Individual Analysis of Mice Vaccinated against a Weakly Immunogenic Self Tumor-Specific Antigen Reveals a Correlation between CD8 T Cell Response and Antitumor Efficacy[1]

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The weakly immunogenic murine P1A Ag is a useful experimental model for the development of new vaccination strategies that could potentially be used against human tumors. An i.m. DNA-based immunization procedure, consisting of three inoculations with the P1A-coding pBKCMV-P1A plasmid at 10-day intervals, resulted in CTL generation in all treated BALB/c mice. Surprisingly, gene gun skin bombardment with the pBKCMV-P1A vector did not induce CTL, nor was it protective against a lethal challenge with the syngeneic P1A-positive J558 tumor cell line. To speed up the immunization procedure, we pretreated the tibialis anterior muscles with cardiotoxin, which induces degeneration of myocytes while sparing immature satellite cells. The high muscle-regenerative activity observable after cardiotoxin inoculation was associated with infiltration of inflammatory cells and expression of proinflammatory cytokines. A single pBKCMV-P1A plasmid inoculation in cardiotoxin-treated BALB/c mice allowed for sustained expansion of P1A-specific CTL and the induction of strong lytic activity in <2 wk. Cardiotoxin adjuvanticity could not be replaced by another muscle-degenerating substance, such as bupivacaine, or by MF59, a Th1 response-promoting adjuvant. Although this vaccination schedule failed to induce tumor rejection in all immunized mice, the analysis of CD8 T cell responses at an individual mouse level disclosed that the cytotoxic activity of P1A-specific CTL was correlated to the antitumor efficacy. These results highlight the critical need to identify reliable, specific immunological parameters that may predict success or failure of an immune response against cancer. *The Journal of Immunology,* **2003, 171: 5172–5179.**

The identification of tumor-associated Ags (TAA)[4] constituted a major breakthrough in the immunological approach to cancer research and opened promising horizons for treatment based on active immunization strategies against well-defined targets expressed exclusively, or principally, by neoplastic cells. The vast majority of TAA identified thus far are represented by antigenic peptides recognized by CTL in the context of MHC class I molecules (1–3). Two categories of TAA are particularly attractive for cancer vaccine development, namely differentiation Ag and cancer-testis (CT) tumor-specific shared Ag. The components of this latter group, the prototypes of which are those encoded by *MAGE* genes in humans, are shared not only by different individuals but also by neoplasia of histologically diverse origins, thus representing a broader target for immunological intervention.

The P1A Ag, a major tumor rejection Ag of the mouse mastocytoma cell line P815, may be considered the murine counterpart of human TAA encoded by the *MAGE*, *GAGE*, and *BAGE* gene families. This CT Ag contains an antigenic epitope recognized by specific CTL in the context of the MHC class I molecule L^d that has been mapped to aa 35–43 of the P1A protein (4).

Induction of strong antitumor CD8 T cell responses represents a cornerstone of cancer immunotherapy and constitutes the experimental scientific basis of most ongoing clinical trials. In the specific case of murine P1A, we previously showed that a DNA-based immunization approach with a plasmid encoding the P1A Ag induced the generation of tumor-specific CTL against the relevant TAA in DBA/2 mice and led to partial protection against a challenge from the P1A-expressing P815 mastocytoma tumor cell line. However, results were biased by high interindividual variability and nonresponsiveness of some mice and did not provide a clear-cut correlation between the generation of P1A-specific CTL and the final outcome of tumors in vaccinated animals (5).

Attempts to increase the efficacy of vaccination procedures and to obtain a more pronounced antitumor activity are not straightforward. In same cases, it was shown that the strength of vaccine-induced immune responses against a foreign tumor Ag strongly correlated with tumor rejection (6). In contrast, the nature of the tumor Ag seems to influence the therapeutic impact of the immune response. In a transgenic mouse model in which an overwhelming majority of T cells carry a P1A-specific TCR, growth of the P1A-expressing J558 plasmacytoma was similar to that observed in nontransgenic littermates (7). In this regard, the present report shows that an optimized DNA vaccination procedure against a weakly immunogenic self tumor Ag resulted in a CTL response in

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[4] Abbreviations used in this paper: TAA, tumor-associated antigens; MLPC, mixed leukocyte-peptide culture; TA, tibialis anterior; HBsAg, hepatitis B surface Ag; PCNA, proliferation cell nuclear Ag; MIP-2, macrophage-inflammatory protein-2; MCP-1, monocyte chemoattractant protein-1.

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all vaccinated mice. Even though this protocol of immunization failed to confer complete protection, analysis of CD8 T cell response in individual mice disclosed that lytic efficacy detected in vitro was directly correlated with a more efficient antitumor in vivo effect. These results directly underscore the critical role played by CD8 T cells in tumor destruction and point out the important need to identify T cell-specific functional parameters that may predict the eventual success or failure of cancer vaccine therapy.

Materials and Methods

Mice

Female BALB/c mice (H-2^d), 6–8 wk old, were purchased from Charles River Laboratories (Calco, Como, Italy). Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies.

Cell lines

J558 is a myeloma cell line induced in BALB/c mice by mineral oil injection. P815 mastocytoma and L1210 lymphoma are tumor cell lines obtained from DBA/2 mice treated with methylcholanthrene. J558 and P815 cells are positive for P1A Ag. IR5P1A$^-$ is a P1A loss variant isolated from a P815 tumor that grew in a DBA/2 mouse immunized with irradiated P815 cells (5). All tumor cell lines were cultured in DMEM (Life Technologies, Paisley, U.K.) supplemented with 2 mM L-glutamine (Life Technologies), 10 mM HEPES (PAA Laboratories, Linz, Austria), 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 150 U/ml streptomycin (Bristol-Myers Squibb, Sermoneta, Italy), 200 U/ml penicillin (Pharmacia & Upjohn, Milan, Italy), and 5% heat-inactivated FCS (Life Technologies).

Expression vectors and DNA-based immunization protocols

The P1A-expressing vector pBKCMV-P1A has been described previously (5). All plasmid DNA preparations were made using Qiagen purification columns (Qiagen, Hilden, Germany) according to the supplier's protocol. Plasmid DNA was analyzed for endotoxin content with the *Limulus* amebocyte lysate kit (BioWhittaker Walkersville, MD). Plasmid DNA was administered according to different protocols. Mice were anesthetized by ethyl ether inhalation and inoculated i.m. with 100 μg of plasmid in 100 μl of saline solution, 50 μl in each tibialis anterior (TA) muscle, three times at 10-day intervals. Alternatively, to enhance muscle cell uptake of plasmid DNA, the TA of each hind leg was injected with 100 μl of 2 μM cardiotoxin solution (Latoxan, Rosans, France) or 100 μl of 0.5% bupivacaine solution (Sigma-Aldrich) in saline solution. Five days after cardiotoxin or bupivacaine inoculation, 50 μl of saline solution containing 25 μg of plasmid DNA were injected into each TA for a total of 50 μg/mouse. Detailed methods and references can be found online at http://www.dnavaccine.com. Plasmid DNA injection in MF59 adjuvant, a gift of Chiron, Siena, Italy, was conducted by inoculating 50 μl of a 1:1 suspension containing 25 μg of plasmid and 25 μl of MF59 in each TA. Gene gun DNA vaccination was performed with a hand-held, helium-driven Helios gene delivery system (Bio-Rad, Hercules, CA). Plasmid DNA was precipitated onto gold particles with a 1.6-μm average diameter as specified by the manufacturer. The inner surface of a Tefzel tubing was coated with the DNA-gold particle preparation with a tube loader (Bio-Rad), and tubing was then cut into 1-cm segments to result in delivery of 0.5 mg of gold and 1 μg of plasmid DNA per shot. Each animal received a gene gun shot at the abdominal epidermis with a helium pressure setting of 400 ψ.

Synthetic peptides and MLPC culture setup

The MHC class I L^d-restricted peptide, corresponding to aa 35–43 of P1A protein (LPYLGWLVF), was synthesized and purified by Technogen (Naples, Italy) and was >95% pure, as indicated by HPLC analysis. The lyophilized peptide was dissolved in DMSO (stock solution 10 mM; Sigma-Aldrich) and stored at −80°C before use. Cell cultures were set up as described previously (8). Briefly, spleens were removed, and 2.5×10^7 splenocytes were restimulated in vitro in a mixed leukocyte-peptide culture (MLPC) with P1A peptide diluted in culture medium at a final concentration of 1 μM. Cultures were set up in 10 ml of DMEM-10% FBS, maintained in 25-cm^2 Falcon tissue culture flasks (BD Biosciences, San Jose, VA) at 37°C and 5% CO_2; after 5 days of incubation, cells were analyzed by flow cytometry and tested for their lytic activity in a ^{51}Cr release assay.

Chromium release assay

Cytolytic activity was measured as described elsewhere (8). Briefly, ^{51}Cr-labeled target cells were incubated with effector cells at various E:T ratios in 96-well Falcon microplates. After 4 h of incubation at 37°C, supernatants were harvested, and radioactivity was counted in a microplate scintillation counter (Top-Count; Packard Instruments, Meriden, CT). For peptide pulsing, ^{51}Cr-labeled target cells (10^6/ml) were incubated with relevant peptides (1 μM final concentration) for 30 min at 37°C and then washed twice before use. Cytotoxicity was expressed either as percent of lysis or as LU_{30}. One LU was defined as the number of effector cells capable of killing 30% of the target cells, and results were expressed in number of LU per 10^6 responder cells (9). Statistical analysis of data was conducted by Mann-Whitney test.

Synthesis of MHC-peptide tetrameric complexes

A detailed description of tetramer construction has been reported elsewhere (10). Briefly, monomeric MHC-peptide complexes were formed by combining the MHC L^d, β_2-microglobulin, and the peptide in an arginine-folding buffer. The refolding reaction was dialyzed and concentrated for purification of folded protein on a Superdex 75 gel filtration column (Amersham Pharmacia Biotech, Piscataway, NJ). The MHC-peptide complex was enzymatically biotinylated with the BirA enzyme (Avidity, Denver, CO), following the manufacturer's instructions, and separated from free biotin by gel filtration chromatography. Extravidin-R-PE-conjugate (Sigma-Aldrich) was mixed with the biotinylated proteins at a molar ratio of 1:4 to form the peptide-MHC tetramer-PE.

Cell staining and flow cytometry analysis

Fresh splenocytes (3×10^6/sample) were incubated in 100 μl of FACS buffer (0.9% NaCl solution containing 2% BSA and 0.02% NaN_3, both from Sigma-Aldrich) with anti-mouse Fc-γR 2.4G2 mAb ascites (ATCC HB-197) for 10 min at room temperature to reduce nonspecific staining. After a washing, cells were resuspended in 50 μl of FACS buffer and labeled with L^d-P1A$_{35-43}$ tetramer-PE (5 μg/ml) for 20 min at room temperature. Each sample was then stained with rat anti-mouse CD8-Tri-color (0.1 μg/10^6 cells, clone CTCD8α; Caltag, Burlingame, CA) and with hamster anti-mouse CD3-FITC (1 μg/10^6 cells, clone 145-2C11; Caltag). In vitro-restimulated spleen cells (10^6/sample) were stained with tetramer-PE and rat anti-mouse CD8-FITC (0.1 μg/10^6 cells, clone CTCD8α). Before analysis, cells were washed twice, resuspended in FACS buffer, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Every sample was also stained with the control tetramer-PE according to the described protocols. Furthermore, untreated mice were used as negative controls for experimental samples. Data analysis was conducted using Cell Quest software (BD Biosciences).

Morphological and immunohistological analysis

For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E. For immunohistochemistry, formalin-fixed, paraffin-embedded or acetone-fixed cryostat sections were incubated for 30 min with anti-CD4, anti-CD8 (both from Sera-Lab, Crawley Down, Sussex, U.K.), anti-Mac-1 (anti-CD11b/CD18), anti-Mac-3, and anti-Ia (all from Boehringer Mannheim, Milan, Italy), anti-polymorphonuclear leukocytes (RB6-8C5, kindly provided by Dr. R. L. Coffman, DNAX, Palo Alto, CA), anti-asialo-GM1 (NK cells; Wako Chemicals, Dusseldorf, Germany), anti-proliferation cell nuclear Ag (PCNA; Ylem, Rome, Italy), anti-dendritic cells (NLDC 145; Cedarlane, Hornby, Ontario, Canada), anti-endothelial cells (Mec-13.324, kindly provided by Dr. A. Vecchi, Negri Nord, Italy), anti-TNF-α (Immuno Kontact, Frankfurt, Germany), anti-IFN-γ, (kindly provided by Dr. S. Landolfo, University of Turin, Turin, Italy), anti-monocyte chemoattractant protein-1 (MCP-1; BD PharMingen, San Diego, CA), and anti-macrophage-inflammatory protein-2 (MIP-2; Serotec, Oxford, U.K.), anti-RANTES (PeproTech, Rocky Hill, NJ) Abs. After a washing, sections were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit or horse anti-goat Ig (Vector Laboratories, Burlingame, CA) for 30 min. Unbound Ig was removed by washing, and the slides were incubated with avidin-biotin complex/alkaline phosphatase (DAKO, Glostrup, Denmark). Quantitative studies of the immunohistochemically stained sections were performed independently by three pathologists in a blinded manner. For each muscle sample, 10 randomly chosen fields were evaluated for each point determination. For cell enumeration, individual cells were counted under a microscope ×400 field (×40 objective and ×10 ocular lens; 0.180 mm^2/field). The expression of cytokines was defined as absent (−), scarcely (+/−), moderately (+), or frequently (++) present on cryostat section stained with the corresponding Ab.

Tumor protection assay

BALB/c mice vaccinated with pBKCMV-P1A plasmid were challenged s.c. with 2×10^6 J558 tumor cells at the times indicated in the text, and then monitored for 60 days. Mice injected with pBKCMV mock plasmid served as negative controls. Splenectomy after vaccination and before tumor inoculation did not alter the response to tumor challenge, as survival rates of vaccinated mice undergoing splenectomy were not significantly different from those of animals having intact spleen (data not shown). The in vivo tumor growth experiments were conducted according to the United Kingdom Co-ordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia (11), and animals were sacrificed by CO_2 overdose. Statistical analysis of data was conducted by Mantel-Haenzel test.

Results

DNA-based vaccination with P1A-encoding plasmid induces high levels of P1A-specific CTL in BALB/c mice after i.m. inoculation but not gene gun

To assess whether a previously described (5) protocol of DNA vaccination could induce an efficient CTL response against the weakly immunogenic P1A Ag in BALB/c animals, 10 mice were injected 3 times i.m. with 100 μg of pBKCMV-P1A at 10-day intervals, 5 mice were inoculated with the same amount of pBKCMV vector lacking the P1A gene as negative controls, and 5 mice were injected with 5×10^6 irradiated P1A-positive J558 tumor cells. Ten days after the last plasmid DNA inoculation, splenocytes from injected mice were stimulated in a MLPC with the $P1A_{35-43}$ peptide. Five days later, the cytotoxic activity was evaluated using J558, L1210, and $P1A_{35-43}$ peptide-pulsed L1210 cells as targets.

As shown in Fig. 1*A*, MLPC from immunized mice displayed elevated lytic activity against the J558 cells bearing the naturally expressed P1A tumor Ag; whereas in the five mice immunized with irradiated J558 cells, the cytotoxicity was very low; mock-inoculated animals were completely negative. The lytic activity elicited by the DNA-based immunization approach was highly specific for the P1A Ag, as MLPC from pBKCMV-P1A-injected mice did not show any appreciable reactivity against P1A-negative L1210 cells, but killed L1210 cells pulsed with the antigenic peptide of the P1A protein.

DNA-based vaccination with a gene gun apparatus is regarded as an efficient and rapid way of inducing strong immune reactions because gold microparticle-delivered DNA has direct access to skin APC (12, 13). This approach was expected to accelerate and amplify the process of CTL induction against the P1A Ag, thus allowing the generation of a more rapid and effective antitumor immune response. To test this hypothesis, we first set up a gene gun vaccination protocol in BALB/c mice by using the pCMV-S plasmid DNA expressing the strong viral HBsAg. As previously reported (14), gene gun immunization was efficient at inducing a highly specific CD8 T cell response against HBsAg after only 1 wk, persisting for at least 3 wk, the last time point evaluated (data not shown).

The rapid induction of a strong CTL response to HBsAg led us to apply the same approach for DNA immunization in the P1A experimental model. Therefore, we shot 10 BALB/c mice with the pBKCMV-P1A plasmid and 10 control animals with the pBKCMV vector lacking the insert. Three weeks after vaccination, five pBKCMV-P1A-immunized mice and five control animals underwent splenectomy and were kept alive for a subsequent challenge experiment. Splenocytes from these mice were stimulated in vitro in a MLPC with the $P1A_{35-43}$ peptide and tested 5 days later for anti-P1A CTL induction. On day 30, all immunized and control mice were challenged with P1A-expressing J558 tumor cells and monitored for survival. Surprisingly, the gene gun vaccination protocol completely failed to generate a CTL response against the P1A Ag (data not shown). More importantly, this immunization approach was also unable to induce a protective effect against tumor challenge, as all vaccinated and control animals died with fully overlapping survival curves (Fig. 1*B*).

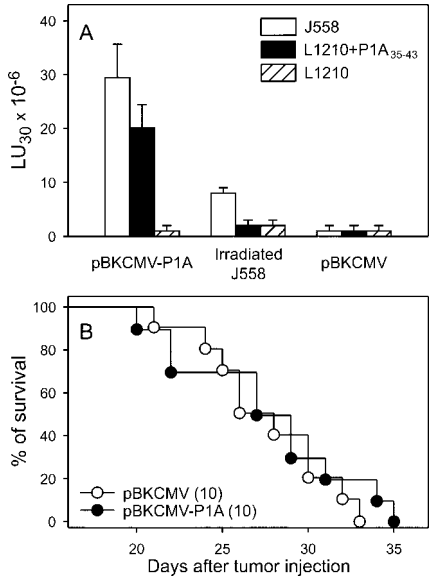

FIGURE 1. P1A-specific CTL generation in BALB/c mice injected with pBKCMV-P1A plasmid. *A*, Splenocytes from 10 BALB/c mice immunized with pBKCMV-P1A plasmid three times at 10-day intervals were collected 10 days after the last DNA inoculation and restimulated in vitro with the $P1A_{35-43}$ peptide (MLPC). Spleen cells from five animals injected with irradiated J558 cells and from five mice injected with pBKCMV control vector were similarly processed. After 5 days of culture, lytic activity was evaluated against ^{51}Cr-labeled target cells indicated in the figure. Cytotoxic activity was reported as $LU_{30}/10^6$ MLPC cells, and data are shown as mean $\pm$ SE. *B*, Ten BALB/c mice were immunized with pBKCMV-P1A by gene gun at day 0; 3 wk later, five of them underwent splenectomy and were kept alive. Thirty days after vaccination, all mice were challenged s.c. with J558 myeloma cells. Survival curves between pBKCMV-P1A-injected mice and mock-treated animals did not significantly differ ($p > 0.5$, Mantel-Haenzel test).

Cardiotoxin-assisted vaccination schedule induces a typical adaptive immune response by exerting a strong adjuvant activity on CTL generation

Based on previous findings, the gene gun vaccination was not a useful approach for the P1A Ag; in contrast, CTL induction obtained in this model by i.m. DNA-based immunization was suboptimal due to the limited responsiveness observed in DBA/2 mice and the length of the vaccination schedule (5). Indeed, this aspect is likely critical in the field of cancer immunotherapy because the immune response must keep pace with the rapid growth of the neoplastic cells. Therefore, we adopted an immunization protocol consisting of i.m. inoculation of plasmid DNA 5 days after pretreatment with cardiotoxin, a potent myotoxin that induces a cycle of muscle degeneration and regeneration. This immunization protocol has been reported to induce strong CTL generation in BALB/c mice after inoculation with pCMV-S (14). To assess the kinetics of anti-P1A CTL induction, five groups of BALB/c mice received cardiotoxin on day 0 and a single inoculation of 50 μg of pBKCMV-P1A plasmid on day 5. Control animals were injected with the empty pBKCMV vector. At different time points after vaccination, spleen cells were restimulated in MLPC with the $P1A_{35-43}$ peptide; after 5 days of stimulation, cultures were tested

against P1A-positive J558 and P815 tumor cell lines and the pulsed or unpulsed IR5P1A$^-$ variant. As shown in Fig. 2, at 4 and 8 days after plasmid inoculation, no cytotoxic activity was observed; at day 12, a striking induction of P1A-specific CTL became apparent in all vaccinated animals; and cytotoxicity values remained maximal up to day 20, the last time point tested. Therefore, cardiotoxin pretreatment strikingly reduced the timeframe for CTL induction in BALB/c mice without interfering with the fine specificity of the lytic activity.

A high CTL lytic activity is associated with expansion of tetramer-positive P1A-specific CD8 T lymphocytes

The results presented thus far prompted us to investigate whether pBKCMV-P1A vaccination induced an increase in the number of Ag-specific CD8 T cells in vivo and whether the induction of lytic activity in culture was accompanied by a parallel increase in the quantity of CTL directed against P1A. Thus, a group of BALB/c mice was immunized with the pBKCMV-P1A construct according to the cardiotoxin-based protocol and sacrificed after 15 days. Splenocytes were directly stained with P1A-L^d tetramers and restimulated in MLPC; 5 days later, cultures were tested for cytotoxicity and expansion of P1A-specific CD8 T cells. As shown in Fig. 3, results from in vivo analysis revealed only small increases (up to $<0.3\%$) in the number of tet$^+$ CD8 T cells. In contrast, restimulation with antigenic peptide in vitro allowed a sustained, although variable, expansion of P1A-specific CTL in all cultures. Finally, restimulated spleen cells from all mice exhibited high levels of lytic activity, which, however, could not be directly correlated with the expansion of Ag-specific CTL. Spleen cells from nonvaccinated control mice contained only background levels of tet$^+$ T cells, could not be stimulated in vitro, and showed no cytotoxic activity.

Muscle-degenerating substances induce stronger CTL activity than Th1 response-promoting adjuvants

It has been reported that administration of naked plasmid DNA coding for HIV-1 gp160, in conjunction with a Th1-promoting adjuvant like QS-21, was efficient in inducing Ag-specific CTL (15). Therefore, we tested whether this approach would be effective with the P1A self tumor-Ag. In this regard, we chose to analyze the MF59 adjuvant, an oil-in-water microemulsion with

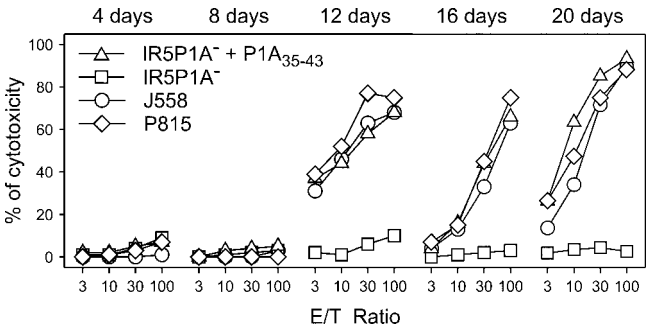

FIGURE 2. Kinetics of P1A-specific CTL development in BALB/c mice pretreated with cardiotoxin and immunized with pBKCMV-P1A. Five groups of 3 BALB/c mice were injected i.m. into the TA with cardiotoxin on day 0 and with pBKCMV-P1A plasmid 5 days later. At different time points from plasmid DNA inoculation, spleen cells were restimulated in MLPC with P1A$_{35-43}$ peptide. After 5 days of culture, lytic activity was evaluated against the ^{51}Cr-labeled target cells reported in the key. Cytotoxicity data are means of triplicates of the percentages of specific ^{51}Cr release at the indicated E:T ratios. Data are from a representative experiment of three that gave similar results.

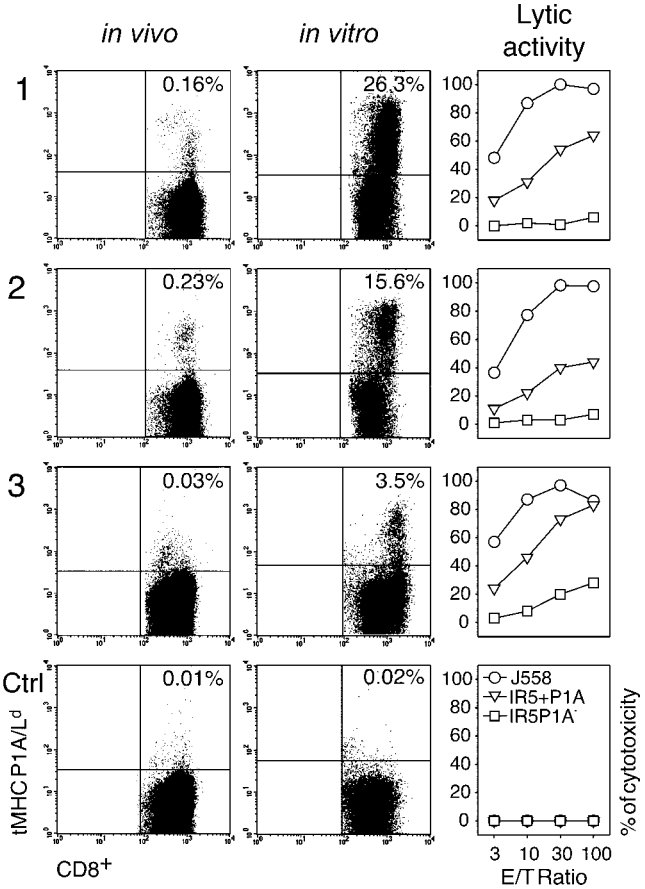

FIGURE 3. In vivo and in vitro analysis of splenocytes from BALB/c mice pretreated with cardiotoxin and immunized with pBKCMV-P1A plasmid. Splenocytes (3×10^6/sample) from three representative DNA-immunized BALB/c mice were costained in vivo with rat anti-mouse CD8 Tri-color mAb, hamster anti-mouse CD3-FITC mAb, and PE-conjugated L^d-P1A$_{35-43}$ tetramer. After in vitro restimulation with P1A$_{35-43}$-specific peptide, 10^6 cells from each MLPC were costained with rat anti-mouse CD8-FITC and PE-conjugated L^d-P1A$_{35-43}$ tetramer and analyzed by FACSCalibur. Percentages of tet-P1A$_{35-43}^+$ cells were calculated within CD8$^+$CD3$^+$ cells for in vivo staining and within CD8$^+$ cells for in vitro analysis. MLPC lytic activity was evaluated against indicated target cells. Splenocytes from a representative nonimmunized control BALB/c mouse was similarly analyzed. Cytotoxicity data are means of triplicates of the percentages of specific ^{51}Cr release at the indicated E:T ratios. Data are from a representative experiment of three that gave similar results.

demonstrated Th1-promoting activity, because it is currently licensed for clinical use (16, 17). Ten BALB/c mice were immunized with 50 μg of pBKCMV-P1A in MF59 at day 0; 2 wk later, five of these animals received a boost with a similar amount of plasmid DNA in MF59. As a positive control, 3 BALB/c mice were pretreated with cardiotoxin and vaccinated as previously described, whereas three normal mice served as negative controls. On day 20, MLPC were set up from all mice using the P1A$_{35-43}$ peptide as stimulator; 5 days later, MLPC cytotoxic activity was evaluated against a panel of relevant targets. Fig. 4*A* shows that mice receiving pBKCMV-P1A in MF59 did not generate any appreciable cytotoxicity against the P1A antigenic epitope, even after a boost treatment; as expected, mice pretreated with cardiotoxin displayed strong CTL activity, whereas control mice and mice injected with only MF59 were negative (data not shown).

On the basis of these negative results, we questioned whether cardiotoxin could be replaced by the local anesthetic bupivacaine, another compound capable of inducing muscle degeneration that

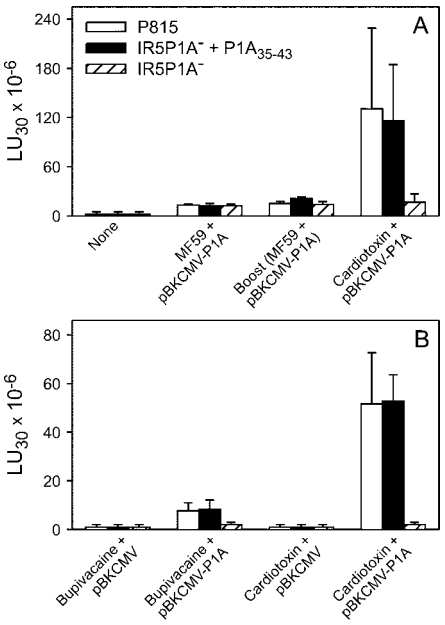

FIGURE 4. Comparative analysis of different adjuvants in facilitating P1A-specific CTL generation. *A*, Ten BALB/c mice were inoculated at day 0 with the pBKCMV-P1A plasmid in MF59 adjuvant, as reported in *Materials and Methods*; 2 wk later, five of these mice were further boosted with the same mixture. Three mice pretreated with cardiotoxin and immunized with pBKCMV-P1A plasmid served as positive controls, and three untreated mice were used as negative controls. At day 20, splenocytes from all animals were restimulated in MLPC with the $P1A_{35-43}$ peptide and tested 5 days later against the target cells shown. Cytotoxic activity was reported as $LU_{30}/10^6$ MLPC cells, and data are shown as mean ± SE. *B*, Groups of three BALB/c mice were injected with cardiotoxin or bupivacaine at day 0 and with pBKCMV-P1A plasmid or the empty pBKCMV vector as control 5 days later. Three weeks after plasmid inoculation, splenocytes were processed as described in *A*. Data are expressed as mean ± SE of $LU_{30}/10^6$ MLPC cells, and target cells are as in *A*.

has been widely used as adjuvant in DNA vaccination protocols (18). Two groups of BALB/c mice were inoculated with either cardiotoxin or bupivacaine at day 0 and with 50 μg of pBKCMV-P1A 5 days later; control animals received the muscle-degenerating substances and the insert-lacking vector pBKCMV. Three weeks after plasmid DNA inoculation, splenocytes from injected mice were restimulated in MLPC and evaluated 5 days later for cytotoxic activity. As shown in Fig. 4*B*, bupivacaine pretreatment promoted Ag-specific CTL generation that was, however, weaker than that observed in mice pretreated with cardiotoxin. On the whole, our data suggest that DNA-based vaccination in regenerating muscle might be an approach to bypass the limited antigenicity of weak self tumor-Ags.

Histological and immunohistochemical features of TA muscles after cardiotoxin and pBKCMV-P1A plasmid injection

To shed some light on the role of cardiotoxin as an adjuvant, we conducted a detailed histological and immunohistochemical analysis of regenerating muscle. The results can be summarized as follows. Interstitial foci of actively proliferating, spindle-shaped myoblasts were observed in longitudinal muscle sections 5 days after cardiotoxin injection. Their nuclear anti-PCNA immunostaining positivity was significantly higher ($p < 0.001$) than in saline-injected muscle (PCNA immunoreactivity: 42.7 ± 9.0% vs 0.0%). Fusion of these myoblasts resulted in unstriated fibers with long central chains of adjacent vesicular nuclei. Macrophages and NK cells were slightly, although not significantly, more numerous among these regenerating fibers than in normal muscle, whereas there was no difference in dendritic cell, lymphocyte, and granulocyte infiltration. The pronounced reactive cell infiltrate that appeared between the necrotic fibers in the first 2 days was drastically reduced by the fifth. Anti-endothelial cell mAb demonstrated that the capillary network was undamaged (data not shown). Eight

Table I. *Immunohistochemical analysis of the tibialis anterior muscle in cardiotoxin-treated mice*[a]

Item	Ctrl[b]	pBKCMV-P1A	Cardiotoxin	Cardiotoxin + pBKCMV-P1A
Infiltrating cells				
NK cells	2.6 ± 1.8[c]	13.6 ± 3.7*	2.8 ± 2.0	23.8 ± 5.1**
Macrophages	3.5 ± 2.2	15.2 ± 4.1*	4.8 ± 2.3	21.3 ± 6.5*
Dendritic cells	1.1 ± 0.6	1.0 ± 0.9	1.5 ± 0.6	0.9 ± 0.6
Granulocytes	2.0 ± 0.8	4.3 ± 1.2*	2.2 ± 1.3	4.7 ± 1.3*
$CD8^+$ lymphocytes	2.1 ± 1.0	3.2 ± 1.4	1.4 ± 0.9	10.1 ± 2.9**
$CD4^+$ lymphocytes	0.9 ± 0.6	1.5 ± 1.1	0.7 ± 0.5	1.3 ± 0.7
PCNA immunoreactivity rate	0.0%[d]	12.6% ± 2.2%*	42.7% ± 9.0%**	41.1% ± 9.5**
Cytokines				
IFN-γ	−	±	−	+
TNF-α	−	+	−	+
RANTES	−	±	−	±
MIP-2	−	+	−	±
MCP-1	−	±	−	+

[a] Mice were injected on day 0 with cardiotoxin and 5 days later with empty pBKCMV vector or pBKCMV-P1A plasmid. Immunohistochemical analysis was conducted 8 days after cardiotoxin inoculation. The expression of cytokines was defined as absent (−), scarcely (+/−), moderately (+), or frequently (++) present on cryostat section stained with the corresponding antibody.

[b] Ctrl, mice injected with saline.

[c] Cell counts were performed at ×400 in a 0.180-mm^2 field on 10 randomly chosen fields per sample. Results are the mean ± SD of positive cells per field.

*, Value significantly different ($p < 0.001$) than that in control mice; **, value significantly different ($p < 0.001$) than that in pBKCMV-P1A-injected mice.

[d] Percent of positive skeletal myofiber nuclei per total skeletal myofiber nuclei.

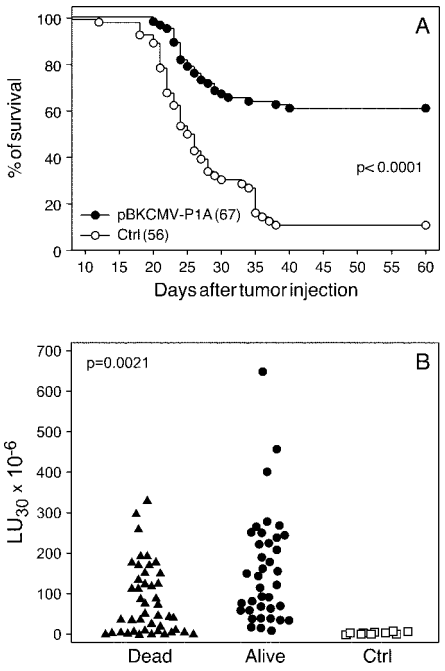

FIGURE 5. Cumulative survival of BALB/c mice vaccinated with pBKCMV-P1A and correlation between CTL activity and antitumor efficacy. *A*, BALB/c mice were vaccinated as reported in legend to Fig. 2. Twenty days after DNA immunization, mice were inoculated with P1A-positive J558 syngeneic tumor cells. Cardiotoxin- and mock plasmid-inoculated animals were used as controls. Immunization with pBKCMV-P1A increased the survival rate following tumor challenge ($p < 0.0001$, Mantel-Haenszel test). *B*, Eighty-two mice vaccinated as in *A* underwent splenectomy at day 14 after DNA inoculation and were challenged with J558 cells 1 wk later. Splenocytes were restimulated in MLPC with the P1A antigenic peptide, and the cytotoxicity was recorded as LU_{30} against P1A-pulsed-IR5P1A$^-$ cells. At the end of the observation period, the cytolytic activity of each vaccinated mouse was plotted as a function of death or survival to tumor challenge. Statistical analysis of data conducted by Mann-Whitney test disclosed a significant difference between regressor and progressor mice ($p = 0.0021$).

days after cardiotoxin treatment (with or without pBKCMV-P1A plasmid injection) regeneration activity was increased as shown by the higher PCNA immunoreactivity rate (Table I) and was accompanied by maturation into cross-striated myotubes. Myoblast regeneration activity was also increased in comparison with saline-injected muscles after bupivacaine treatment (PCNA immunoreactivity, $24.9 \pm 4.0\%$ vs 0.0%); however, it never reached that observed after cardiotoxin treatment (data not shown). Injection of the plasmid in cardiotoxin-pretreated animals resulted in a significant increase in NK cells and CD8 T lymphocytes, in comparison with mice inoculated with the plasmid alone. Expression of the proinflammatory cytokines IFN-γ, TNF-α, RANTES, MIP-1, and MCP-2 was more evident in cardiotoxin/plasmid- than plasmid-injected mice, but almost absent in saline- and cardiotoxin-injected mice. In bupivacaine -treated or in plasmid-injected bupivacaine-pretreated animals, the number of infiltrating reactive cells and the expression of cytokines and chemokines were slightly, but not significantly reduced in comparison with those observed in cardiotoxin- or plasmid-injected cardiotoxin-pretreated animals, respectively (data not shown). The histological and immunohistochemical features of muscles injected with pBKCMV empty vector with or without cardiotoxin were similar to those of cardiotoxin- or saline-injected muscle, respectively.

Survival to tumor challenge correlates with higher CD8 T cell response

We previously observed that in vivo treatment with a depleting anti-CD8 mAb abrogated the partial protection induced by DNA vaccination in DBA/2 and BALB/c mice (data not shown). Therefore, it appeared likely that Ag-specific CD8 T cells play a major role in tumor destruction after DNA vaccination and that their increased generation and/or activity should lead to a greater protective effect. To verify this hypothesis, groups of BALB/c mice were immunized as previously reported and challenged with P1A-expressing J558 cells 20 days after DNA inoculation. Fig. 5*A* shows the cumulative results of survival after challenge in comparison with data obtained in nonvaccinated cardiotoxin-treated control animals. Among 67 BALB/c DNA-vaccinated mice, 41 (61%) rejected the tumor, whereas others ultimately died. Spontaneous tumor regression in control mice was occasionally observed, a feature previously reported by us (5) and other authors (19). Statistical analysis between treated and control mice showed that survival differences were highly significant ($p < 0.0001$). To assess the existence of a possible link between CTL activity in vitro and the antitumor in vivo efficacy, we conducted further survival experiments where mice were vaccinated as previously reported, tested for anti-P1A CTL activity at day 14 by performing splenectomy, and kept alive for a subsequent challenge with J558 cells that was conducted 1 wk later. At the end of the observation period, the cytotoxic activity of each tested mouse was plotted as a function of death or survival to tumor challenge. As reported in Fig. 5*B*, a statistically significant ($p = 0.0021$) difference was observed between cytotoxicities detected in regressor mice (median value, 133.1 LU_{30}) in comparison with progressor mice (median value, 49.1 LU_{30}). Cardiotoxin-treated control mice exhibited no cytolytic capacity, nor did they survive the tumor challenge.

On the whole, these data indicate that lytic activity of Ag-specific CTL may be per se an independent prognostic factor to predict the outcome of tumor challenge in vaccinated mice.

Discussion

Experimental DNA vaccines have been frequently used against infectious diseases, demonstrating their potential power in both experimental research and clinical applications (20, 21). In oncology, however, preliminary human clinical data have not been very promising (22). One of the main reasons cited for the weak immune responses obtained is the poor efficiency of in vivo transfection of naked DNA. Accordingly, we observed that multiple injections of naked DNA were necessary to elicit an immune response against P1A in mice. The poor responses induced in vivo in humans raise an important question about the route of plasmid delivery needed to induce strong and efficacious immune responses. In this regard, direct access to skin Langerhans cells by gene gun injection appears to be an interesting approach. When we addressed this problem in our experimental system, an interesting dichotomy was observed: although gene gun delivery of plasmid DNA was very efficient at inducing a strong CTL response against a heterologous Ag, it completely failed to induce a response to a self tumor Ag. Similar results have been seen in previous studies. For example, gene gun immunization of C57BL/6 mice with plasmid DNA encoding the autologous murine tyrosinase-related protein-2 was unable to induce protective immunity against B16 melanoma cells naturally expressing tyrosinase-related protein-2 (23, 24). Therefore, it appears that a very favorable set of circumstances is needed to ensure the generation of an effective immune response against weakly immunogenic self Ags including TAA. In this regard, cardiotoxin has been reported to promote efficient CTL

generation against a variety of Ags. In the present work, we have confirmed the strong adjuvanticity of cardiotoxin for heterologous proteins but more importantly have found that cardiotoxin treatment exhibits a very high adjuvant activity even with P1A, bypassing the limited responsiveness of some mice and leading to CTL generation in all vaccinated animals. Cardiotoxin pretreatment also shortened the time required for CTL induction, given that maximal results were obtained in <2 wk. Concerning the possible mechanisms of action of this myotoxin, histological findings showed that degeneration of muscle cells by cardiotoxin resulted in pronounced myoblast proliferation with synthesis of myosin and the regeneration of muscle fibers as early as 3 days after injection. The immature muscle fibers might be more able to take up plasmid DNA, and high biosynthetic activity could increase the expression of the injected plasmid in the cells. The inflammatory environment of plasmid-injected regenerating muscle tissue might also be a favorable setting for one or more factors capable of stimulating the immune response. Nonspecific reactive cell infiltration generated by the plasmid alone coupled with the cardiotoxin-mediated increase in myoblast proliferation led to a significant increase in $CD8^+$ T cells, together with pronounced cytokine expression. The chemoattractants RANTES, MIP-2, and MCP-1 produced by endothelial and reactive cells play a key role in leukocyte recruitment and accumulation, whereas TNF-α and IFN-γ are probably involved in the generation of a P1A-specific immune memory, because it has been shown that their presence in the rejection area of s.c. injected tumor cells engineered to release several cytokines coincides with the establishment of a specific antitumor immune reaction (25, 26).

Although at first sight it seems questionable whether the use of a venomous substance would be feasible in clinical practice, snake-derived drugs or potent bacterial toxins such as the botulinum toxin are currently used in humans (27, 28).

In contrast, under our experimental conditions, neither another myotoxic substance (bupivacaine) nor the Th1-promoting agent MF-59, which had been previously reported to exert a strong adjuvant activity (17), was able to replace cardiotoxin in inducing a CTL response against P1A. This might be related to a different profile of toxicity on muscle fibers by bupivacaine in comparison with cardiotoxin (29, 30) or to the necessity of developing a MF59 emulsion to adsorb plasmid DNA to improve intracellular delivery of plasmid DNA upon immunization (31).

Human clinical trials are revealing a poor correlation between objective clinical responses to cancer vaccines and the ability of the vaccine to induce a T cell response in vitro (22). These controversial findings may depend on the techniques used for monitoring the patient's immune status and raise the question of whether the immunological surrogate endpoints chosen for the evaluation of immunotherapy protocols are relevant to the antitumor activity of cancer vaccines (32). In this regard, it has been previously reported that the simple detection of a measurable immune response does not directly imply antitumor reactivity but that the strength of the immune reaction is critical to achieve this aim (6). Accordingly, we reported a direct correlation between the in vitro cytolytic efficacy of anti-P1A CTL and tumor outcome in vaccinated animals, thus reinforcing the concept that CTL play a fundamental role in tumor destruction and providing further support to class I epitope-focused vaccination strategies. Nevertheless, in vitro analysis of cytotoxic function of anti-TAA CTL does not completely reflect their impact on the tumor; in fact, a discrepancy is still observable between some mice exhibiting high cytotoxicity and tumor in progression and others having regressed the neoplasia while displaying low cytolytic efficacy. This dichotomy is likely dependent on the fact that other effector mechanisms may play a critical role in mediating the antitumor effects of Ag-specific CD8 T cells, a prominent candidate molecule being IFN-γ (33). Therefore, the development of reliable assays for efficient monitoring of the immune response to TAA is a critical objective that will undoubtedly contribute to the success of cancer vaccination (34).

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