Vaccination with Poly-L-Arginine As Immunostimulant for Peptide Vaccines: Induction of Potent and Long-Lasting T-Cell Responses against Cancer Antigens


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ABSTRACT

Vaccines that induce high numbers of sustained T cell responses are urgently needed for the treatment of numerous diseases including cancer. Antigen-presenting cells (APCs), the most important of which are dendritic cells, orchestrate antigen-dependent T cell responses in that they present antigens to T cells in an appropriate environment. Here we present evidence that after vaccination with a simple mixture of the cationic poly-amino acid poly-L-arginine and tumor antigen-derived peptide antigens, large numbers of antigen-specific T cells are induced and APCs mediate the generation of T lymphocytes. We observe that after s.c. injection, MHC class II+ cells infiltrate injection sites and are loaded with large amounts of antigen in vivo under the influence of poly-L-arginine. Consequently, numerous antigen-charged APCs can be detected in draining lymph nodes of vaccinated animals. Antigen-specific T cell responses induced are systemic and were readily detected more than 4 months after the last vaccination, the latest time point we measured. By contrast, even after repeat injections, we were consistently unable to detect antibody responses against poly-L-arginine, allowing this compound to be used for numerous booster injections.

Clinical trials in cancer patients using poly-L-arginine as immunostimulant will be carried out in the near future.

INTRODUCTION

The development of vaccines has saved more lives than any other medical intervention (1). Most, if not all established vaccine technologies exert their effects via the induction of high titer neutralizing antibodies. For the rejection of tumors, however, it is thought that cellular immune responses are very important (2, 3).

APCs are, and, in particular, DCs, have the unique capacity to prime T cells (4, 5). One obvious prerequisite for the successful generation of T cells in a vaccine context, therefore, is to charge APCs with sufficient antigen, which in turn will result in the activation of antigen-specific T cells.

Thus far, the most widely used approach to charge APCs with antigen is to isolate these cells and load them in vitro with either peptide or protein antigens before reinfusing them into patients or experimental animals (reviewed in Ref. 5). Results obtained in early stage clinical trials are encouraging (reviewed in Ref. 5); however, simpler strategies allowing charging of APCs in vivo are needed to make such vaccines widely applicable in the future.

Cationic poly-amino acids, including poly-L-lysine and poly-L-arginine, mediate protection against challenge with live tumor cells in animal models when cojected with tumor antigen-derived peptides (6). However, the mode of action of this vaccine and, in particular, the involvement of T cells, which are thought to be the most important anticancer cell type (2, 3, 7), is not known. In this article we present data extending our previous findings with special focus on optimal conditions and effector mechanisms for vaccinations using poly-L-arginine as adjuvant: we provide evidence that large numbers of T cells are generated after vaccination of animals with poly-L-arginine and a tumor antigen-derived peptide, and that the generation of T cells is mediated by APCs that are charged with antigen in vivo under the influence of poly-L-arginine.

MATERIALS AND METHODS

Animals. Female C57BL/6 (H-2b) and DBA/2 (H-2d) mice were obtained from Harlan Winkelmann (Borchen, Germany) and were used at the age of 6–8 weeks. All animal experiments were carried out following Austrian guidelines.

Reagents. Peptides were synthesized by solid phase synthesis using standard N-(9-fluorenyl)methoxycarbonyl chemistry. Crude peptides were purified by reverse-phase high-performance liquid chromatography, and the identity and purity were confirmed by mass spectroscopy. Peptides used in individual experiments are indicated in the respective figure legends.

For fluorescein (FITC) labeling of peptide GYKDGEYI (8), the peptide was incubated with an 8-fold molar excess of FITC (Molecular Probes, Eugene, OR). Unreacted dye was separated by gel filtration.

Poly-L-arginine was obtained from Sigma (Vienna, Austria; Lot 68H5903; average degree of polymerization 60) and dissolved in endotoxin-free water (Mayrhofer Pharmazeutika, Linz, Austria) at 10 mg/ml. Aliquots were stored frozen at −20°C.

Recombinant enhanced green fluorescent protein (GFP) was produced using a commercially available baculovirus-based expression system (Bac-To-Bac Baculovirus Expression System; Invitrogen, Vienna, Austria).

IFN-γ ELISPOT assays. IFN-γ ELISPOT assays were carried out as described (9). Purified monoclonal antibodies from clone R46A2 (coating antibody) and biotinylated antimouse IFN-γ mAb from clone AN18.17.24 (detection antibody) were generously provided by Dr. Edgar Schmitt, Institute of Immunology, Mainz, Germany. Values shown are means of duplicate measurements. Error bars represent SDs.

Immunofluorescence Analyses of Injection Sites and Draining Lymph Nodes. Seven days after s.c. administration of poly-L-arginine/FITC-conjugated peptide mixtures, thin cryosections (<2.5 μm) were prepared from excised, fixed, and polyvinylpyrrolidone-embedded injection sites. Specimens were fixed with 8% buffered paraformaldehyde to retain antigens in sections. MHC class II antigen was detected using purified anti-I-Aβ (clone B21.2; American Type Culture Collection, Manassas, VA) and a Texas red-conjugated goat-antirat antibody (Cedarlane Laboratories, Hornby, Ontario, Canada). Staining with the B21.2 antibody was not impaired by this fixation procedure. However, antibodies against CD11c (clone N418) or DEC-205 (clone NLDC-145) could not be used if cryosections were fixed with 8% paraformaldehyde.

Lymph node single-cell suspensions were prepared 5 days after the third vaccination (100 μg of poly-L-arginine/100 μg of GFP/dose) and centrifuged onto glass slides. Cytospin preparations were fixed with 0.5–1% buffered paraformaldehyde, permitting the use of the NLDC-145 antibody (Serotec, Oxon, Oxford, UK).
Oxford, United Kingdom), which recognizes the DEC-205 epitope expressed by DCs (10).

**Vaccination Experiments.** Mice were vaccinated with a mixture of peptide or protein and poly-l-arginine in a total volume of 100 μl three times, either at weekly, biweekly, or monthly intervals (as indicated in the figure legends). Vaccines were prepared as described previously (6).

All components of the vaccine tested negative in Limulus Amebocyte Lysate Assays (BioWhittaker, Aspen, Germany).

**RESULTS**

**Poly-l-Arginine Promotes in Vivo Charging of MHC Class II+ APCs with Large Amounts of Antigen.** To address whether poly-l-arginine enhances capture of antigen by APCs in vivo, animals were given s.c. injections of a mixture of fluorescence-tagged peptides and poly-l-arginine. Cryosections of excised injection sites were examined microscopically: whereas peptides injected alone disappeared within 8–16 h, a deposit of antigen was detectable for as long as 10 days in the presence of poly-l-arginine. Similar results were obtained with protein antigens (β-galactosidase, GFP; not shown). In time course experiments it became evident that MHC-class II+ cells, a marker commonly used for DCs/Langerhans cells of the skin (4, 11), gradually infiltrate injection sites and take up the antigen. The maximum number of antigen-charged, MHC class II+ cells infiltrating injection sites was observed between 5 and 7 days after antigen/poly-l-arginine administration (Fig. 1). At these time points a massive infiltrate of MHC class II+ cells was observed with more than 80% of antigen-loaded cells expressing MHC class II molecules. Confocal microscopy analysis revealed that almost all antigen-charged MHC class II+ cells contained large amounts of intracellular antigen, which colocalized either with MHC class II compartments (Fig. 1, yellow staining) or occurred free in the cytoplasm (Fig. 1, green fluorescence).

**Poly-l-Arginine Facilitates Migration of Antigen-charged APCs to Lymphoid Organs.** To demonstrate that APCs charged with antigen by poly-l-arginine migrate to secondary lymphoid organs, where priming of T cells takes place (12, 13), animals received s.c. injections three times with a mixture of poly-l-arginine and GFP serving as easily detectable “reporter” antigen. Five days after the last vaccination, cytospins of draining inguinal lymph node cells (10^5 cells/slide) were prepared and examined by fluorescence microscopy. As shown in Fig. 2, GFP-charged APCs, detected by double staining with MHC class II antibodies, were readily detected. Similar results were obtained using antibodies directed against DEC-205, which recognizes the NLDC-145 molecule expressed on DCs (Ref. 10; data not shown). Enumeration of MHC class II+ (and DEC-205+, not shown), antigen-charged APCs revealed that approximately 80% of antigen-charged APCs were detectable per microscopic slide (10^5 cells/cytospin preparation) after injection of a mixture of GFP and poly-l-arginine, corresponding to approximately 0.08% of total lymph node cells. After three injections of GFP alone, no antigen-charged cells were detectable. Assuming that 1% of lymph node cells are APCs (14), our data indicate that a substantial proportion (on the order of 8%) of the APC fraction in draining lymph nodes were GFP-charged. Injection of peptides as antigens yielded similar results (data not shown).

**Vaccination of Animals with a Mixture of Tumor Antigen-derived Peptides and Poly-l-Arginine Induces Sustained, Antigen-specific T Cell Responses.** We chose the well-defined, H-2Kb-restricted tumor peptide antigen TRP-2\_181–188, derived from murine TRP-2 (15) to address the question of whether peptide-specific T cells could be induced after injection of a mixture of TRP-2\_181–188 and poly-l-arginine. TRP-2 is a tumor rejection antigen for the murine melanoma B16/F10 that forms tumors in C57BL/6 mice (15). In addition, this antigen is recognized by T cells from human melanoma patients (16).

As shown in Fig. 3, animals vaccinated three times with a mixture of poly-l-arginine and TRP-2\_181–188 develop strong, TRP-2\_181–188-specific immune responses detectable in lymph nodes (Fig. 3A), spleen (Fig. 3B), and peripheral blood (Fig. 3C). Typically, after three vaccinations, the number of cells secreting IFN-γ upon exposure to TRP-2\_181–188 ranged from 200 to 1000, or more, among 10^6 unseparated input cells as judged by ELISPOT analysis. In control ELISPOT assays with an irrelevant peptide, we did not observe IFN-γ-secreting cells in numbers significantly above background levels obtained in untreated animals or unstimulated cells. The anti-TRP-2\_181–188 response observed is entirely within the CD8+ T cell compartment, because in cell sorting experiments, only the CD8+ fraction responded with IFN-γ production upon stimulation with TRP-2\_181–188 in ELISPOT assays (data not shown). T cell responses induced with this vaccine are sustained and long-lasting: TRP-2\_181–188-specific IFN-γ ELISPOTs could easily be detected more than 4 months (137 days) after the last vaccination, the latest time point we measured (Fig. 3D).

Under certain circumstances it may be advantageous to use longer peptide fragments possibly containing one or more T cell epitopes. To examine whether protein fragments or large peptides can be used together with poly-l-arginine, we injected a mixture of poly-l-arginine and the TRP-2\_181–205 peptide containing the minimal TRP-2\_181–188 T cell epitope. Again, high numbers of T cells specific for TRP-2\_181–188 could be generated (Fig. 3E). Thus, short minimal T
minimal T cell epitopes can be evoked with large peptides, animals were immunized three times with TRP-2 141–205 peptide alone. T cell responses in animals vaccinated with peptide alone declined rapidly and were not detectable anymore at time points >20 days. D, the longevity of the T cell response was measured by vaccinating animals as above and carrying out ELISPOT T cell assays, using splenocytes, on days 10, 20, 60, and 137 after the last vaccination (three total). T cell responses in animals vaccinated with peptide alone declined rapidly and were not detectable anymore at time points >20 days. E, to assess whether T cell responses against minimal T cell epitopes could be evoked with large peptides, animals were immunized three times with TRP-2 141–205 in conjunction with poly-L-arginine. TRP-2 141–205 contains the known T cell epitope TRP-2 181–188 (15). On day 10 after the last injection, ELISPOT assays were carried out using TRP-2 141–205 (60 mer) or TRP-2 181–188 (TRP2 188) peptide. Negative controls were two peptides contained in TRP-2 (TRP-2 170–205 and TRP-2 239–280). F, the route of injection determines the magnitude of the immune response. C57BL/6 mice (five per group) were immunized three times at weekly intervals with a mixture of poly-L-arginine (100 μg/mouse) and peptide TRP-2 181–188 (100 μg/mouse) s.c. into the flank or the pinna. Ten days after the third injection, single-cell suspensions of spleens were prepared and antigen-specific T cell responses were enumerated by ELISPOT assays.

We next compared the influence of different injection sites on vaccine efficacy; the highest number of IFN-γ-secreting cells was consistently observed after intrapinnal injection followed by s.c. applications (Fig. 3F), as has been described for other vaccines (17). Injection of the vaccine into the footpad resulted in a similar number of IFN-γ-secreting cells as compared with s.c. injection into the flank (data not shown). These observations further add to the above findings, in that it is known that in mice very high concentrations of Langerhans cells are found in the ears (11, 18).

Finally, in B16/F10 challenge experiments, we were able to achieve protection in a large proportion of animals in poly-L-arginine/ TRP-2 181–188-vaccinated groups but not in control animals (not shown), confirming previous findings (6).

DISCUSSION

We show here that with a simple, defined, and entirely synthetic vaccine consisting solely of a mixture of the cationic poly-amino acid poly-L-arginine and a tumor antigen-derived peptide, a potent and long-lasting T cell response is induced.

One obvious prerequisite for specific priming of T cells is that APCs are loaded with antigen. We present evidence that, indeed, MHC class II+ cells are charged in vivo with peptide antigens at the site of injection under the influence of poly-L-arginine. Surprisingly, we observed that at the site of injection, more than 80% of antigen-charged cells also expressed MHC class II antigens (day 7). One explanation for this finding is that the highly mobile APCs rapidly invade the vaccine injection site where, under the influence of poly-L-arginine, they capture large amounts of antigen, as they do in vitro (19). We were unable to use markers other than MHC class II antigens for the characterization of APCs, because sections of injection sites had to be fixed with 8% paraformaldehyde to retain antigens in specimens. Nevertheless, we believe that, indeed, APCs, and very likely DCs/Langerhans cells are the cell type stained with the MHC class II marker, because these cells displayed typical morphology and they migrated to lymph nodes. For the analysis of lymph nodes, the DC-specific marker DEC-205 (10) could also be used for detection and enumeration of antigen-charged APCs, yielding comparable numbers than did staining with the MHC class II antibody (not shown).

By using confocal microscopy, we were able to show that APCs also take up, rather than simply bind, the antigen. This forms the basis for the use of longer fragments or proteins rather than the minimal peptide epitopes themselves for vaccination, because APCs could trim antigens to the appropriate length. Alternatively, APCs could process antigens extracellularly (20). Indeed, we show that long polypeptides, such as the TRP-2 141–205 fragment, containing one or more minimal T cell epitopes, can be used for vaccination. Furthermore, after injection of the long TRP-2 141–205 peptide in conjunction with poly-L-arginine, we observe CD4-restricted T cell responses against an as yet uncharacterized MHC class II-binding peptide.4

The high number of antigen-charged APCs in lymph nodes (on the order of 8% of the total lymph node APC fraction) is reflected by a high, sustained, and antigen-specific T cell response. We consistently detect 200–1000, or more, spot-forming cells/10⁶ nonseparated input cells in ELISPOT assays and only CD8+ cells are responsible for IFN-γ production in this setting. Assuming that approximately 10% of lymph node cells, splenocytes, and peripheral blood mononuclear cells are CD8+, we calculate that reproductively more than 1/500 of the CD8+ T lymphocytes respond specifically to the TRP-2 peptide

4 Unpublished observation.
ELISPOT assays. These numbers are in the same range as precursor frequencies measured during viral infections (21–24).

We observed in our vaccination studies with various peptides and poly-l-arginine that, in particular, hydrophobic peptides (like the TRP-2-derived peptide YVDFVWL used here) are good inducers of peptide-specific T cells in combination with poly-l-arginine. In contrast, more hydrophilic peptides (e.g., the ovalbumin-derived peptide SIINEKEL; Ref. 25) are inferior in inducing peptide-specific T cells after vaccination with poly-l-arginine.

Thus far we have not been able to measure any antibody or T cell response against poly-l-arginine even after repeat vaccinations, which is of particular importance for booster injections.

The strategy we present here for APC-based vaccines relies entirely on defined components. If peptides are used as antigens, the vaccine is fully synthetic, thus greatly reducing the risks involved using live organisms. All components can be synthesized relatively easily at low cost according to standards required by regulatory authorities. The steadily increasing number of tumor antigens recognized by T cells (26) has formed the basis for several clinical studies with encouraging results (27–29). Vaccine efficacy may further be enhanced if peptides are injected in conjunction with poly-l-arginine, warranting clinical trials using this compound.

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