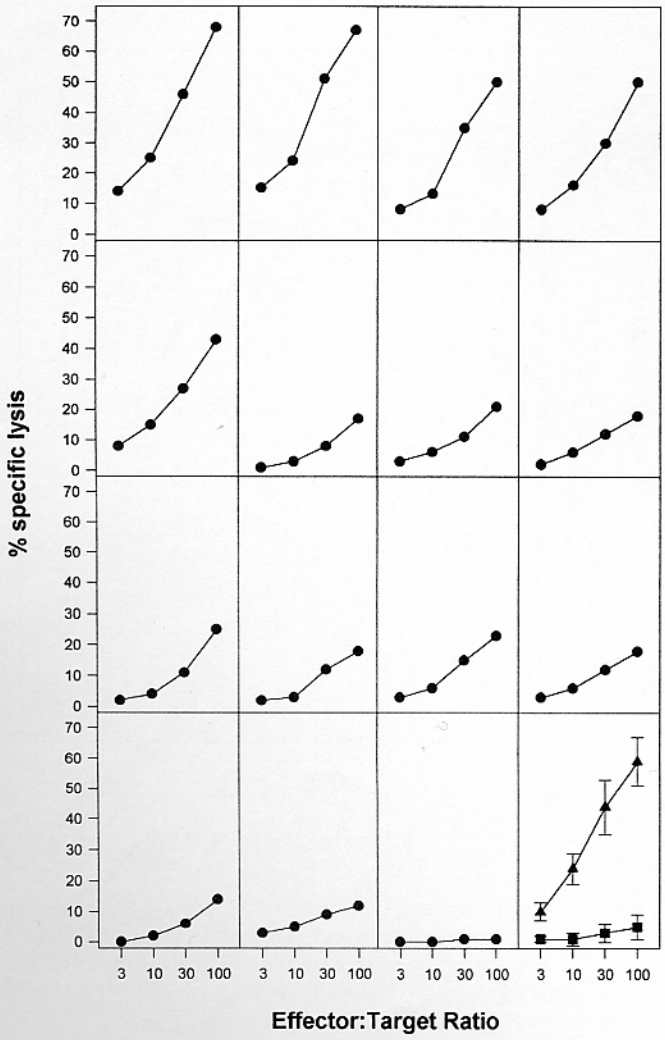

FIG. 3. Lysis of P815 target cells by CTL from DBA/2 mice injected three times with pBKCMV-P1A. Spleen cells were collected 10 days after the last plasmid inoculation and restimulated *in vitro* in MLTC for 5 days in the presence of irradiated P815 cells. Each plot representes the cytotoxic activity obtained with spleen cells of 1 mouse against ^{51}Cr-labeled P815 cells. The lytic activities of splenocytes from 15 mock-injected mice (squares) and from 5 animals inoculated three times with irradiated P815 cells (triangles) are shown as mean ± SD in the bottom right-hand plot. Mice immunized with pBKCMV-P1A were considered positive if their cytotoxic activity was 3 SD greater than that shown by mock-inoculated animals.

ing and presentation of the AU1-tagged P1A protein was correct, as indicated by the specific lysis of L1210 cells stably transfected with pBKCMV-P1A by a P815A-specific CTL clone. Thus, epitope tagging may provide a powerful and time-saving tool to study biological properties of TSA.

A prominent feature of DNA vaccination is the long-lasting expression of the antigen at the site of inoculation. In fact, using a luciferase reporter system, persistent expression was demonstrated in muscle tissues for several months (Wolff *et al.*, 1992). Consistent with this report, we observed expression of P1A mRNA for more than 4 months following a single DNA inoculation. One possible concern associated with i.m. injection of plasmid DNA is that the myocytes expressing the antigen may themselves become targets for CTL, eventually leading to the development of autoimmune myositis (Robertson, 1994). To test this, muscle samples from 5 vaccinated mice that had shown a high anti-P815A CTL response were analyzed for the presence of pBKCMV-P1A DNA and mRNA, and for the possible infiltration of muscle tissue by mononuclear cells. Two months after the third plasmid inoculation, 4 of 5 mice showed detectable levels of pBKCMV-P1A plasmid DNA and P1A mRNA. Histological analysis of the same muscles revealed that mononuclear cell infiltration was minimal or absent in four of the five analyzed muscles. Only one muscle, from a P1A-positive mouse, showed a single cluster of mononuclear cells around a blood vessel (data not shown). These observations tend to argue against the possibility that myocytes would become targets of P815A-specific CTL with subsequent development of autoimmune myositis. Accordingly, Mor *et al.* (1997) were unable to induce an autoimmune disease by using a variety of DNA vaccines.

Raz *et al.* (1994) reported that three injections of DNA were more efficient in inducing a CTL response against influenza nucleoprotein. By using a similar immunization procedure, a P815A-specific CTL response was induced in the majority of the 15 mice immunized with pBKCMV-P1A. It is interesting to note that while 5 of these mice exhibited a strong CTL response, which was comparable to that of mice receiving repeated immunizations with irradiated P815 tumor cells, 7 mice had intermediate CTL activity, and 3 showed no significant cytotoxicity. A similar variation in the strength of the CTL response was observed by Warnier *et al.* (1996) using a different gene therapy approach consisting of injection of a recombinant adenovirus coding for the P815A antigen. These authors reported that 55% of immunized mice failed to generate CTL in MLTC that had been set up using *P1A*-transfected L1210 stimulator cells. A possible explanation for the great variability in the response to vaccination with the *P1A* gene is suggested by a recent report demonstrating individual differences in the CTL response of mice inoculated with living P815 tumor cells. In fact, while some animals were more prone to mount a CTL response against the P815A antigen, others responded better to antigens (*e.g.*, C, D, E) distinct from that encoded by the *P1A* gene (Brichard *et al.*, 1995). Accordingly, analysis of TCR usage *in vivo* demonstrated that only 70% of P815 tumor-bearing mice developed T cells with a characteristic TCR rearrangement specific for the P815A antigen (Levraud *et al.*, 1996).

The individual variability in immune response among animals immunized with pBKCMV-P1A was also observed when resistance to P815 tumor cell challenge was evaluated. In fact, 6 of 10 vaccinated mice were fully protected following pBKCMV-P1A immunization, and 2 mice showed a prolonged survival time; the eventual growth of the tumor in these 2 mice might be explained by the emergence of stable P815A antigen-loss variants (Uyttenhove *et al.*, 1983). However, 2 immunized mice showed P815 tumor cell growth resembling that of control mice, thus suggesting that in these mice vaccination was not able to elicit a cell-mediated immune response. As also reported by others (Brichard *et al.*, 1995; Levraud *et al.*, 1996), the levels of cytotoxicity elicited in vaccinated animals did not always match the final outcome of tumor cell challenge. Some vaccinated animals with low CTL activity rejected the tumors, and a few showing high CTL activity did not (data not shown). The lack of a strict correlation between CTL response and tumor behavior might be explained by the emergence of antigen-loss variants in the case of tumor progression in mice having high levels of CTL, or to the involvement of different effector cell populations (*e.g.*, Th1 effector cells) when tumors regress despite low cytotoxicity.

In conclusion, our studies indicate that DNA immunization with the *P1A* gene generates a highly specific CTL response that protects mice against tumor cell challenge, thus supporting the use of this immunization approach as a promising method for treatment of human cancers expressing tumor antigens of the MAGE, GAGE, and BAGE families (Boon and Van der

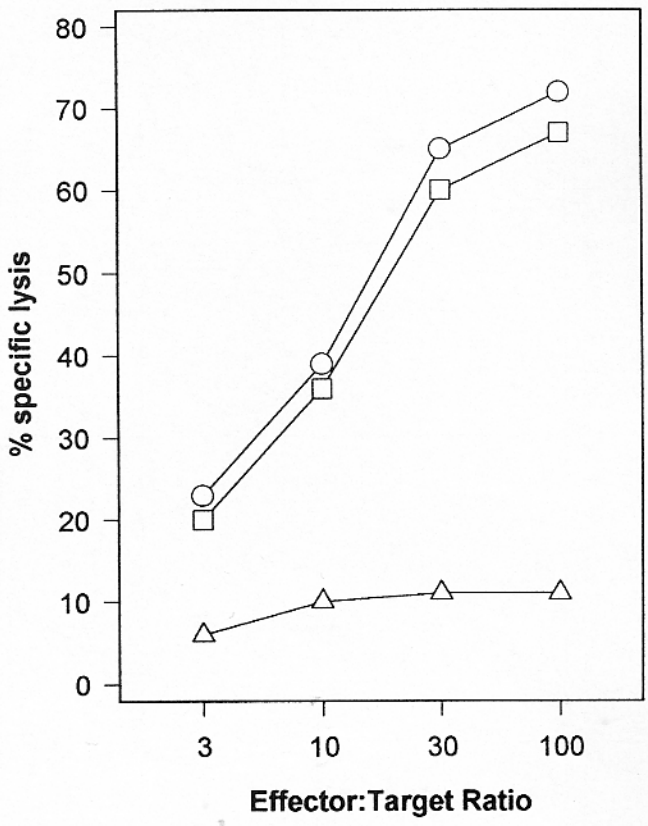

FIG. 4. Representative lytic activity of splenocytes obtained from DBA/2 animals immunized three times with pBKCMV-P1A. MLTC were set up as reported in Fig. 3 and tested against ^{51}Cr-labeled P815 cells (circles), peptide-pulsed IR5P1A$^-$ cells (squares), or IR5P1A$^-$ cells (triangles).

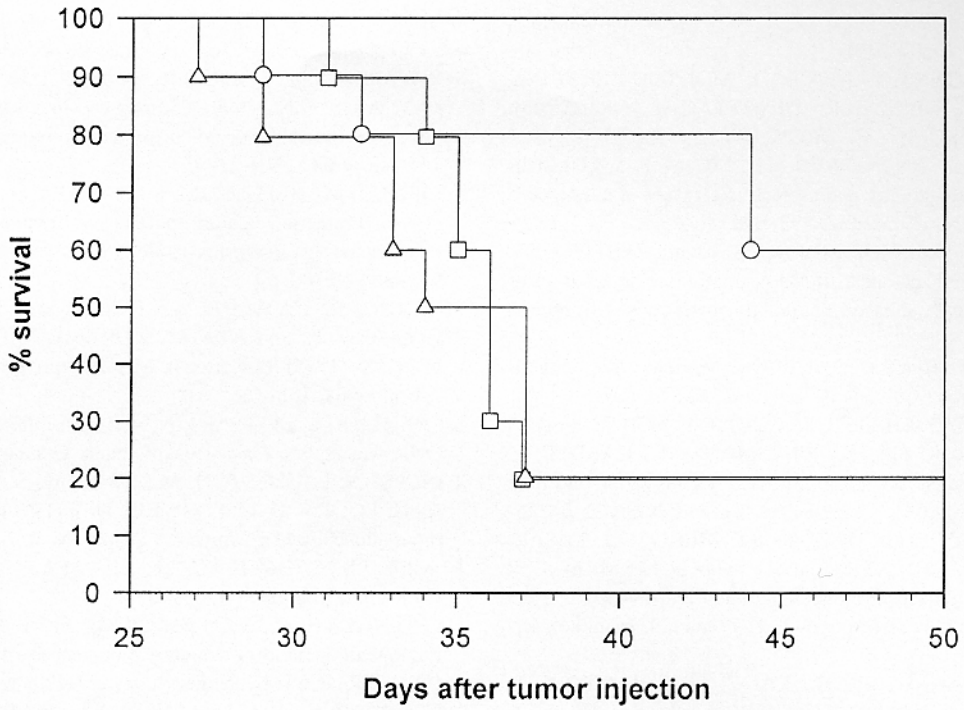

FIG. 5. Survival of DNA-immunized mice after challenge with P815 tumor cells. Ten DBA/2 mice were injected three times i.m. in the exteriorized quadriceps muscles with pBKCMV-P1A, and challenged with 10^6 P815 tumor cells s.c. 3 weeks after the last plasmid inoculation (circles). Ten mice that received pBKCMV vector lacking insert (squares) and 10 nontreated animals (triangles) served as negative controls. Although the mice were observed for a total of 120 days, no change in survival was noted beyond 50 days. Immunization with pBKCMV-P1A increased the survival rate following tumor challenge ($p = 0.06$; log-rank test) compared to mice injected with the vector only or noninjected animals.

Bruggen, 1996; Warnier *et al.*, 1996). Experiments are currently in progress to evaluate whether the individual differences in the orientation of the cellular immune response to the P815A antigen might be bypassed by using more efficient immunization procedures such as the combined administration of cytokines, either as recombinant proteins or coinjected plasmids (Xiang and Ertl, 1995; Conry *et al.*, 1996), the use of polycistronic vectors expressing the *P1A* gene in association with costimulatory molecules (Conry *et al.*, 1996), or by altering pBKCMV-P1A by the insertion of short immunostimulatory DNA sequences that have been reported to enhance the immunogenicity of plasmid DNA (Sato *et al.*, 1996).

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