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DNA-Based Vaccination against Tumors Expressing the P1A Antigen

Antonio Rosato,[1] Gabriella Milan, Dino Collavo, and Paola Zanovello

Immunology Section, Department of Oncology and Surgical Sciences, University of Padova, Via Gattamelata 64, I-35128 Padova, Italy

Based on experience acquired in the last few years, we describe some technical steps and provide suggestions on how to induce an immune response against tumors expressing the weakly immunogenic antigen P1A by means of a DNA-based vaccination approach. P1A is the product of a normal mouse gene, which shares many characteristics with already identified human tumor-associated antigens, and therefore represents a useful experimental model to evaluate the efficacy of new vaccination strategies potentially applicable to the field of human tumors. Information gained with this model has been applied with success in other experimental settings, and thus we think that the procedure described herein may constitute a valid platform that can be implemented and further refined. © 1999 Academic Press

The term *vaccination* in oncology is somewhat misleading, because patients already have cancer when they undergo vaccination. Contrary to vaccines against most infectious agents, antitumor vaccines should be naturally endowed with the capacity to eliminate a disease, rather than to prevent it. Nonetheless, the term *tumor vaccine* has become widely used in basic and clinical research approaches to cancer immunotherapy to indicate immunization procedures aimed at increasing the response of the immune system against neoplastic cells. Most protocols of active immunization developed thus far have achieved prophylactic responses against tumor take in a vast variety of experimental models, but little therapeutic effect against already established neoplastic lesions. Efforts aimed at inducing an immune response against tumors have focused mainly on the generation of tumor-specific cytotoxic T lymphocytes (CTLs), which are currently thought to have a prominent role in neoplastic cell destruction (1). This goal has been achieved by increasing the immunogenicity of intact cancer cells with adjuvants or by engineering tumor cells with cytokine or costimulatory molecule genes. Other lines of research have explored immunization with tumor-associated antigens (TAAs), which result from derepressed or mutated genes in tumor cells, and are recognized by CTLs in the form of peptides associated with class I major histocompatibility complex (MHC) molecules. Immunization with purified TAA peptides or with antigen presenting cells, such as dendritic cells, pulsed with TAA peptides has been proposed. In addition, TAA-coding genes have been inserted into recombinant viral vectors and used to infect the host's cells to induce the generation of an immune response against the transgene product (2).

DNA-based immunization, a novel procedure that has already proved successful in inducing immune responses to a vast variety of viral, bacterial, and parasitic agents (3), represents an innovative approach to active cancer immunotherapy, as it could allow the delivery and rapid testing of the large number of recently described TAAs in the form of genes. Moreover, TAA synthesis within host cells favors the presentation of TAA epitopes to CTLs in the context of the host's MHC class I molecules, and allows the generation of a strong antigen-specific CTL response.

Although we cannot propose a magic recipe to cure experimental tumors by DNA-based vaccination, this article provides some technical guidelines and suggestions for the generation and monitoring of an antitumor immune response induced by injection of a DNA plasmid expressing a well-defined TAA. In particular, the experimental model described here is represented by tumors expressing the product of the normal mouse gene *P1A*, originally identified in the mastocytoma cell

[1] To whom correspondence should be addressed. Fax: +39 (049) 8072854. E-mail: arosato@ux1.unipd.it.

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line P815 (4), and recognized by specific CTL clones in the context of the MHC class I molecule L^d. The P1A antigen has many characteristics in common with the group of human shared TAAs (5), and thus it is a useful experimental model in which to analyze the efficacy of new vaccination strategies that potentially could be applied to the therapy of human tumors. On the other hand, the information provided here may be generalized to other model antigens, such as the xenogenic proteins artificially introduced into a tumor cell that have been extensively used to date in cancer immunotherapy (6, 7). Genes encoding each of these different model TAAs might be cloned into a plasmid vector and used as a DNA vaccine to induce an immune response capable of exerting therapeutic activity against tumors bearing the relevant antigen.

DESCRIPTION OF METHOD

Choice of Plasmid Vector and *in Vitro* Analysis of Protein Production

A detailed description of the construction of the P1A-encoding pBKCMV-P1A plasmid we currently use is beyond the scope of this article, the aim of which is to provide a general approach to cancer immunotherapy through DNA-based vaccination. However, while details can be found elsewhere (8), some considerations can be made to ensure a successful procedure. A large panel of different expression vectors driven by different promoters have already been employed. In our hands, the best results have been consistently obtained with cytomegalovirus (CMV)-promoter-based expression vectors both *in vitro* and *in vivo,* in terms of levels of TAA expression, and capacity to induce strong CTL generation, respectively. Readily available plasmids, such as pcDNA3 (Invitrogen BV, The Netherlands), can provide a starting point for cloning the TAA-encoding gene of interest. After the cloning step, two features of the newly constructed plasmid must be checked carefully: the sequence of the plasmid insert, and plasmid expression of the TAA protein by means of Western blotting or immunofluorescence analysis on transiently transfected cells with the use of specific polyclonal or monoclonal antibodies. To bypass the lack of specific antibodies that recognize the TAAs of interest, the "epitope tagging" procedure may be employed. This method consists of adding a short sequence coding for a tag epitope recognized by a commercially available antibody to the TAA-encoding gene. In our experience, the addition of a 6-amino-acid tag (AU-1) recognized by anti-AU-1 monoclonal antibody (BabCO, Richmond, CA) did not alter the physical properties of the TAA, nor modify the correct processing and presentation of P1A antigenic epitope (8).

Vaccination Procedures

The P1A model has been widely studied to gain insights into the optimal immunization schedule. Concerning the routes of plasmid administration, we failed to induce a CTL response by skin bombardment with a gene gun apparatus or by subcutaneous or intradermal inoculation of the plasmid in saline solution. Our current successful standard procedure consists of the transcutaneous inoculation of the tibialis anterior (TA) muscle after removing the fur with a trimmer. After anesthesia, mice (20 g body wt) are inoculated in each TA to a depth of about 2 mm with 100 μl of a 10^{-5} M solution of cardiotoxin (Latoxan, Paris, France) using a 1-ml syringe and a 27-gauge needle. Five days later, muscles are injected in the same way with 50–100 μg of plasmid DNA resuspended in 0.9% NaCl sterile saline solution (50 μl per each TA). The plasmid DNA is purified using endotoxin-free Qiagen columns (Qiagen GmbH, Hilden, Germany) following the supplier's protocol. Further informations and technical details about cardiotoxin pretreatment and plasmid inoculation can be found on-line (9).

Monitoring the CTL Response *in Vitro*

1. Mixed Leukocyte–Tumor Cell Culture (MLTC) and Mixed Leukocyte–Peptide Cell Culture (MLPC) Preparation

Reagents

Complete culture medium. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml each penicillin and streptomycin, 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 5×10^{-5} M β-mercaptoethanol. Different batches of FCS should be tested for capacity to support cell proliferation without inducing nonspecific cytotoxic activity.

Incomplete culture medium. DMEM supplemented with 3% (v/v) FCS, 100 U/ml each penicillin and streptomycin, and 10 mM Hepes.

Peptide (95% purity) stock. 1 mM solution in molecular biology-grade dimethyl sulfoxide (DMSO); filter-sterilize, aliquot at 100 μl/vial, and store at −80°C.

Procedure

Although very sensitive methods to evaluate antigen-specific CTLs, such as the use of ELISPOT (10) analysis and tetramers (11), were recently reported, a readily performed procedure to study CTLs from mice immunized with a TAA-coding plasmid DNA consists of setting up MLTC and MLPC bulk cultures from splenocytes. High levels of lytic activity in these cultures strongly indicate potent CTL generation *in vivo.* In MLTC, splenocytes are restimulated with irradiated tumor cells bearing the relevant TAA; in MLPC, the stimulus is the antigenic peptide. To prepare lymphoid cells for restimulation, spleens are removed aseptically

from mice and transferred onto a cell strainer or a 10-cm Petri dish. Splenocytes are dissociated from the spleen stroma by gentle pressing with a syringe plunger; the stroma is removed, and the cell clumps are broken by careful pipetting of the cell suspension. After two washings with incomplete medium, viable cells are counted, cells are washed again, and the pellet is resuspended in complete culture medium, adjusting the cell concentration to 10^7/ml. To prepare stimulator tumor cells, cells are collected from the culture and washed once with incomplete medium. To block tumor cell proliferation, the cell pellet is irradiated with 60–100 Gy; alternatively, mitomycin C can be used to inhibit tumor cell division, but in this case cell recovery and viability are decreased and stimulating capacity is reduced. After irradiation, the cells are washed twice with incomplete medium and counted, and their concentration is adjusted to 2×10^5/ml in complete culture medium. To set up MLTC, 2.5×10^7 splenocytes and 0.5×10^6 irradiated stimulator tumor cells are added to a 25-cm^2 culture flask with 15-ml final volume of complete culture medium. A responder (splenocytes)-to-stimulator (irradiated cancer cells) ratio of 50, as described, is usually optimal for most experimental tumors. For MLPC setup, 2.5×10^7 splenocytes and 1 μM final concentration of sterile peptide stock solution are added to a 25-cm^2 culture flask with 15-ml final volume of complete culture medium. The flasks are positioned vertically and cultured for 5 days in an incubator at 37°C, with 5% CO_2 and 95% humidity. Vertical positioning of the flasks allows better cell–cell interactions.

2. ^{51}Cr-Release Assay

Reagents

^{51}Cr as sodium chromate (Na_2CrO_4) in normal saline solution at 1 mCi/ml.

Triton X-100: 5% (v/v) solution in saline solution.

Procedure

CTL lytic activity is measured using a ^{51}Cr-release assay. Cells from the bottom of the MLTC or MLPC flasks are resuspended and viable cells are counted. After one washing, cell concentration is adjusted to 1.8×10^6/ml in incomplete medium. To prepare target cells, 1×10^6 cells are pelletted and resuspended with 10 μl of FCS. Cells are labeled with 100 μl ^{51}Cr solution for 1 h at 37°C, washed three times, and then resuspended to 2×10^4/ml. When cells loaded with the antigenic peptide are the targets, peptide is added at 0.5–1 μM final concentration after the labeling step and two washes. The cells are then incubated at 37°C for 30 min, washed three times, and resuspended as above. To set up the test, 100 μl of the effector cell suspension is first distributed in a round-bottom 96-well plate, running all samples in triplicate and realizing a threefold dilution (4 to 6 points). One hundred microliters of target cell suspension is added, and plates are centrifuged for 3–5 min at 150*g* and then incubated for 4 h at 37°C, with 5% CO_2 and 95% humidity. Triplicate wells for spontaneous and maximum release are set up by adding 100 μl of incomplete medium or Triton X-100 solution, respectively, to target cells. After incubation, 100 μl of supernatant is collected and radioactivity is counted in a gamma counter. Lytic activity is calculated according the following formula: percentage specific release = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

Monitoring the Antitumor Activity *in Vivo*

The final step of a vaccination protocol against a defined TAA consists of evaluating the induction of protective immunity against a lethal challenge with tumor cells bearing the relevant antigen. In this regard, the P1A antigen is shared by tumors of different histotypes and, thus, is particularly useful to determine the cross-protective role of the immunization procedure. At least three different tumor cell lines—mastocytoma P815, plasmocytoma J558, and fibrosarcoma Meth A—express P1A (12). Particular attention must be given to Meth A, however, because not all lines available from different laboratories are positive for this antigen. P815 cells are syngeneic in DBA/2 mice, while Meth A and J558 cells are derived from BALB/c animals. To evaluate tumor take or rejection, neoplastic cells in PBS are inoculated subcutaneously, provided the fur is removed with a trimmer, on a flank at doses of $1–5 \times 10^5$ for Meth A and J558 and $2–3 \times 10^6$ for P815 in syngeneic DNA-immunized and control recipient mice. Tumor growth is then monitored by measuring tumor diameter with a caliper (13), and in terms of survival according to international regulations (14).

CONCLUDING REMARKS

The P1A antigen is a very good physiological model for studies of immunointervention; indeed, we advance it may become a standard for the development and comparison of antitumor immunological strategies against neoplasias expressing antigens belonging to the class of shared TAAs. P1A antigen, moreover, might prove useful for the testing and validation of new plasmid DNA delivery procedures for optimal CTL induction and therapeutic efficacy (15). In particular, it might be instrumental in analyzing one of the most interesting features of DNA-based vaccines, i.e., their inherent adjuvant properties. In fact, bacterial DNA contains unmethylated CpG dinucleotides that have immunostimulatory ca-

pacity, and are able to directly trigger B-cell activation and stimulate monocytes, macrophages, and dendritic cells, thus inducing the upregulation of costimulatory molecules and the secretion of cytokines, predominantly interleukin-12 and interferon γ (16). The induction of cytokines strongly biased toward a Th1-like pattern, which play a major role in CTL induction, together with the intracytoplasmatic synthesis of the antigen encoded by the plasmid, makes DNA-based vaccines the ideal candidates for active cancer immunotherapy approaches aimed at inducing a specific immune response against well-defined tumor antigens. In our hands, CpG sequences appeared to have an important role in CTL induction against P1A; cloning of the P1A-coding sequence into a plasmid DNA vector devoid of immunostimulatory sequences failed to generate a detectable immune response. On the other hand, it must be kept in mind that the presence of immunostimulatory sequences in the pBKCMV-P1A plasmid did not suffice to achieve complete protection against a P1A-expressing tumor (8). Therefore, it is quite likely that further refinements in the construction of the plasmid DNA backbone, such as determining the amount and type of CpG motifs to introduce in a DNA vaccine for optimal efficacy, as well as their location within the plasmid, and the need to supply them either in the form of cloned sequences in the vector or as synthetic oligonucleotides, will strengthen DNA vaccines, endowing them with full therapeutic activity even against the weakly immunogenic antigen P1A.

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