Ann Hematol (2001) 80:255–266
DOI 10.1007/s002770100324

REVIEW ARTICLE

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Clinical approaches to vaccination in oncology

Received: 25 March 2001 / Accepted: 29 March 2001 / Published online: 24 April 2001
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Abstract Immunotherapy of cancer is still in the early stages of development although almost a century has passed since initial attempts were made to stimulate the immune system in order to destroy malignant cells. Historically, a variety of specific and non-specific immunostimulatory strategies have been administered with only modest clinical success. However, recent advances in tumour immunology, most notably the identification of new tumour antigens and the better understanding of antigen processing and presentation to avoid or break immune tolerance, have paved the way for the development of a variety of novel and specific vaccine approaches. The most important and widely used are whole-cell vaccines, dendritic-cell-based immunotherapy and peptide vaccines. The first wave of clinical trials has revealed that, in general, such vaccination strategies are safe. However, clear examples of clinical responses, especially in conjunction with vaccine-induced immune responses, are still rare. Most clinical trials are too small to allow for comments on the efficacy, and the cohort of patients studied is too heterogeneous with regard to immune status. Therefore, standardised techniques for the accurate assessment of the individual immune phenotype before and during the trial are needed to allow for the identification of the sub-group of patients who will respond favourably to treatment. The precise definition of immune parameters in these patients will then lead the way for optimised treatment procedures that might even be beneficial for a larger group of cancer patients.

Keywords Dendritic cells · Gene transfer · Hybrid cell vaccine · Tumour peptides · Tumour vaccine

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Introduction: setting out to design a cancer vaccine

Although the idea of fortifying or stimulating the immune system to fight cancer has been around for nearly 100 years [17], the combination of new technologies and a better understanding of immune-system mechanisms have resulted in an explosion of basic research in recent years and, finally, a transition of this work into the clinic where trials in cancer patients have been initiated. The individual immunotherapeutic approaches chosen differ significantly from each other; however, they all have one goal in mind: to kick-start the immune system in order to destroy the malignant cells.

When a pathogen invades the human body, the immune system usually responds in force. In contrast, cancer-cell-associated antigens that stimulate an immune response induce in most instances a weak reaction, if at all. To bolster the immune response, cancer patients are given genetically engineered, synthetic, or natural antigens, which are altered to become more recognisable to the immune system. The reasons why the response of the immune system to cancer cells is impaired compared to the strong response against infectious pathogens are manifold. One important point which regained scientific interest only recently [60] and always has to be considered is that tumour antigens, for the most part, are normal self-antigens, and tumour immunity is mostly autoimmunity [61]. The old concept that the immune system can discriminate between self and non-self seems to be inadequate and has many shortcomings. Moreover, the task of the immune system is not to distinguish between ‘that which is to be destroyed’ and ‘that which is not to be destroyed’ [16]. Instead, the immune system seems to be able to discriminate between dangerous and non-dangerous signals as defined in Matzinger’s danger model [4]. An immune system acting under danger-model principles categorises antigens into those associated with danger (to be attacked) and those not associated with danger (to be tolerated) and the latter set includes most self-antigens because self is usually not dangerous.

For a tumour antigen to be recognised as immunogenic, the antigen by itself or the immune complex which it is part of should surpass a certain level of immunogenicity paired with the danger signal. Under normal circumstances, the level of endogenous immunity against a tumour antigen is below a critical threshold necessary for efficient anti-tumour immunity. In addition, a common rule of the immune system is that the stringency of tolerance against a particular antigen correlates with the endogenous immunity level which is usually below the (auto-)reactivity threshold level. If one chooses an antigen for cancer-vaccine purposes against which immune tolerance is stringently maintained, even a strong adjuvant (or danger signal) will not raise the level of immunity above the critical threshold and the vaccine will fail. If one chooses an antigen against which immune tolerance is less stringently maintained, but uses a weak adjuvant, the vaccine also will fail. However, if one uses a strong adjuvant to present an antigen against which endogenous tolerance is relatively non-stringent, then it will be easier to elevate the level of immunity against that antigen above the critical threshold and the vaccine will be successful [61]. According to this theory, the choice of a cancer vaccine always depends on the nature of the combination of antigens and adjuvants used.

In a recent review [63], we dealt with the principles of tumour vaccines and discussed a number of advances made over the last years in the area of defining tumour antigens, the development of new delivery systems and, finally, new approaches for monitoring immunological responses in vaccine patients. The current review is an extension of this initial review, with emphasis on trials and concepts that are of importance for the transfer of cancer-vaccine strategies into the clinical practice. Again, the vaccine field is so broad and diverse that not all ideas and concepts can be discussed. We focused on those we believe will have a major influence on the design of cancer vaccine studies in the future.

Whole-cell vaccines: a black-box approach

Developing a vaccine strategy as described above, one comes almost immediately to the important question about the kind of vaccine to be used. The scientific community working on this issue is split into two parties: those developing vaccines utilising whole tumour cells and those working on vaccines targeting defined antigens. The advantage of tumour-cell-based vaccines is that these, in principle, comprise all relevant tumour antigens, especially if autologous tumour cells are used. As a consequence, there is, with respect to the vaccine design, no need for prior identification of the tumour antigens to be included in the vaccine [57]. Historically, tumour-cell-based vaccines were one of the first concepts to be developed for cancer-vaccine purposes, because with the lack of information about specific tumour antigens, the tumour cell appeared to be the best source of antigens for activating the immune system.

Initial attempts to produce vaccines focused on various forms of tumour-cell preparations, including whole tumour cells (either autologous or allogeneic), tumour-cell lysates, or cell extracts. In early studies, tumour-cell vaccines were given following irradiation and often admixed with non-specific adjuvants (e.g., BCG). The addition of an adjuvant seemed to be necessary, as initial attempts to immunise with irradiated autologous tumour cells met with little success [47, 51]. The failure was explained by the observation that the immunising tumour cells were poorly immunogenic and generally failed to induce long-lasting anti-tumour immune responses in vivo; this supported the "danger model" as described above. Therefore, subsequent studies adopted a variety of immunologic adjuvants or modified the tumour cells to increase the tumour's immunogenicity.

Increasing immunogenicity by ex vivo cytokine gene transfer

Introduction of cytokine genes into tumour cells, an approach described as ex vivo gene therapy, allows the sustained local release of cytokines capable of enhancing the intensity and quality of the immune response to a tumour. Studies in mouse tumour models have established that tumour cells engineered to secrete IL-1, -2, -4, -6, -7, 12, -18, as well as TNF-α, G-CSF, GM-CSF, or IFN-γ can lead to tumour rejection by stimulating both specific and non-specific anti-tumour responses. Rejection depends on a high level of cytokine production by the gene-modified cells and is caused either by stimulation of host anti-tumour effector responses [23, 85] or by alteration of the immunological environment of the tumour, allowing the complete rejection of a tumour and even providing protection of the host against subsequent challenge with unmodified tumour cells [28].

Despite the considerable interest in this approach, ex vivo engineering of autologous cells suffers from several major drawbacks. Isolation of primary autologous cells in which the expression of high levels of the therapeutic gene remains stable is not only cumbersome and expensive, but also poorly reproducible [2]. Batch-to-batch variation of engineered cells complicates analysis of the biological effects observed in each patient. In addition, the therapeutic efficacy of cytokine gene-transduced tumour cells is low. The picture emerging from phase I studies of vaccination of cancer patients with transduced human tumour cells is not very encouraging [89]. Even though the approach itself is safe, the published data show that less than 10% of patients achieve an objective response [64].

Some of the limitations of ex vivo gene therapy may be addressed by the use of replicating viruses and a combination of multiple cytokines. Several recent studies have shown that the therapeutic activity of vaccines consisting of tumour cells transduced with multiple genes is greater than that of single-gene vaccines. Combinations of GM-CSF and IFN-γ [7]; IL-2 and IL-4 [58]; GM-CSF

and IL-4 [87,]; IFN-γ, IL-4, and IL-6 [56]; IL-2 and IL-12 [1]; and IL-12, pro–IL-18, and IL-1β-converting enzyme [59] have been shown to significantly augment anti-tumour effects. The objective of such "multiple" gene therapies is to orchestrate an effective multicellular response. Hence, the order, the type, the dose, and the duration of produced cytokines can have distinct biological effects in anti-tumour gene therapy, reflecting in part the complexities of the underlying immune response [70].

Replicating viruses for gene transfer to tumour cells have attracted interest because a higher transduction efficiency and oncolysis of transduced tumour cells can be achieved [90]. Adenoviral vector systems were among the first to be developed and clinically tested for this purpose. Despite their promise, adenovirus derivatives have several limitations. First, as with other adenoviral vectors, the robust immune response makes the readministration of a given viral serotype after an initial infection impractical. That 90% of the adenovirus vector is eliminated from the organism within 24 h after intravenous administration indicates the involvement of innate immune mechanisms [92, 93]. A rapid $CD8^+$ cytotoxic T lymphocyte (CTL) response to viral proteins eliminates virus-infected cells, and a long-lasting humoral response to viral epitopes further limits the readministration of the virus. Initial studies with adenoviral vectors have established that there is an in vivo correlation between serum levels of antiviral antibodies and inhibition of viral transduction [37, 93].

The first two human clinical trials of genetically engineered cancer-cell vaccines using retroviral delivery systems were in kidney and prostate cancer [54]. In both trials, cancer cells were removed at surgery and then genetically modified to secrete high levels of GM-CSF by ex vivo transduction with the retrovirus MFG-GM-CSF. After being irradiated, the cells were inoculated into the skin every 28 days with dose escalation; three dose levels were used for the kidney cancer patients (4×10^6 cells, 4×10^7 cells and 4×10^8 cells) and two dose levels were applied in the prostate cancer patients (1×10^7 and 5×10^7). Between three and five patients were entered at each dose level. Side effects associated with vaccine administration were minimal at all levels and limited to pruritus, low grade fever, chills and malaise. No replication-competent retrovirus was detected in any of the vaccinated patients at any time. Biopsies of vaccine sites demonstrated recruitment of immune effector cells to sites of autologous cancer-cell antigen deposition. The intensity and character of immune-cell infiltration increased with increasing vaccine dose and with the efficacy of the retroviral gene transfer. Of the 18 patients with kidney cancer participating in the trial, one out of three patients treated on dose level 2 exhibited regression of multiple pulmonary metastases following treatment which lasted 7 months. In the prostate trial, all eight treated patients ultimately had progressive disease. The major limitation of these trials was associated with the preparation of autologous cancer-cell vaccines: the recovery, expansion and viability of cancer cells limited both the frequency with which vaccines could be created from resected tumour specimens and the quantity of vaccine cells available for administration. At the highest dose level, cell expansion to the specified numbers was successful only in 20% for the renal cancer patients and in 43% for the prostate cancer patients.

Hybrid cell fusion

Hybrid cell vaccination is a new cancer-immune-therapy approach that aims at recruiting T cell help for the induction of tumour-specific cytolytic immunity. The vaccines are generated by fusion of the patients' tumour cells with allogeneic MHC class II bearing cells [73]. The basic idea is that the hybrid cells thus generated will display the full antigenicity of the tumour cell and be highly immunogenic by the effect of allogeneic MHC class II and co-stimulatory molecules contributed by the fusion partner cell [15, 32]. This concept has been tested in animal models for thymoma [73], hepatocarcinoma [32] and adenocarcinoma of various origins [29].

Two clinical trials, in patients with melanoma and renal cell cancer, have been reported so far. In the melanoma trial, 16 patients (nine female and seven male) with metastatic melanoma at advanced stages were enrolled [82]. For six of the 16 patients, the hybrid cell vaccines were prepared from freshly isolated tumour cells fused with activated allogeneic B cells. In the remaining ten patients, autologous tumour cell lines were used as the tumour-cell source and fused with activated allogeneic B cells under the same conditions. The fusion efficiencies usually ranged between 20 and 30% for heterologous hybrids. Patients received three subcutaneous (s.c.) injections of the vaccine with at least 3×10^7 tumour cells (range $3–10\times10^7$) at two separate sites distant from tumour lesions, usually the lower abdomen and the upper thigh. The response status was established 4 weeks after the third vaccination. In the case of a clinical response, the vaccination treatment was continued beyond the primary study end point until progression or relapse occurred. The treatment was well tolerated, causing only minor side effects that in most cases were classified as grade I and in no case exceeded grade II. Side effects were strongest within 24 h after vaccination and waned within 72 h, with the exception of one case. In two cases, regionally restricted vitiligo occurred after vaccination, suggesting the induction or expansion of a melanoma-specific T cell response. The authors reported an average survival time of the responders of 16.1 months, exceeding the average 6-month life expectation of patients with advanced stage metastatic melanoma.

In the second trial, 17 patients with metastatic renal cell carcinoma staged pT1–4N*x*M1 were enrolled [40]. Inclusion criteria were bi-dimensionally measurable metastatic lesions, an ECOG score of less than 3, life expectancy of more than 3 months and a positive result in the DTH test for common recall antigens, indicating in-

tact cellular immune reactivity. All patients had tumour nephrectomy or metastatic surgery and tumour samples were processed within 12 h after surgery. Hybrids of 5×10^7 autologous tumours and 5×10^7 allogeneic dendritic cells were generated by electrofusion techniques, with an average final yield of 10–15% hybrid cells. Eligible patients received at least two s.c. injections of hybrids, close to inguinal lymph nodes, with a 6-week interval. Patients with measurable clinical response after 12 weeks received a booster vaccination every 3 months. The hybrid cell vaccination was well tolerated by all patients. There were no serious adverse effects or any clinical signs of autoimmune reaction. In some patients, mild fever for 1–2 days or a transient erythema and induration at the site of injection occurred. Six patients reported pain at the metastatic sites. There were no substantial changes in the results of routine blood tests or in the ratios of lymphocyte populations in peripheral blood after multiple immunisations.

HLA-A2 restricted $CD8^+$ T cell responses against Muc1 [11], and HER2/neu [12] epitopes were studied to define the specificity of the immune reaction induced. No reactivity against Muc1.1 and Muc1.2 [11] or the HER-2/neu-derived antigens GP2 and E75 [12] was detected before hybrid cell vaccination therapy. Two patients had a Muc1.2 peptide-specific reaction after the booster vaccination indicating the induction of specific CTLs and the establishment of a tumour-directed immune response in vivo. $CD4^+$ T cell responses were analysed by delayed-type hypersensitivity (DTH) tests in which 5×10^5 irradiated autologous tumour cells were injected intracutaneously in the forearm. No positive DTH reactions were obtained before vaccination. However, 11 of 17 patients tested after 12 weeks presented a positive reaction 1–3 days after tumour challenge. Seven patients developing positive DTH reactions had a clinical response of complete or partial rejection of metastatic lesions.

Seven of 17 patients (41%) responded to hybrid cell vaccination, with four complete tumour remissions, two partial remissions and one ‘mixed response.’ The mean follow-up time was 13 months (range 3–21 months). Three of the four patients with complete remission successfully rejected all metastases within the first 12 weeks, with a total of two injections, and remained free of any detectable tumour lesions for up to 21 months. Typically, the reduction of the tumour mass occurred within the first weeks after the first immunisation. All other patients (8 of 17) suffered progressive disease or died from their disease.

The problem of both studies is the small number of patients included, a bias in patient selection, and that spontaneous tumour regression caused by cellular immune responses is known to occur for melanoma and renal cell cancer [31, 48]. The frequency of spontaneous regressions of metastases in patients after cytoreductive nephrectomy remains undetermined, although they have been documented by various groups [49]. Moreover, in the renal cancer study, only patients with a positive DTH test for common recall antigens as an indicator for intact cellular immune reactivity were included. It cannot be ruled out that this selection process for patients with a favourable immune status is responsible for therapy in this group leading to more tumour regressions than in historical control groups.

The overall published data shows, from our point of view, that the use of whole tumour cells as the basis for vaccine development has major limitations. Since the antigenic composition of tumour-cell-based vaccines is very complex and not fully characterised, it is very difficult to understand their therapeutic effect, or lack thereof, on the disease. In various murine tumour models, the protective effect of tumour-cell vaccination was shown to involve defined T cell responses. In contrast, it has so far proven to be very difficult to establish such causal relations in clinical vaccination studies: the number of variables is much higher and extensive analysis of immune responses is less feasible. Only in selected cases did the detection of T cell immunity against defined antigens coincide with clinical responses, suggesting that these T cells were involved in the clinical effects observed [40, 55, 74]. Again, the renal cancer study is such an example, as only two patients, one with complete remission and one with stable disease, developed cytotoxic T cells specific for a defined tumour antigen. The impact of this T cell response on the observed tumour remission or stabilisation remains elusive, as it is unclear if these T cells were involved in tumour-cell lysis in vivo. In addition, no immunological data could be obtained that would explain the tumour regressions in the other patients with tumour responses. So, even if the studies were to reveal that vaccination induces clear-cut antitumour effects in a small fraction of the patients, it would be very difficult to use the available data to develop an improved vaccination protocol that would work in a larger fraction of patients. In the case of tumour-cell-based vaccines, a step-by-step, rational improvement of vaccine design is hard to envision.

We do favour, in contrast, the use of vaccines comprising defined antigens which enable the improvement of vaccine strategies based on experimental findings: it allows the systematic analysis of vaccine-induced immunity in relation to clinical responses. In our opinion, this notion strongly argues for the development of antigen-specific rather than tumour-cell-based anticancer vaccines. A first step has to be the identification of tumour-related antigens by cellular- or humoral-based technologies, and their subsequent characterisation under the concept of a peptide- or protein-specific vaccine. The immune response to these pre-defined peptides or proteins can be followed in the patient and correlated with other immunological parameters or even the clinical response.

3. Target-specific approaches: peptide/protein vaccines

From a conceptual point of view, peptide- or protein-based approaches and cell-based concepts have opposite

origins. The identification of tumour-associated immunogenic peptides or proteins and the characterisation of a specific immune response directed against these structures are the primary goals of the peptide- or protein-based approach. Therefore, after potential candidate antigens have been identified by cellular- [22, 83] or humoral-based assays [65], they are analysed in a second step for their in vitro capacity to stimulate a $CD4^+$- and/or $CD8^+$-restricted immune response. The disadvantage of this approach is that the identification of suitable target antigens is cumbersome and the vaccine is restricted to a single epitope in the case of peptides or to a few epitopes if proteins are used. To be immunogenic in vivo and induce or amplify a target-specific immune response, peptides or proteins have to be presented in the context of MHC molecules by antigen-presenting cells (APC). This can be achieved either by injection of the peptides or proteins into the skin or by drainage of lymph nodes where professional antigen-presenting cells such as dendritic cells (DCs) will take them up, process and present them to the immune system. A second option is the ex vivo loading of DCs with the peptides or proteins and their readministration to the patient via suitable sites.

In vivo administration of peptides or proteins

The direct injection of peptides or proteins into patients is easy and safe in terms of the preparation and handling of the vaccine and, therefore, the most commonly used approach. As many tumour antigens were characterised initially in melanoma patients and cellular immune responses are known in this cohort of patients, melanoma is the tumour prototype to which many forms of immunotherapy have been applied extensively over the past two decades. The largest pool of data is for the use of melanocyte-differentiation antigens such as Melan A/MART-1, gp100 and tyrosinase; this supports the concept that normal differentiation antigens can serve as tumour-rejection antigens.

MART-1 was used as a target antigen in 25 patients with high-risk resected stages IIB, III, and IV melanoma, who were immunised with a vaccine consisting of the minimal epitope, the immunodominant peptide comprising nine amino acids (AAGIGILTV), mixed with incomplete Freund's adjuvant [88]. Patients were immunised with increasing doses of the peptide in a phase I trial to evaluate the toxicity, tolerability, and immune response to the vaccine. Immunisations were administered every 3 weeks for a total of four injections, preceded by leukapheresis to obtain peripheral blood mononuclear cells for immune analyses, followed by a post-vaccine leukapheresis 3 weeks after the fourth vaccination. Overall, the vaccine was well tolerated with only grade I or II toxicities such as local pain and granuloma formation as well as fever or lethargy occurring. No vaccine-related grade III/IV toxicity was observed. Of the 25 patients, 12 were anergic to skin testing against the specific MART-1 peptide at the initiation of the trial, and 13 developed a positive skin-test response to the MART-1 peptide. Immune responses were measured by release of IFN-γ in an ELISA assay by effector cells after multiple restimulation of peripheral blood mononuclear cells and by ELISPOT assay. Of 22 patients, 10 demonstrated an immune response to peptide-pulsed targets or tumour cells by an ELISA assay after vaccination, as did 12 of 20 patients by an ELISPOT assay. From a clinical perspective, nine of 25 tumour-resected patients have relapsed with a median of 16 months of follow-up, and three patients in this high-risk group have died.

Especially in melanoma, metastatic tumour cells are likely to lose expression of the respective differentiation antigens used for immunisation [5]. This loss of melanocytic tissue differentiation antigens often coincides with loss of pigmentation in these metastatic deposits which presents clinically as vitiligo. In some of their patients, Knuth and co-workers [33] observed that after an initial phase of tumour regression, progressive disease occurred in certain areas of tumour manifestation, although peptide-specific CTLs that lysed efficiently HLA-matched melanoma cell lines in vitro were readily detected. Biopsies from lesions in the phase of progressive tumour growth were compared with biopsies taken prior to entry into the study and revealed a highly heterogeneous antigen expression pattern or even loss of antigen expression. Therefore, other target antigens beside the group of differentiation antigens have to be studied.

Recently, a strategy utilising spontaneous antibody responses to tumour-associated antigens (SEREX) has led to the identification of a new cancer test antigen called NY-ESO-1 [65, 72]. NY-ESO-1, which is regarded as one of the most immunogenic antigens known today induces spontaneous immune responses in 50% of patients with cancers expressing NY-ESO-1 [36]. In a first phase I clinical trial, twelve patients with cancers positive for NY-ESO-1 were treated with HLA-A2 restricted NY-ESO-1 derived peptides. Out of the 12 patients, nine had progressive melanoma. All nine melanoma patients developed a cellular $CD8^+$ immune response against the NY-ESO-1 peptide used for immunisation. Seven out of the nine patients had positive results in the DTH and cytotoxicity assay. The overall implication of these data is that most patients have a combined $CD4^+$ and $CD8^+$ response to vaccine treatment. From a clinical point of view, two mixed responses, five stable and two progressive diseases were observed. Larger, randomised trials are needed to justify the attribution of the observed disease stabilisation to the vaccine treatment.

Besides melanoma, haematological cancers such as B cell non-Hodgkin's-lymphoma (NHL) and certain leukaemias expressing truly tumour-associated antigens are attractive targets and were the subjects of clinical trials. The idiotypic determinants of the immunoglobulin synthesised by a clonal B cell malignancy are unique and can thus serve as tumour-specific antigens [71]. A decade ago, first trials using purified autologous idiotypic protein as a vaccine started in B cell NHL patients [8, 34, 42], with the largest study including 41 patients [34].

All patients received a series of injections with a vaccine consisting of tumour idiotype (Id) protein coupled to keyhole limpet haemocyanin (KLH). Subjects were observed for toxicity, immune responses, and tumour status. The median duration of follow-up of all patients was 7.3 years from diagnosis and 5.3 years from the last chemotherapy given before vaccine treatment. Of all patients treated, 20 (49%) generated a specific humoral response but only seven (17%) had a specific cellular immune response against the idiotypes. Two patients who had residual disease experienced complete tumour regression in association with the development of these immune responses. The median duration of freedom from disease progression and overall survival of all 20 patients with an anti-Id immune response was significantly prolonged compared to that of the patients who did not have an immune response. Before the vaccine treatments commenced, 32 patients were in their first remission and nine were in subsequent remissions. Analysis of the 32 first-remission patients also showed an improved clinical outcome in the patients with a specific immune response compared to those who did not (freedom from progression 7.9 years vs 1.3 years, $P = 0.0001$).

The results of this study have to be interpreted with caution because all patients had been pretreated by chemotherapy before they entered the vaccine trial and no prospective randomised trial followed to confirm the data. The disadvantage of the Id approach in general relates mainly to the problem of protein production and the development of Id-loss variants allowing the malignant B cell to escape immune recognition and destruction [27]. Since the tumour Id is unique for every B cell lymphoma, protein production for vaccine purposes has to be performed for every patient individually, requiring between 2 to 6 months.

In the field of leukaemias, chronic myelogenous leukaemia (CML) represents a unique opportunity to develop therapeutic strategies using vaccination against a truly tumour-specific antigen that is also the oncogenic protein required for neoplasia. CML is characterised by the t(9;22) that results in the bcr-abl fusion oncogene and in the expression of a chimeric protein product p210. Peptides derived from amino acid sequences crossing the b3a2 fusion breakpoint in p210 elicit class I restricted cytotoxic T lymphocytes or class II responses in vitro [94]. Such sequences were evaluated for safety and immunogenicity in a multidose vaccine trial in 12 adults with chronic-phase CML [62]. Cohorts of three patients each received either 50 µg, 150 µg, 500 µg, or 1500 µg total peptide mixed with 100 µg QS-21 as an immunological adjuvant. Delayed-type hypersensitivity (DTH), humoral responses, and cytotoxicity responses were measured. All 68 vaccinations were well tolerated without significant adverse effects. In three of the six patients treated at the two highest dose levels of vaccine, peptide-specific, T cell proliferative responses (n=3) and/or DTH responses (n=2) that lasted up to 5 months after vaccination were generated. However, the size of the trial is too small to allow conclusions to be drawn about the value of bcr-abl specific vaccination for the treatment of CML.

Trial size is a general problem for most peptide- and protein-based vaccine trials. Efficacy data of a treatment regimen are usually not supported by statistical data but refer to case reports. Another limitation of these trials is the reproducibility of the approach. As outlined before, tumour antigens in the form of peptides or proteins have to be processed by the APC after subcutaneous or intradermal administration and presented by the corresponding MHC complex to effector or helper lymphocytes. In addition, there are preliminary data indicating that the site of antigen injection in relation to the afferent lymphatics and draining lymph nodes is crucial for the induction of T and B cell immune responses [96]. According to the model proposed by Zinkernagel, the antigen administered has to reach the local lymph node in sufficient quantities and remain there for a certain time period to induce and not to delete MHC class I restricted T cell responses. If this model holds to be true, the current strategies for peptide and protein administration have to be regarded as insufficient, as they are not standardised in terms of the site of administration with regard to afferent lymphatics and consistency regarding the dosage reaching the local lymph node.

Moreover, data from Melief's group indicate that some peptide antigens can induce peripheral T cell tolerance instead of activation [80]. Immunisation with these particular peptides administered the same way and at the same doses as protective peptide vaccines [24, 81] caused systemic peripheral CTL tolerance; this demonstrates that the route and dose of peptide administration causing CTL priming cannot be generalised for all peptides delivered. More importantly, these findings indicate that clinical trials using synthetic peptide-based vaccines for the reinforcement of the host's immune response against tumours should be conducted with caution, because they might lead to an effect opposite to that intended, namely T cell tolerance, resulting in enhanced tumour growth rather than protective T cell immunity. In their study [80], ex vivo peptide-loaded DCs represented the only formulation tested that was capable of inducing CTL-mediated immunity leading to tumour protection, whereas all other tested modes of synthetic peptide delivery caused enhanced tumour growth.

Ex vivo loading of dendritic cells

DC-based cancer vaccines offer the potential for an effective, non-toxic, and outpatient-based approach to cancer therapy [6]. The evidence that DCs can mediate the in vivo rejection of established tumours in murine models and the relative ease with which it is possible to generate large numbers of DCs in vitro has made them feasible components in human cancer vaccine protocols [30]. While the majority of DC-based clinical trials has been in patients with melanoma, such trials could eventually serve as a basis for future DC-vaccine trials incorporat-

ing patients with more common malignancies such as breast, colon, lung and prostate cancer.

One of the earliest studies with peptide-pulsed DCs was performed by Mukherji and co-workers [53]. Three immunocompetent patients (judged by their ability to exhibit a DTH reaction to one or more microbial antigens) with advanced melanoma who were HLA-A1+ and whose tumours expressed the MAGE-1 gene were immunised with a vaccine consisting of a MAGE-1 nonapeptide (EADPTGHSY) pulsed onto DCs. Vaccination induced autologous melanoma-reactive and peptide-specific CTL responses. In particular, the frequency of circulating autologous melanoma-reactive CTL precursors was increased [35]. Although the number of patients included did not allow any conclusions on the efficacy of DC-based vaccines to be drawn, this clinical trial paved the way for other studies following it and demonstrated the feasibility of this approach in cancer patients.

Clinically relevant data on the efficacy of this approach were published recently by Nestle and co-workers [55], who reported on the vaccination of 16 melanoma patients with peptide (tyrosinase, gp100 and MART-1/Melan A) or tumour-lysate-pulsed DCs. Keyhole limpet haemocyanin was added as a CD4 helper antigen and immunological tracer molecule. Vaccination was well tolerated and no physical sign of autoimmunity was detected in any of the patients. DC vaccination induced a positive peptide-specific DTH response in 11 patients. Five of 16 patients demonstrated objective responses to the DC vaccine (two complete responses, three partial responses) with regression of metastases in various organs (skin, soft tissue, lung, and pancreas). A drawback of this study is the use of fetal calf serum (FCS) for cell culture and that peptides derived from this may have been loaded on DCs. Immune responses to peptides derived from the FCS might account to some degree for the positive DTH reactions observed.

A similar approach was chosen by Chakraborty and co-workers [13], who entered 17 patients (11 with metastatic disease) with malignant melanoma into a phase I vaccine trial with tumour-cell-lysate-loaded DCs. All patients were immunised intradermally with the vaccine in a phased dose escalation (10^5–10^7 cells/injection) monthly for 4 months. Out of the 17 patients, 13 completed all four immunisations showing no toxicity. One patient had a partial regression of a s.c. nodule. Nine patients had a DTH response at the vaccine site. The immunohistochemical analysis of vaccine-infiltrating lymphocyte (VIL) specimens revealed that the lymphocyte infiltrate consisted predominantly of $CD8^+$ cells. Antigen-specific $CD8^+$ T cell responses were detected in three of five $CD8^+$ VIL specimens. However, since no controls of lymphocyte specimens obtained from lesions prior to treatment or from patients not being treated were included, it is impossible to attribute either the infiltration of tissue by lymphocytes or the antigen-specific T cell response to the vaccine administered.

A series of trials in prostate cancer on DC-based immunotherapy was performed by Salgaller and co-workers [66, 79]. Their first phase I clinical trial assessed the administration of autologous DCs pulsed with an HLA-A0201-specific peptide of prostate-specific membrane antigen (PSMA) in 51 patients with metastatic, hormone-refractory prostate carcinoma. Patients received six infusions every 6 weeks of up to 6×10^6 monocyte-derived DCs. The DCs were generated from adherent precursors following 7-day treatment with GM-CSF and IL-4. Two high-affinity binding peptides, nine amino acid fragments designated PSM-P1 and -P2, from each end of the PSMA molecule, were studied. Five cohorts were established, with patients given (1) PSM-P1 only, (2) PSM-P2 only, (3) DC only, (4) DC + PSM-P1, or (5) DC + PSM-P2. During this safety study, subjects received escalating doses of 0.2, 2.0 or 20 μg/mL peptide. An average decrease in PSA was observed only in group 5, with partial responses in seven men. Based on these data, a second study was conducted which included 33 men with advanced hormone-refractory disease and 41 men with locally recurrent disease [66]. All participants received both PSMA-derived peptides and up to 2×10^7 DCs, as six intravenous infusions every 6 weeks. An overall response rate of 25–30% was achieved, including complete responders (CR) in the two groups that had not received prior vaccine therapy. Among hormone-refractory patients, there was a relation between the number of DCs administered and the duration of response, with all surviving subjects continuing to receive ongoing follow-up examinations.

Only limited data from pilot trials are available on the treatment of haematological malignancies by peptide- or protein-loaded DCs. Lim and Wood [46] recently published results from six patients with IgG myeloma treated with autologous DCs. These cells were pulsed with the autologous Id or KLH (as control) and re-infused i.v. back to the patients on three separate occasions. Immune responses to KLH and autologous Id were measured and clinical responses were monitored. The treatment was well tolerated without any side effects. All patients developed both B- and T cell responses to KLH, indicating the ability of the host immune system to mount immune responses to an antigen delivered by this vaccination strategy. Id-specific responses were also observed. Proliferative responses of peripheral blood mononuclear cells to Id were observed in five of the six patients after treatment. In two patients, the responses were associated with the production of IFN-γ. There were also increases in cytotoxic T cell precursor frequencies for Id-pulsed autologous targets in three patients. B cell responses characterised by the production of anti-Id IgM occurred in three and anti-Id IgG in four of the five evaluated patients. In a larger study by Titzer and co-workers [78], 11 patients with advanced MM were treated with $CD34^+$ stem-cell-derived DCs that were pulsed with Id peptides. Subsequently, the patients received three boost immunisations every other week with a combination of Id and GM-CSF (nine patients) or with Id peptide-pulsed DCs (two patients). Treatment was well tolerated with no side-effects. Immunological effects of the Id vaccination

were analysed by the monitoring of changes in anti-Id antibody titres and Id-specific T cell activity. After vaccination, an increase in anti-Id IgM or IgG serum titres was observed in three out of ten analysed patients. The Id-specific T cell response detected by ELISPOT was increased in four out of ten analysed patients after vaccination. In terms of clinical responses, one patient from the study by Lim presented with a modest (25%) but consistent drop in the serum Id level and one patient from the Titzer study [78] showed a decreased plasma cell infiltration in the bone marrow.

All of these studies demonstrate that the administration of the various DC vaccines is safe, with little or no toxicity. It is premature at this stage to attempt to draw conclusions on which DC-based approach to cancer treatment is best. The approaches chosen differ significantly in terms of number of DCs used; the published studies differ greatly, over a two-log concentration. Other key questions to be resolved include which development stage of DCs and which route of administration to use. These are important issues, as even DC-based vaccine strategies can induce tolerance [18].

As an alternative to dendritic cells, spontaneous lymphoblastoid cell lines (Sp-LCL) were recently tested as a source of APC for an autologous cancer vaccine [39]. Sp-LCL were easily obtained from latently EBV-infected cancer patients and were transfected with the point-mutated p21 ras (muRas) as a model tumour antigen. It was shown that these muRas-LCL can efficiently present tumour antigens to the immune system and induce antigen-specific cytotoxic T cell responses in vitro and in vivo [Kubuschok et al., unpublished results]. This strategy may therefore become an addition or an alternative to the repeated administration of ex vivo pulsed dendritic cells.

Broadening the target-specific immune response: polyepitope vaccines

Single-epitope-based approaches have the disadvantage that an HLA-restricted CTL can respond to one antigen only [9]. CTL responses specific for multiple antigens and restricted by multiple HLA alleles would clearly be desirable for cancer immunotherapy, given the variable expression of tumour antigens [10, 45] and MHC alleles [26] by tumours and their metastases. Targeting multiple antigens and MHC alleles might be achieved by use of multiple recombinant antigens or mixtures of synthetic peptide epitopes. The former would require complex recombinant vaccine antigen mixtures or constructs and is not very practicable. The latter is complicated by problems associated with equivalent peptide solubility, chemical modifications of certain amino acids, and inter-peptide interactions [21]. Polyepitope vaccines that contain multiple conjoined minimal HLA-restricted CTL epitopes, derived from a variety of tumour antigens, in one single recombinant construct offer a feasible alternative [50].

Suhrbier and co-workers [77] have demonstrated that despite the large number of epitopes restricted by the same allele, multiple epitopes within the vaccine construct were either recognised by epitope-specific CTL from melanoma patients and/or generated epitope-specific CTL in an HLA-A2 transgenic mouse system [50]. The polyepitope approach thus allows multiple antigens to be simultaneously targeted and should therefore increase a patient's spectrum of anti-tumour CTL responses. As more tumour antigens and target epitopes are identified, a panel of polyepitope vaccines might be envisaged, with each vaccine containing multiple epitopes restricted by one HLA allele. An appropriate HLA-matched mixture might then be delivered to cover all the HLA alleles expressed by any individual patient. The argument that the administration of multiple epitopes in the same vaccine could block each other in binding to the respective MHC and inhibit the generation of a broad immune response to the entire spectrum of epitopes administered seems to be incorrect [3]. Work done by Sherritt and co-workers [69] demonstrates that pre-existing CTL specific for one epitope in a vaccine did not influence the ability of that vaccine to prime and expand CTL responses specific for several new epitopes. This inability of dominant pre-existing CTL to significantly suppress the priming of new epitopes suggests that immunodomination or suppressive effects may be generally weak or subtle and/or may have little measurable influence when reasonably potent vaccines are used [67]. Only the presence of large numbers of pre-existing CTL populations inhibited vaccine-induced responses to epitopes restricted by the same MHC gene as the pre-existing CTL in some mouse strains. Importantly, this type of inhibition did not extend to epitopes restricted by other MHC genes. In some cases, the CTL response to peptides presented in non-identical MHC complexes was even enhanced; this might be explained by increased cross-presentation of antigen to APC. The polyepitope approach thus allows multiple antigens to be targeted simultaneously and should increase a patient's spectrum of anti-tumour CTL responses.

Where will the field go from here?

We are, to be honest, reluctant to comment on this question where progress in the vaccine area is rapid and diverse. New technologies related to the development of vaccines, their administration in patients and the monitoring of immune responses are emerging and will lead the field into new directions that cannot be envisaged today. However, we believe that the systematic analysis of a defined tumour-associated peptide/protein which might be administered by any route or concept described above will be the most rational way to follow. Monitoring the specific immune response in patients under treatment is the crucial step for the development of new vaccines. This can be achieved only if the target sequence is known and techniques are established to assess the dis-

tribution of the target sequence in tumour or normal tissues and subtle methods to analyse the cellular and humoral response after vaccination can be applied.

The first question of the tissue distribution of the target sequence used for immunisation is not trivial, since most tumour antigens identified are intracellular proteins and their ability to be presented by the respective MHC complex in sufficient quantity on the surface of the tumour cell remains unknown. Recently, Hoogenboom and co-workers [14] used a phage display library to isolate antibodies with specificity for a defined tumour antigen (MAGE-A1) presented by the respective HLA-A1 MHC molecules. The antibodies established recognised only the complex formed by the peptide and the MHC molecule and bound neither to the peptide or MHC molecule alone nor to the MHC molecule in combination with an irrelevant peptide. The widespread application of this technique will allow, for the first time, the systematic analysis of the distribution pattern of tumour antigen/MHC-complexes in tumour tissues and will answer the question about which antigen is expressed in sufficient numbers and specificity by the respective tumour cells.

The development of new monitoring techniques will – in our opinion – lead the way for the design of future vaccine trials. Most studies thus far have measured T cell responses to peptide/protein-specific vaccination by comparative assessment of pre- and post-vaccination peripheral blood cells in in-vitro T cell bulk cultures after stimulation with relevant and irrelevant peptides/proteins. The generated tumour-specific T cells were subsequently tested for their function by the assessment of proliferation, cytokine production or their ability for cytolysis. These assays are excellent for the qualitative assessment of T cell responses at certain time points in an individual's life or for non-parametric comparison of treatment outcomes in different patient groups, but cannot provide quantitative insight about the strength of the observed response [25]. Therefore, monitoring strategies have been implemented to evaluate the frequency of tumour-specific T cells including limiting dilution assay, ELISPOT assay and intracellular FACS analysis [38, 68, 75, 86]. However, these tests also rely on long- or at least short-term in vitro stimulation with cytokines or co-stimulatory factors, which may alter the functional phenotype of the cells, yielding results that on the one hand may overestimate the strength of the immune reaction of the subject in vivo. On the other hand, they underestimate the actual frequency of CTL precursors by not identifying T cells with a threshold for cytokine expression/proliferation above the stimulus applied [76]. Moreover, naive T cells, less responsive to peptide/protein-specific stimulation, might be missed [19].

In contrast, the use of soluble HLA/peptide complex tetramers, which has recently been proposed for the monitoring of vaccine trials, has the appeal of allowing direct enumeration of T cells specific for a particular antigen within relevant samples such as peripheral blood, lymph nodes and tumours [44]. The combination of HLA/peptide tetramer (tHLA) together with FACS analysis of surface and intracellular antigens establishes a direct and comprehensive strategy for the assessment of antigen-specific immune responses in vivo. Real-time reverse-transcription PCR for monitoring cytokine expression might be an important method for their additional characterisation.

The application of these techniques will be especially helpful to elucidate the reasons for the failure of a vaccination approach. For example, Lee and co-workers [44] could directly analyse the cytolytic function of tyrosinase-specific T cells in melanoma patients after cell sorting on the basis of tetramer staining. To their surprise, tetramer-positive cells sorted from a patient after specific peptide vaccination failed to lyse either target cells pulsed with the relevant peptide or HLA-matched tyrosinase-positive melanoma cell lines even after IL-2 activation. Thus, the absence of cytolytic activity observed in the peptide-specific T cells indicates that these cells were non-cytolytic in vivo. However, T cells from this patient were perfectly able to mount a specific and adequate immune response against EBV-pulsed targets, indicating a T cell dysfunction restricted to the tyrosinase-specific population.

Clonal deletion, exhaustion or senescence have been implicated as possible reasons for the induction of systemic, epitope-specific immune tolerance [20, 43, 84]. By the tetramer technology described, T cells can be readily identified after vaccination indicating that deletion of tumour-reactive T cells may not be as significant in humans as suggested by preclinical models [43, 52]. This notion is supported by another study where the frequency and activation status of melan-A specific lymphocytes in the peripheral blood of melanoma patients and normal controls was analysed by a modified tetramer-staining protocol. The results of these phenotyping experiments were then compared with assays of CTL function ex vivo. In terms of quantification, it is important to note that circulating CTL specific for melan-A were detectable ex vivo in 13 of 26 patients with malignant melanoma and 5 of 10 healthy controls, but even the highest tetramer frequency seen in this patient series (0.3% of CD8 cells) was relatively low compared to those seen in acute viral infections [41, 52]. In some patients, melan-A tetramer positive cells were readily detected, but there was no phenotypic evidence that these cells had ever responded to antigen. This phenomenon was observed even in tumour-infiltrated lymph nodes (TILN), in which melan-A-expressing tumour cells were effectively adjacent to melan-A specific CTL precursors. These data suggest that many melan-A-specific CTL that appear anergic ex vivo may in fact be naive or unprimed.

As indicated in these studies, inadequate immune responses in patients with cancer and other chronic illnesses might be due to a quantitative problem, that is, the number of tumour-specific T cells is too low, or, else, by a more qualitative problem, characterised by inadequate priming, a decreased T cell receptor signalling capacity or circulating immune-suppressive cytokines [91,

95]. These questions have to be addressed systematically in future clinical trials so that one can learn how T cells can be activated optimally in vivo against pre-defined tumour antigens; this then, in turn, will lead us to a new generation of vaccine trials.

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