Anticancer vaccination strategies

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Introduction

Tumors often upregulate the expression of molecules that are normally suppressed or expressed at much lower levels in adult tissues, the so-called tumor-associated antigens (TAAs) [1]. Some ‘self-reactive’ T lymphocytes capable of T-cell receptor (TCR)-mediated recognition of these antigens and of mediating tumor rejection survive thymic selection and can be detected in peripheral blood and lymphoid tissues. Indeed, cases of spontaneous immune-mediated tumor regressions are reported, especially for melanoma, renal cell carcinoma and tumors presenting with neurological paraneoplastic syndromes, especially non-small-cell lung cancer [1–3]. However, in most cases, the immune system fails to recognize and destroy tumor cells, possibly due to the inefficiency of these as antigen-presenting cells (APCs), and to the lack of contact between tumor cells and the immune system [4, 5].

Now that knowledge of the immune system has improved, new perspectives for the development of active antitumor immunization strategies have been proposed. The main goal of these approaches is to expand in the patients CD8+ cytotoxic T lymphocytes (CTLs) capable of rejecting tumor cells via recognition of tumor-associated antigenic epitopes expressed by human leukocyte antigen (HLA) class I molecules on the cancer cells. However, optimal immunotherapeutic approaches should probably also prime CD4+ helper T cells, given the key role that these cells play in the control of immune responses [1]. The antigenic material used in anticancer vaccinations can be in the form of whole tumor material (either allogeneic or autologous) or of specific TAAs, which are delivered as DNA (naked or comprised in recombinant viruses), RNA, protein or HLA class I/II restricted peptide epitopes [1]. Many of these antigenic materials can be injected directly, often coupled to immunostimulatory cytokines or adjuvants, or used for \textit{ex vivo} loading of APCs, usually dendritic cells (DCs) (Figure 1). These are specialized APCs with the capacity to initiate an immune response by presenting antigen-derived epitopes to T lymphocytes in a highly immunogenic fashion [6–9]. All of the antitumor vaccination approaches are believed to ultimately depend on the presentation of tumor-derived antigen(s) to T cells by DCs in the patient’s lymphoid tissues [10]. For the biology of DCs and the applications of \textit{ex vivo} manipulated DCs to cancer immunotherapy we refer the reader to the review by Lesterhuis et al. [11] in this Supplement. Here, we mainly focus on the non-DC-based approaches that are currently evaluated for specific cancer immunotherapy.

Autologous/allogeneic tumor cells as a tumor vaccine

Injection of whole tumor cells or cell lysates of allogeneic source is one of the first approaches that was evaluated for the induction of an antitumor immune response [1, 10]. One of these vaccines, named Melacine (Corixa Corporation, Seattle, WA, USA), a lyophilized preparation from two melanoma cell lines, produced clinical responses in \(\sim 10\%\) of patients, whereas stabilization of disease was noted in 10% to 20% of patients [12]. A multicenter phase III trial of low-dose cyclophosphamide plus Melacine versus a four-drug chemotherapy regimen showed no difference in response rates and survival, with fewer and milder side-effects due to Melacine [12]. A similar polyvalent vaccine called Canvaxin was administered to patients with stage III melanoma after surgical resection. This group of 935 patients was compared with another cohort of patients who received chemotherapy but not the vaccine after surgery. This study showed a significant survival advantage in patients receiving Canvaxin compared with the non-vaccine group: median survival was 56.4 months in the vaccine group versus 31.9 months in the non-vaccine group, whereas overall survivals at 5 years were 49% and 37%, respectively [13].

More recently, vaccination studies have tended to make use of autologous tumor material. In addition, in order to increase the immunogenicity of these preparations, tumor cells are usually co-injected with an adjuvant and/or cytokines or growth factors. Alternatively, tumor cells can be engineered to express immunostimulatory cytokines [such as interleukin (IL)-2, IL-4, granulocyte–macrophage colony-stimulating factor (GM-CSF)] or co-stimulation molecules (B7.1, B7.2 and related molecules). Preclinical experimental data indicate that direct presentation of antigens by tumor cells to T cells may be a minor pathway compared to the capture and cross-presentation of tumor-derived material by APCs such as DCs. Consistent with this, co-administration or transduction of tumors with cytokines aimed at APCs seems to lead to the most potent induction of systemic antitumor immunity. These factors include GM-CSF, a myeloid growth factor that favors DC differentiation and activation, and CD40 L, another signal which drives DC differentiation and activation from circulating precursors [14].

Among the reported clinical studies, a seminal work has been published by Vermorken et al. [15], who vaccinated...
patients with stage II and III colon cancer after surgical resection. The vaccine consisted of $10^7$ irradiated autologous tumor cells mixed with $10^7$ viable BCG organisms. Three weekly intradermal vaccinations were administered starting 4 weeks after surgery, with a booster vaccination at 6 months. The study enrolled 254 patients who were randomly assigned to postoperative immunotherapy or no adjuvant treatment. The authors report that a sufficient amount of tumor tissue for vaccine preparation was generally obtained in $\sim 80\%$ of the patients. Whereas no significant benefit of vaccination in stage III disease was detected, immunotherapy was associated with a significantly longer recurrence-free period ($P=0.011$) and $61\%$ risk reduction for recurrence in patients with stage II disease.

More recently, Jocham et al. [16] performed a large randomized study for an autologous tumor cell vaccine after radical nephrectomy in patients with non-metastatic renal-cell carcinoma. The 558 patients enrolled in the trial were randomized before surgery to receive the vaccine or no adjuvant treatment. The vaccine was prepared by incubating autologous tumor cells in the presence of interferon $\gamma$ (IFN$\gamma$), which results in upregulation of HLA molecules and of the antigen presentation apparatus. Cells were subsequently killed by repeated freezing–thawing cycles and divided into aliquots. The vaccinated patients received six intradermal applications of the vaccine ($\sim 5 \times 10^6$ cells) at 4-week intervals. The final intention to treat population of this study was 379 patients, given the withdrawal of $\sim 30\%$ of the patients because of non-fulfillment of postoperative inclusion criteria (histology, correct tumor stage), or because of the inability to prepare the vaccine. This study detected a significantly reduced risk of tumor progression for the vaccinated patients. This was associated with an increased survival at 5 years and at 70 months: $77\%$ and $72\%$, respectively, in the vaccine group, and $68\%$ and $59\%$, respectively, in the control group.

An example of gene-modified autologous tumor cells as a vaccine is the study performed by Salgia et al. [17], who vaccinated 35 patients affected by non-small-cell lung cancer with autologous tumor cells infected with a replication-defective adenoviral vector encoding human GM-CSF, and subsequently irradiated. Vaccines were successfully prepared for 34 of 35 patients enrolled in the study. The number of cells per vaccine administration ranged from $1 \times 10^6$ to $1 \times 10^7$, depending on the initial cell recovery. The cell preparations were injected intradermally and subcutaneously weekly for 3 weeks, and then every other week. Immunization stimulated the development of delayed type hypersensitivity (DTH) reactions to irradiated autologous tumor cells in 18 of 22 patients. Two patients who had no evidence of disease because of surgical resection at enrollment remained free of disease during the study. Five patients showed stabilization of disease and one mixed response was observed.

Finally, tumor cells can be fused with autologous or allogeneic DCs (see review by Lesterhuis et al. [11] in this Supplement). This approach is based on the assumption that the generated hybrid cells express the whole antigen repertoire of the tumor and simultaneously acquire enhanced immunogenicity, as they carry the DC antigen presentation machinery [18].

Marten and co-workers [19] have recently made use of this kind of approach to treat 12 patients with metastatic renal cell carcinoma. In this study, patients were administered three monthly injections of an average of $3 \times 10^7$ tumor cells fused with $2 \times 10^7$ DCs. The tumor cells were of either autologous (four patients) or allogeneic (eight patients) source. DTH to the tumor cells used for the vaccine was found to be enhanced in seven out of 12 patients. Also, the cytotoxicity of peripheral blood lymphocytes against renal cell carcinoma cells was found to increase during treatment. Four stabilizations of disease were reported during this trial, this effect being associated with the development of antitumor immunity.

Peptide-based vaccinations

Over the past 15 years, several peptide epitopes have been identified that are derived from tumor antigens through the intracellular antigen processing apparatus and are presented at the surface of tumor cells by HLA molecules [20]. Inducing an immune response targeted to one or more of these peptide epitopes can lead to selective tumor rejection. Two different approaches can be used to identify such epitopes: the first approach (termed forward immunology) makes use of T cells reacting to tumor material (usually the tumor infiltrating lymphocytes) to track and finally identify the molecules and even the peptide epitopes for which these lymphocytes are specific. This approach allowed the identification of the first TAAs [1]. The second possible path (termed reverse immunology) takes advantage of the identification of characteristics (motifs) that peptides must have in order to bind defined HLA molecules. Based on these criteria, algorithms have been generated that allow predicting epitopes derived from a defined protein antigen. These epitopes can subsequently be synthesized and tested for HLA-binding capacity and for their immunogenicity, and epitope-specific CTLs can be used to verify that these peptides are actually presented by tumor cells that express the corresponding antigen [21, 22]. In addition, mass spectrometry coupled to microcapillary liquid chromatography can help directly sequence HLA-binding peptides eluted from tumors, thus facilitating the identification of TAA-derived presented T cell epitopes [23]. In some cases, the amino acid composition of a defined epitope has been modified in order to enhance its capacity to bind the HLA molecules. This translates into stabilization of the peptide–HLA complex and sustained TCR triggering. The so-modified epitopes often lead to enhanced activation of T cells that are specific for the original epitope [10].

For most of the proteins that have been shown to be upregulated or functionally deregulated in cancers, and thus appear as suitable target antigens, HLA-binding peptides have been identified. These antigens include telomerase, tyrosinase, gp100, MAGE, Melan-A/MART, MUC1, CEA, p53, Her-2/neu, survivin, Ras, etc. For some of these antigens, both HLA class I- and HLA class II-restricted peptides have been
Peptides per se are poorly immunogenic and hardly induce a response when injected alone. Hence, they are usually injected together with an adjuvant. Examples of such compounds include BCG, incomplete Freund’s adjuvant (IFA), diphtheria toxoid, tetanus toxoid peptide epitopes, GM-CSF and IL-12. The most suitable immunological adjuvant for this kind of immunization has not yet been defined. However, a recent study comparing IFA, QS-21 (a purified saponin) and GM-CSF suggested that QS-21 and GM-CSF would be superior to IFA as adjuvants for vaccination with peptides [24]. Alternatively, peptides can be loaded onto peripheral blood mononuclear cells (PBMC) or onto ex vivo generated DCs that are subsequently re-injected into the patient, whereby the latter approach involves the expensive and time-consuming procedures required to generate DCs from the patient’s circulating precursors [6–9, 25].

Numerous peptide-based vaccination studies have been performed for different types of malignancies, especially for melanoma. Altogether, the results of these studies are encouraging and peptide-based vaccines are presently being evaluated in several phase III clinical studies. In this field, Marchand et al. [26] treated 39 patients with metastatic melanoma with three subcutaneous injections of the MAGE-3.A1 peptide (100–300 μg) at monthly intervals. Of the 25 patients who completed the treatment, seven presented significant tumor regressions (including two complete responses). However, no evidence for peptide-specific CTL response was found in the PBMCs of the patients who were analyzed, including the two who displayed a complete response. A recent study by Cebon and co-workers [27] evaluated the side-effects and efficacy of a vaccination regimen for stage III and IV melanoma patients, which combined a Melan-A and an influenza matrix peptide together with IL-12 as an adjuvant. Twenty-eight patients were enrolled, of whom 24 were evaluable for clinical and immunological responses. IL-12 was administered either subcutaneously or intravenously at dosages ranging from 0 (placebo) to 100 ng/kg. Treatment was well tolerated. One complete response was observed in a subject with subcutaneous disease, and a partial response in a subject with hepatic metastases was also reported. Biopsies of accessible tumors showed infiltration with CD4+ and CD8+ Melan-A peptide-specific lymphocytes. However, no clear dose-dependent effect of IL-12 was demonstrated. Finally, Rosenberg and co-workers [28] have reported on the vaccination of patients with metastatic melanoma with an immunodominant gp100-derived peptide (g209-2M), which was modified to increase binding to HLA molecules. The peptide was administered together with low doses of IL-2. Different from the previous study, this group was able to detect a peptide-specific immune response in 91% of patients. Thirteen of 31 patients (42%) receiving the vaccine had objective clinical responses, and four additional patients had mixed or minor responses.

The potential of modified TAA-derived epitopes is also suggested by a study performed by Fong et al. [29] at Stanford. They reported on the vaccination of 12 colon cancer patients with a modified CEA-derived peptide (610D) that was pulsed onto ex vivo manipulated peripheral blood DCs. In this study, patients were given Flt3 ligand in order to expand in vivo the DCs and increase DC harvest. DCs were loaded with keyhole limpet hemocyanin (KLH) as an immunologic tracer, and with the peptide. Patients underwent two intravenous DC administrations 1 month apart. An expansion of CEA-specific CTLs was detected in seven out of 12 enrolled patients. Two complete responses, one mixed response and two cases of stable disease were reported. However, one possible bias of this study is represented by the administration to the patients of Flt3 ligand, which is capable of eliciting antitumor immunity per se [30]. Thus, the immunological and/or clinical results observed may possibly be ascribed, at least in part, to the effect of this growth factor.

Finally, we have vaccinated a series of 10 patients with advanced breast or ovarian cancer with tumor-associated peptides of HER-2/neu or MUC1 that were loaded onto mature monocyte-derived DCs [31]. DC administration (median of 6.5 × 10⁶ per vaccine) was performed every 2 weeks and repeated thereafter every 4 weeks until tumor progression. In five of 10 patients, peptide-specific CTLs were detected after three vaccinations. Interestingly, following vaccination we detected in two patients an increase in CTLs specific for different TAAs such as CEA and MAGE-3. One patient obtained a partial response after vaccination and two other patients experienced short periods of disease stabilization.

The issue of whether peptides plus adjuvant would be more or less immunogenic than peptides loaded on DCs remains controversial [32]. In this context, a recent trial comparing the injection of peptide-loaded DCs with peptides injected in an emulsion containing GM-CSF and an adjuvant (Montanide ISA-51 adjuvant) found that the latter vaccine formulation was possibly more immunogenic than the peptide-loaded DCs in melanoma patients [33]. However, the DCs used for this trial were likely to be in an immature state, since they were generated by incubating the monocytes in the presence of GM-CSF and IL-4 without addition of maturation stimuli. In fact, some evidence suggests that immature DCs may be less immunogenic than mature DCs or may even favor tolerance induction [6–9]. Hence, the outcome of this study may have been influenced by the reduced immunogenicity of the ex vivo generated DCs. Further investigation is required to elucidate this important aspect.

Finally, one major drawback associated with targeting a single (or few) epitope(s) is the increased chance of favoring immune escape of tumor cell clones that downregulate the antigen. However, as mentioned before, the emergence of an immune response to antigens not included in the vaccine may occur in some cases (a phenomenon called ‘antigen spreading’), possibly due to cross-presentation of TAAs from...
apoptotic tumor cells [7, 31]. This would reduce the chances of clonal tumor escape. In addition, it has recently been reported that antigen-specific CTLs can also clear variant tumor cells with downregulated antigen expression [34]. This effect would be dependent on CTL-mediated lysis of the stromal cells present in the tumor, which cross-present tumor-derived antigenic material.

**Protein-based vaccines**

Whole TAAs in the form of a protein can also be used to elicit antitumor immunity. When injected into the patient, these proteins are supposed to be captured by tissue resident DCs, leading to presentation in the context of HLA class II and possibly class I molecules. Again, in order to increase the immunogenicity of the antigen, this is usually conjoined with an adjuvant or loaded onto ex vivo generated DCs [1, 7].

Marchand and co-workers [35] have recently published the results of a vaccination study for melanoma and bladder cancer patients, which made use of a recombinant MAGE-3 protein combined with an adjuvant [a mix of the QS21 saponin and of monophosphoryl lipid A (MPL)]. Patients were immunized with escalating doses of recombinant MAGE protein given at 30, 100 and 300 μg. Four intramuscular injections were given at 3-week intervals. Patients whose tumor stabilized or regressed received two booster vaccinations at 6-week intervals. The treatment was well tolerated without significant side-effects. Among the 33 melanoma patients who were evaluable, two partial responses, two mixed responses and one stabilization of disease were observed. Additionally, a partial response was observed in one of the three metastatic bladder cancer patients included in the study.

Within this category also falls the use of idotype peptide for the immunization of patients affected by lymphoma or multiple myeloma [8, 36–39]. These represent unique types of malignancies, since they produce (at least in the majority of cases) high levels of a protein, namely an antibody, that is only expressed by the malignant clone. Whereas the monoclonal antibody can easily be obtained from the serum of patients affected by multiple myeloma, the generation of sufficient amounts of idotype protein from lymphoma patients is trickier and requires the generation of hybridoma cell lines obtained from primary lymphoma cells [8, 36, 37]. The purified idotype peptide is subsequently administered with an adjuvant such as KLH or GM-CSF. Alternatively, it can be fed to DCs ex vivo for subsequent reinfusion of these APCs into the patient.

This kind of approach has been pioneered by the group of Ron Levy at Stanford. In the pilot study published by this group in 1992, nine B-cell lymphoma patients with minimal residual disease or a complete remission after chemotherapy received a series of subcutaneous injections of the tumor-derived idotype, which had been conjugated to a protein carrier and mixed with an immunologic adjuvant [39]. The induction of an idotype-specific immunologic response was detected in seven patients (humoral response in two patients, cell-mediated response in four patients and both in one patient). In addition, the two patients with measurable disease exhibited complete regression.

The same group subsequently reported on the vaccination of follicular lymphoma patients by means of idotype protein, which had been loaded onto ex vivo manipulated DCs. This represents the first published DC-based vaccination study. The preliminary report by Hsu et al. [36] has recently been updated by Timmerman and co-workers [37]. In total, 35 patients with low-grade non-Hodgkin’s lymphoma were immunized intravenously with idotype-loaded DCs. Patients received three infusions at monthly intervals, followed by a fourth, administered 2–6 months later. Two weeks after each infusion, patients received subcutaneous injections of 0.5 mg soluble idotype and KLH proteins without DCs. The numbers of DCs ranged from $12 \times 10^6$ to $69 \times 10^6$. Side-effects were mild. An idotype-specific immune response was detected in 23 of 35 patients. The observed immune response was a T-cell proliferative response (14 patients), an antibody-mediated response (six patients) or a combination of both (three patients). In seven of 28 patients an objective response to DC vaccination was recorded.

Administration of idotype-loaded DC has also been evaluated for the treatment of multiple myeloma following high-dose chemotherapy and peripheral blood precursor cell transplantation. However, the efficacy of this immunotherapeutic approach for multiple myeloma remains uncertain [38].

**Heat shock proteins**

Heat shock proteins (HSPs) are intracellular proteins that work as chaperones for intracellular peptides. They include gp96, HSP70, calreticulin and HSP110 [1, 10]. Once these proteins are released from a cell, they can be captured by APCs that will present the HSP-associated peptides in the context of HLA class I molecules, although the mechanism(s) of cross-presentation still remains unclear [40]. A candidate receptor for HSPs, namely for gp96, is the surface receptor CD91 [41]. Cumulating evidence also suggests that DCs may receive an activating signal from extracellular HSPs, which triggers DC maturation [42]. HSPs for the production of antitumor vaccine can either be engineered to transport defined TAAs (such as the E7 protein from HPV or the MAGE antigen) or purified from patients’ tumor specimens for subsequent reinfusion [10]. In the latter case, the HSPs will be complexed with a broad spectrum of tumor-associated peptides.

The autologous, tumor-derived HSP gp96–peptide complexes (HSPPC-96, Oncophage; Antigenics, Woburn, MA, USA) have been tested in stage IV melanoma patients by Belli et al. [43]. Autologous HSPPC-96 vaccine was prepared from tumor samples of each patient. Out of 64 patients enrolled in the study, 42 were able to receive the vaccine, and 39 patients received one or two complete cycles of vaccination consisting of four weekly injections. Out of 28 patients with measurable disease, two had a complete response and three
had stable disease. A significantly increased number of post-vaccination melanoma-specific T-cells was detected by enzyme-linked immunospot (ELISpot) assay in the PBMC of 11 out of 23 patients, whereby the clinical responders displayed a higher frequency of specific T-cell activity.

**DNA-based vaccines**

This vaccination form consists of the intramuscular injection of naked DNA plasmids encoding tumor antigens (gp100, MART1, MUC1, etc.) under the control of a constitutively active promoter, such as CMV [10]. In the recipient, the DNA encoding the tumor antigen(s) is transcribed and translated leading to the production of tumor antigen. The mechanism of antigen-specific immunity induction, in this case, is believed to be dependent on cross-presentation of antigenic material by APCs *in vivo*. Given the intrinsic weak immunogenicity of the naked DNA, plasmids are often engineered by including genes coding for co-stimulatory molecules, cytokines or HSPs [10]. Immunization with DNA vaccines by gene gun represents an attractive approach in which gold particles coated with expression plasmid DNA encoding target genes are ‘bombarde’ into the skin [44, 45]. This procedure has been suggested to transfect plasmid DNA directly into the DCs present in the skin. Transfected DCs express the encoded antigen and present the processed peptides to the antigen-specific T cells to initiate an immune response. The results reported by Sudowe et al. [44] and Garg et al. [45] in animal models indicate that this approach is a potentially effective method for antitumor immunity induction.

An example of DNA-based vaccination is the study performed by Klencke et al. [46] in patients with high-grade dysplasia induced by human papillomavirus (HPV). These authors vaccinated 12 HPV-16+ anal dysplasia subjects with four intramuscular injections of 50–400 µg of plasmid DNA encapsulated in biodegradable polymer microparticles at 3-week intervals. The plasmid DNA encoded for multiple epitopes and was intradermally administered, whereas avipox-CEA (2.5 × 10⁷ p.f.u./vaccination) was given subcutaneously. All vaccinations were administered 4 weeks apart and tumor responses were evaluated every two treatment cycles. An increase in CEA-specific T-cell precursor frequency was observed in six of six patients in the VAAA group compared with two of five patients in the AAAV group after four vaccination cycles, suggesting a superiority of the first regimen. In the course of the study, the effect of simultaneous administration of cytokines (GM-CSF alone and GM-CSF plus IL-2) together with the avipox vaccine was also evaluated in patients who had no evidence of progressive disease after four cycles of vaccine administration. Local GM-CSF strongly increased the elicited CEA-specific immune response as similarly, did low-dose IL-2 plus GM-CSF, thus indicating that these cytokines work as a potent adjuvant in this kind of immunization strategy. However, very limited clinical activity of the vaccine was detected in this patient population.

In another trial, a live recombinant vaccinia virus expressing the human MUC1 and IL-2 genes was tested in nine patients with advanced inoperable breast cancer [49]. The patients were vaccinated intramuscularly with a single dose of vaccine. Virus doses ranged from 5 × 10⁵ to 5 × 10⁷ p.f.u. No serious side-effects were detected in this study. One patient showed a proliferative response to MUC1 peptide, which further increased after a booster injection. This patient had a concomitant decrease in CEA serum levels and remained clinically stable for 10 weeks. Evidence of MUC1-specific CTLs was detected in two patients.

**Recombinant viruses as vaccine vectors**

Different types of viruses can be engineered to express a defined tumor antigen and used to induce an antigen-specific immune response: these include attenuated replication-deficient poxviruses such as avipox, fowlpox and canaripox virus, adenovirus, herpes virus, and Venezuelan equine encephalitis virus [1, 10]. The major advantage related to the use of viruses is their strong immunogenicity, which leads to significantly greater immune response to the encoded antigen compared with other immunization strategies such as peptides or protein antigen administered with standard adjuvant. On the other hand, two major obstacles limit the application of viral vectors in anticancer vaccinations: first, the presence of the development of neutralizing antibodies in individuals undergoing vaccination. Patients who have never been exposed to a particular virus before vaccination develop such antibodies after the first vaccination, and this precludes the use of the same vector for subsequent vaccinations. However, this obstacle can be circumvented by cycling different vectors. Another concern related to this kind of approach is represented by the safety issues, given that in some studies heavy side-effects have been associated with virus administration [47].

Several clinical studies have made use of recombinant virus to elicit immunity to TAAs. For instance, Marshall et al. [48] have tested two different vaccination schedules, where the vaccine formulation consisted of recombinant vaccinia (rV)-CEA or avipox-CEA virus. The patients enrolled in this study were randomized to receive either rV-CEA followed by two avipox-CEA vaccinations (VAAA), or avipox-CEA (three times) followed by one rV-CEA vaccination (AAA V). rV-CEA [1.0 × 10⁷ plaque-forming units (p.f.u.)/vaccination] was administered intradermally, whereas avipox-CEA (2.5 × 10⁷ p.f.u./vaccination) was given subcutaneously. All vaccinations were administered 4 weeks apart and tumor responses were evaluated every two treatment cycles. An increase in CEA-specific T-cell precursor frequency was observed in six of six patients in the VAAA group compared with two of five patients in the AAAV group after four vaccination cycles, suggesting a superiority of the first regimen. In the course of the study, the effect of simultaneous administration of cytokines (GM-CSF alone and GM-CSF plus IL-2) together with the avipox vaccine was also evaluated in patients who had no evidence of progressive disease after four cycles of vaccine administration. Local GM-CSF strongly increased the elicited CEA-specific immune response as similarly, did low-dose IL-2 plus GM-CSF, thus indicating that these cytokines work as a potent adjuvant in this kind of immunization strategy. However, very limited clinical activity of the vaccine was detected in this patient population.

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**Monitoring the immune response to anticancer vaccination**

Detection of the immune response to tumor antigens after vaccination represents one of the major endpoints of clinical
immunotherapy studies. In most cases, the lymphocytes reacting to the tumor antigens are detected in the PBMC. The T cells specific for a defined antigen can be monitored by different approaches, which include ELISPOT assay, intracellular staining for IFN-γ, tetramers, proliferation assays, enzyme-linked immunosorbent assay (ELISA) for IFN-γ, tumor necrosis factor-α, IL-4, IL-10, cytotoxicity assays and real-time PCR for IFN-γ [6–8].

In some cases the DTH assay has demonstrated immune reactivity to tumor-derived material following vaccination. It is usually performed by intradermal injection of antigenic material or DCs loaded with tumor antigen(s) before and after vaccine administration [6–8].

In those patients showing regression of tumor masses following vaccination, the demonstration of T lymphocytes and/or inflammatory cells infiltrating the tumor allows better correlation of the clinical outcome with the elicited immune response [50]. In some cases, the tumor infiltrating lymphocytes can be isolated and analyzed.

Some methods are available for the isolation of antigen-specific lymphocytes, including FACS or MACS technology. These effectors can be expanded and further characterized. This goal can be achieved by combining tetramer staining with antibodies to surface markers such as CD28, CD25, CD45RA, CD45RO, CCR7 and/or with intracellular staining for cytokines (IFN-γ, IL-4, IL-10).

Finally, including a tracer antigen such as KLH or an immunogenic peptide (i.e. influenza peptides or CD4 epitopes) in the vaccine formulation allows evaluation of the efficacy of the vaccination approach in vivo and the responsiveness of the immune system to vaccine administration [6–8, 31]. Thus, these antigens are supposed to work as ‘positive controls’ in the immunization procedures. These immunogens can be particularly useful when comparing the efficacy of different vaccine administration routes/schedules or different immunization strategies.

**Some perspectives: prophylactic vaccinations and vaccinations in allogeneic bone marrow transplants**

The immunization of patients against TAAs as an anticancer prophylaxis is, at present, extremely controversial [10]. Some high-risk individuals may be considered for prophylactic anticancer vaccination, even though, in these cases, the potential advantages should be balanced with the potential side-effects of the procedure (for instance autoimmunity induction) and also evaluated in terms of costs/benefits. An exception is represented by those tumors caused by infectious agents, particularly viruses. An example is the study performed by Koutsky and co-workers [51], who randomly assigned 2392 young women to receive placebo or an anti-HPV-16 vaccine made of virus-like particles. Women were monitored for infection with HPV-16 and for cervical intraepithelial neoplasia. The incidence of persistent HPV-16 infection was 3.8% woman-years in the placebo group and 0% woman-years
in the vaccine group (P<0.001). Importantly, all nine cases of HPV-16-related cervical intraepithelial neoplasia occurred among the placebo recipients, suggesting an important effect of the vaccine in preventing tumor development. Similarly, anti-HBV vaccinations will contribute to reduce the incidence of hepatocarcinoma induced by this virus [52].

Finally, antitumor vaccinations may intersect other therapeutic approaches such as the allogeneic bone marrow/peripheral blood precursor cell transplantations, whose antitumor activity is dependent, at least in part, on the (still poorly characterized) graft-versus-tumor effect [53]. Recent advances in transplantations, particularly the use of reduced-intensity conditioning regimens, have led to a reduction in the treatment-related mortality associated with these procedures. This has permitted the evaluation of allogeneic transplantation and donor lymphocyte infusion in cancers other than leukemia, also including solid tumors such as renal cell carcinoma and breast cancer [54–57]. In this setting, the availability of methods to manipulate the immune response could be harnessed to selectively target the alloreactive response to tumor antigens, while possibly sparing normal tissues and organs [58].

Conclusions

The clinical studies performed so far have demonstrated that anticancer vaccine formulations can generally be safely administered without significant side-effects, except for some cases of vitiligo observed in melanoma patients. These approaches have been shown to induce antigen-specific immunity in vivo in a significant fraction of the patients, being effective also in heavily pretreated individuals. Nevertheless, clinical responses have often been limited to a tiny minority of patients, possibly due to the advanced stage of disease of the patients enrolled in these studies. The results of studies performed in colorectal cancer and renal cell carcinoma patients suggest that anticancer vaccinations may indeed be clinically useful in earlier disease stages such as in the adjuvant setting, when the elicited immune response is more likely to contribute to eliminate residual tumor cells [15, 16]. However, the results of these studies need to be confirmed and the types of malignancies as well as the clinical settings for which specific immunotherapy may be beneficial need to be identified.

Major obstacles to carrying out large randomized studies, which would help in defining the possible role of vaccinations are: (i) the necessity to coordinate the approaches of different groups used to different vaccination techniques (as exemplified in this article); (ii) the need for standard methods for the monitoring of the immune response to vaccinations, which would allow better comparisons between different immunization strategies; (iii) the rising costs and the increasingly demanding GMP guidelines, which restrict the clinical evaluation of anticancer vaccines to a limited number of specialized centers. Overcoming these obstacles and, in particular, establishing optimized approaches to be used for multicenter trials are necessary steps for the near future of anticancer vaccinations.

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