Idiotypic vaccination for B-cell malignancies as a model for therapeutic cancer vaccines: from prototype protein to second generation vaccines

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Background and Objectives. Cancer vaccines are aimed at inducing tumor-specific immunity by immunizing patients with tumor cells or their antigenic components, known as tumor-associated antigens (TAA). Antigens which are either mutated or selectively or abundantly expressed in malignant, but not in normal, cells are considered as TAA. Each patient's B-cell malignancy is usually derived from a single expanded B-cell clone, which expresses an immunoglobulin (Ig) with a unique idiotype (Id, variable regions of Ig). Therefore, Id can be regarded as a TAA and a potential target in clinical vaccination approaches. Although use of tumor-derived Id as an immunogen to elicit antitumor immunity against B-cell malignancies is an attractive idea, the broader use of idiotypic vaccines has been hampered by the fact that autologous Id is not only a weakly immunogenic, self antigen, but is also patient-specific so that the vaccine must be individually prepared for each patient. In this review we will first summarize the latest data from the clinical tests of experimental idiotypic vaccines and discuss issues relevant to the clinical application of cancer vaccines in general; we will then critically review new trends and achievements in the development of the *second generation* vaccine formulations.

Evidence and information sources. The authors of the present review are currently working in the field of B-cell tumor immunotherapy and have contributed original papers to peer-reviewed journals. The material analyzed in the present review includes articles and abstracts published in journals covered by the Science Citation Index and Medline.

State of Art. The results from a number of experimental models and clinical trials have demonstrated that vaccination with tumor-derived Id can induce immune responses directed against the tumor. Idiotypic vaccines can be divided into two types, although both are at the experimental stage: traditional and second generation, based on the methods of production and vaccine delivery. Second generation vaccines utilizing genetically engineered protein and DNA formulations have, for the first time, opened up the possibility of streamlining production of simpler and effective custom-made idiotypic vaccines. The use of various adjuvants and exogenous carriers is being replaced by more potent genetic carriers which target Id and various co-stimulatory molecules to professional antigen presenting cells (APC), particularly dendritic cells (DC).

Perspectives. Id is the only widely accepted tumor marker and is a promising therapeutic target for immunotherapy of B-cell malignancies. It has been unequivocally established that Id vaccination of patients with follicular lymphoma administered when patients have minimal residual disease, has antitumor effect and potential to improve the clinical outcome. Consequently, the applicability of Id vaccines for other B-cell malignancies such as chronic lymphocytic leukemia, mantle cell lymphoma and multiple myeloma needs to be tested. Idiotypic vaccines should be tailored to target preferentially various subsets of immune cells, such as DCs, which would up take and properly process and present Id, activating both arms of the immune system, humoral and cellular. Moreover, the vaccine should induce the production of a milieu of inflammatory cytokines and lymphokines at the delivery site to elicit a T helper type 1 (Th1) immune response. Components of the inflammatory response can be used to target DCs *in vivo*, activating the so-called *danger* signal for circumventing the poor immunogenicity of self-tumor antigens. For example, chemotactic factors of innate immunity are able to deliver Id to APC and render this otherwise non-immunogenic antigen immunogenic. The strategies developed for Id vaccines can be used as a general strategy for eliciting T-cell immunity to other weakly immunogenic, clinically relevant self-tumor antigens.
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It is becoming clear that an effective antitumor immunity depends on activation of both arms of the immune system, humoral and cellular. In particular, the importance of CD8+ T-cells in inhibition of tumor growth and eradication of cancer cells has been often emphasized.[1,2] In addition, CD4+ T-cells are required for generation and main-

tenance of potent antitumor immunity.[3] Since most T-cell tumor-associated antigens (TAA) identified so far are self-antigens,[4] the ability to generate potent T-cell-mediated responses against self-antigens is a prerequisite for the validation of cancer vaccines in humans. There are no widely accepted TAA for B-cell malignancies except B-cell-derived immunoglobulin, a clonal marker for each patient's tumor. The specific antigenic determinants of immunoglobulin (Ig) variable (V) regions, termed idiotype (Id), are unique and produced by a single B-cell clone. Since the first demonstration by Sirisinha, Eisen and Lynch that syngeneic tumor-derived Ig could induce idiotype-specific antibody production and subsequently anti-MOPC-315 tumor response, Id vaccination remains an attractive approach.[5] The idea is further supported by numerous successful experiments conducted both in animal models of B-cell malignancies and in patients with B-cell lymphoma. However, unlike other TAA, a wider utilization of idiotypic vaccines is hampered by the fact that they have to be custom-made for each patient. However, the second generation of more effective idiotypic vaccines, which are simpler to produce and more potent, are being explored extensively, primarily in animal models. It is practically feasible and relatively simple to generate artificial recombinant molecules which are much smaller in size and retain all the unique features of parental tumor-derived Ig, not shared with other molecules. For example, unique fragments of Ig can be cloned from an individual malignant B-cell into a so-called single chain antibody (scFv), consisting solely of VH and VL fragments linked together in frame with a short peptide linker sequence. Although fragile conformation of the scFv can be disrupted by cross-linking with adjuvants, alternative approaches such as genetic fusion to various carriers, which bear T-helper epitopes and xenogeneic immunogenic fragments from human Ig Fc,[6] pathogens and toxins[7] have been successfully used to improve immunogenicity of scFv-based vaccines. Moreover, effective antigen-specific immunity can be elicited by targeting antigens to antigen-presenting cells (APC) via ligands for cell surface receptors such as mannose receptor,[8] FcR[9] and DEC205.[10] Recently, we have proposed a novel strategy to enable more specific attraction and targeting of subsets of APC, particularly immature dendritic cells (DCs), utilizing chemokine receptors. We demonstrated that both arms of immunity, cellular and humoral, can be induced by vaccination with fusion proteins consisting of scFv and chemokine.[11,12]

Idiotypic vaccination for B-cell malignancies with prototype protein vaccines: results and lessons from clinical trials

Follicular lymphomas (FL) are tumors of germinal center B-lymphocytes.[13] The FL cells are relatively well-differentiated B-cells, corresponding to the resting, Ag-responsive B-lymphocytes with a clonal origin, which express unique Ig variable regions, a tumor antigen/marker.[14] Surface expression of a clear tumor-specific antigen associated with a relatively indolent course of the disease provides a favorable clinical setting for idiotypic vaccination. However, the vaccine utilizes a self-antigen naturally protected by tolerance mechanisms as an immunogen to induce anti-idiotypic, and consequently antitumor immunity. Moreover, prior to vaccination, patients are treated with several cycles of chemotherapy which may affect their immunocompetence. Therefore, the first basic question is whether it is even possible to immunize these patients against a weakly immunogenic self-protein such as Id. The second question is whether immunization can produce clinical benefit. Although earlier clinical studies demonstrated that Id vaccination elicits specific anti-Id antibodies and some cellular responses which correlated with improved survival of patients with B-cell lymphoma,[15,16] the direct role of tumor-specific T-cells, particularly CD8+ cells, has been demonstrated only recently.[17] For example, autologous tumor-specific cytotoxic CD8+ T-cells as well as antigen-specific helper CD4+ T-cells were detected in 19 out of 20 previously untreated patients with follicular lymphoma (FL) in chemotherapy-induced first complete remission (CR), treated with a series of five monthly vaccinations with autologous Ig protein conjugated to an exogenous carrier, keyhole limpet hemocyanine (KLH), together with local subcutaneous administration of granulocyte-macrophage colony-stimulating factor (GM-CSF). Evidence of antitumor immunity correlated with eradication of minimal residual disease (MRD). At diagnosis and after chemotherapy, despite being in CR, eleven of 20 patients had tumor cells with unique *bcl-2* rearrangements at the major breakpoint region (MBR) detectable in their blood by polymerase chain reaction (PCR). However, eight of the eleven (73%) converted to sustained molecular remissions following vaccination. These results provide the definitive and affirmative answer to the first question of whether it is possible to immunize against a self-tumor antigen, whereas the second question about the clinical benefit could not be
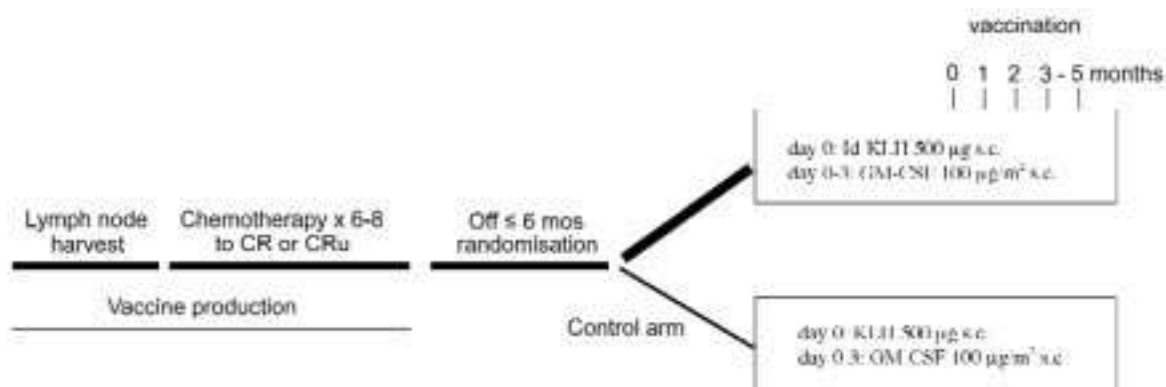
Figure 1. Study design for the phase III clinical trial of Id-KLH vaccine in follicular lymphoma. Patients with a CR or CRu after uniform chemotherapy are randomized in a 2:1 ratio. After a 6-month interval to allow for immune reconstitution, patients are injected with five rounds of Id-KLH + GM-CSF (test arm) or KLH + GM-CSF (control arm) vaccine.

answered by a single arm study without a concurrent control group of patients, and it is currently being addressed in a randomized, controlled phase III trial sponsored by the National Cancer Institute (Figure 1).

A major goal in idiotypic vaccination for FL is to streamline the production of these individualized vaccines while maintaining or improving their ability to generate T-cell-mediated responses and antitumor effects. A novel formulation of Id vaccine in which KLH is replaced by a more uniform liposomal carrier containing recombinant human IL-2 has recently been tested in a pilot clinical trial in FL patients in CR or PR after uniform chemotherapy. Approximately 6 months after chemotherapy, patients received 5 doses of the liposomal Id/IL-2 vaccine subcutaneously at monthly intervals. Preliminary results show that this vaccine formulation is well tolerated and immunogenic in all 9 patients evaluated.[18]

Overall, as summarized in Table 1, meaningful immunologic responses and antitumor effects have been reported in FL patients using different formulations of Id vaccine. However, the idiotypic vaccination in B-cell tumors other than FL is less advanced. In multiple myeloma (MM), the vigorous Id-specific immune responses reported in lymphoma have not been detected yet (Table 2). This may be explained by immunosuppression such as functional defects observed in peripheral blood DC,[19-21] and/or large burden of circulating MM-derived Id, which could neutralize anti-Id humoral responses. Unlike lymphomas, Id produced by myeloma cells is mostly secreted into the serum

and the malignant plasma cells express very little surface Id as a potential target for anti-Id humoral responses and ADCC. However, it has recently been shown that MM patients can mount T-cell responses to tumor idiotype, for example, autologous monocyte-derived DC pulsed with Id protein induced cytotoxic T lymphocytes (CTL) that are specific for the autologous primary myeloma cells and secretion of Th1 cytokines *in vitro*.[22,23] Therefore, these data support possibility and importance of induction of antitumor cellular responses in patients with MM treated with Id-vaccine.

Id vaccines for aggressive lymphomas have just begun to be tested. This subgroup of lymphomas display less favorable features, such as lower levels of surface-expressed Id and a more rapid clinical course. However, preliminary data from a phase II study demonstrate that patients with different subtypes of aggressive lymphoma in their first chemotherapy-induced CR, vaccinated with recombinant Id-KLH and GM-CSF developed tumor-specific immune responses, although almost exclusively humoral.[24]

Dendritic cells, a major regulator of immune responses, have been shown to effectively process and present pulsed tumor antigens and elicit potent CTL and antitumor responses both in animal models and clinical trials.[25,26] Pilot studies have been initiated also for patients with FL[27,28] and MM[29,30] by vaccinating patients mostly at the MRD state with autologous DC pulsed with tumor-derived Id, alone or coupled with KLH. DC vaccines were well tolerated without significant side effects and elicited anti-KLH humoral responses. Importantly, in FL the

Table 1. Summary of recent Id-vaccine clinical trials in follicular lymphoma.

Treatment	Clinical setting	Immune responses	Antitumor effect	References
Id-KLH + GM-CSF	1st clinical complete remission	19/20 tumor-specific T-cell cytokine release 6/6 tumor-specific CTL 15/20 Id-specific antibodies	8/11 molecular remissions	(17)
Id-KLH	residual disease; clinical complete remission	17/41 Id-specific antibodies 7/41 Id-specific T cell proliferation 11/16 tumor-specific CTLp ↑	2/20 CR	(15) (16) (96)
Id-KLH or Id-KLH + GM-CSF	residual disease; clinical complete remission	8/9 Id-specific antibodies	2/4CR 3/5 molecular remissions	(97)
Id-pulsed DCs	residual disease	8/10 Id-specific T-cell proliferation 2/10 Id-specific antibodies	2/10 CR, 1/10 PR 1 molecular remission	(28) (27)
Id- or Id-KLH-pulsed DCs	1st remission	6/18 Id-specific T-cell proliferation 5/18 Id-specific antibodies	4/18 CR	(27)
Id-pulsed DCs →Id-KLH	1st remission	12/17 T-cell responses n.o.s 12/17 Id-specific antibodies	n.e.	(98)

CR, complete response; PR, partial response; CTLp, cytotoxic T lymphocytes precursor frequency; n.e., not evaluated; n.o.s, not otherwise specified; → followed by.

Table 2. Summary of recent Id-vaccine clinical trials in multiple myeloma.

Treatment	Clinical setting	Immune responses	Antitumor effect	References
Id-KLH + GM-CSF or IL-2	1st remission after HDCT and PBSCT	2/11 Id-specific T-cell proliferation 8/10 Id-specific positive skin test	0/12	(99)
Id-KLH + GM-CSF	remission after HDCT and PBSCT	6/8 Id specific T-cell responses n.o.s	n.e.	(100)
Id-KLH + GM-CSF	1st remission after HDCT and PBSCT	4/5 Id-specific T-cell cytokine release 1/5 Id-specific antibodies	2/11 CR	(101) (102)
Id + GM-CSF	IgG MM	1/5 Id-specific T-cell proliferation 5/5 Id-specific T-cell cytokine release 5/5 Id-specific antibodies	1/5 decrease M protein	(103)
Id-pulsed DCs → d-KLH	1st remission after HDCT and PBSCT	2/12 Id-specific T-cell proliferation	1/12 decrease M protein	(29)
Id-pulsed DCs →Id-KLH + GM-CSF	remission after HDCT and PBSCT	2/10 Id-specific T-cell proliferation 1/10 Id-specific CTL	3/10 decrease M protein	(104)
Id-pulsed DCs	remission after HDCT and PBSCT	Id-specific T cell proliferation in responding pts	3/17 CR, 2/17 PR	(105)
Id-pulsed DCs	remission after HDCT and PBSCT	4/5 Id-specific T-cell proliferation 2/5 Id-specific T-cell cytokine release 5/5 Id-specific antibodies	1/5 decrease M protein	(106)
Id- or Id-KLH pulsed DCs →Id-KLH	remission after HDCT and PBSCT	4/26 Id-specific T-cell proliferation	2/21 CR 8/21 decrease M protein	(30)
Id-KLH-pulsed DCs	IgG MM	5/6 Id-specific T-cell proliferation 2/6 Id-specific T-cell cytokine release 3/6 Id-specific CTLp ↑ 4/5 Id-specific antibodies	1/6 decrease M protein	(32)
Id-pulsed DCs →Id +GM-CSF	Advanced pts	3/10 Id-specific antibodies 4/10 Id-specific T-cell cytokine release	1/10 decrease plasma cell infiltration in bone marrow	(107)
Id-pulsed DCs	Advanced pts	n.e.	6/42 decrease M protein	(108)
Id-KLH-pulsed DCs	Advanced pt	Id-specific T-cell proliferation Id-specific T-cell cytokine release Id-specific CTL Id-specific antibodies	decrease M protein	(109)
Id-KLH-pulsed DCs + GM-CSF	Advanced pts	2/2 Id-specific T-cell proliferation 2/2 Id-specific T-cell cytokine release 1/2 Id-specific antibodies	0/2	(110)

HDCT, high dose chemotherapy; PBSCT, peripheral blood stem cell transplantation; CTLp, cytotoxic T lymphocytes precursor frequency; CR, complete response; PR, partial response; n.o.s, not otherwise specified; →followed by.

majority of vaccinated patients raised various levels of anti-Id humoral and/or cellular responses and some had durable tumor regressions.[27] Interestingly, unlike immunotherapy with idiotypic protein, DC-based vaccine elicited Id-specific antibodies in only about one third of the patients treated, and this was not improved by the booster immunization with Ig-KLH protein vaccine.[27] Moreover, the potency of DC + Id vaccine in this study[27] was only slightly improved by incorporation of KLH, though it was essential in the preclinical experiments.[31] Although it is difficult to compare results from this trial[27] with data reported by others due to differences in the stage of measurable disease at the time of vaccination, treatment of patients in CR elicited comparable levels of disease-free survival with Id-KLH + GM-CSF protein vaccine.[17] In MM, DC pulsed with Id-KLH also elicited potentially useful immunologic responses such as Id-specific T cell proliferation detected from 15%[30] to as many as 83% patients.[32] In the latter study, the response was associated with production of IFNγ in 2 out of 6 patients and an increase in CTL precursor frequency in 3 patients. However, a wider use of DC-based vaccines may not be feasible yet for a variety of reasons, as the DC field itself is under development and production of GMP grade autologous DCs for each patient is laborious and complex. Moreover, it is not completely clear which are the best DC subsets to use to induce optimal immune responses, since some DC subsets may induce tolerance rather than activation.[33]

Id vaccination has been investigated so far mostly in the autologous setting. However, preliminary results suggest that this immunotherapeutic maneuver may have a therapeutic effect in the setting of allogeneic stem cell transplantation. In this field, exploitation of the potential antitumor effect of stem cell grafts relies on strategies for enhancing graft-versus-tumor effects without aggravating graft-versus-host disease (GVHD). One such strategy would be to selectively target an immune response against a defined TAA. The exquisite tumor specificity of Id makes it an ideal candidate antigen for donor immunization. Transfer of donor Id-specific T-cell immunity was detected at the time of allografting of Id-immune marrow.[34] Release of high levels of T helper type 1 (Th1) cytokines in an MHC-restricted fashion in response to stimulation with recipients' myeloma cells was detected in two donors immunized with Id proteins obtained from their recipients.[22] Thus, despite the limited number of patients tested, several important conclusions can be drawn which support the

proof of a principle: first, it is possible to immunize healthy donors with a patient's Id; second, it is possible to transfer Id-specific T-cell immunity directly from an immunized donor to a recipient; third, the donor-derived T-cell responses are not blocked by the presence of circulating Id-antigen or by GVHD prophylaxis-induced immunosuppression.[34] These results set the stage for an ongoing phase I/II clinical trial at the *National Cancer Institute* of donor immunization prior to allogeneic stem cell transplantation following a non-myeloablative conditioning regimen for MM. In the same clinical setting, to avoid any potential complications associated with immunization of healthy donors with tumor-derived products, *in vitro* priming of donor T-cells using Id-pulsed DCs may provide an alternative to *in vivo* donor immunization and allow the transfer of highly enriched populations of Id-specific T-cells from donor to recipient (tumor-specific donor lymphocyte infusion).

There are several lessons that can be drawn from these Id-vaccine clinical trials which can potentially serve as a model for cancer vaccine trial design in general. First, it is important to optimize the interpretation of immunologic, molecular, or clinical results by selecting a study population which is homogeneous with respect to underlying disease histology and the amount and type of prior therapy.[17,18] Second, observations in both animal models and clinical studies suggest that vaccines may be more effective in the setting of MRD.[35] Third, the use of vaccines targeting known tumor antigen(s) such as Id may have the methodological advantage over tumor cell-based vaccines of allowing the systematic analysis of vaccine-induced immunity in relation to clinical responses.[36] It is critical to evaluate immunologic and clinical responses systematically by the uniform application of appropriate endpoints across the entire study population. Identification and monitoring of immune responses is one of the major challenges in the setting of antitumor vaccination. For example, the immunologic monitoring of CD8^{+} T-cell responses against autologous tumor targets, when feasible, has several advantages over the use of surrogate targets and should be established as a standard for future vaccine studies. Anti-Id antibodies have been shown to be important,[37] but not essential for antitumor effects of the vaccine. Bendandi *et al.* reported that at least three patients achieved molecular remissions without a detectable antibody response, thus suggesting that a humoral response may not be required.[17] The ability of patient's T lymphocytes to lyse autologous FL cells would suggest

that the tumor antigen (i.e. Id) is naturally processed and presented by FL cells.[17] However, vaccinations with idiotype may lead to induction of CTL responses to other TAA expressed by malignant cells (antigen spreading) as a result of cross priming. Characterization of Id vaccine-induced responses, particularly T-cell responses, is a challenging task that can be accomplished using various manipulations to enhance activity of APC *in vitro* and using more sensitive methods such as intracellular cytokine staining and ELISPOT assay.[38] As regards the evaluation of antitumor effects, using a sensitive tumor marker or molecular probe to measure reduction or disappearance of subclinical tumor mass can be an effective alternative to clinical tumor response criteria. For example, the majority of FL patients carry a t(14,18) translocation of *bcl-2* oncogene to the immunoglobulin heavy chain joining region (IgH). Therefore, PCR-based evaluation of *bcl-2* specific major breakpoint cluster region (MBR) in the extracellular serum DNA to monitor MRD appears to be an ideal surrogate marker for clinical activity and vaccine efficacy.[39]

Models for murine B-cell malignancy to validate human idiotypic vaccines

Most murine tumor models use artificial tumor antigen, often immunogenic by itself. In contrast, syngeneic murine lymphomas and myelomas express clone-specific tumor Id, which is usually a non-immunogenic self-antigen. It is postulated that immunity elicited in these models using Id-vaccines would closely predict a future clinical outcome in humans. In concordance, data from recent clinical Id-vaccine trials also supported the reliability of these murine models. In fact, the ability of GM-CSF to enhance potency of Ig-KLH and activate the T-cell arm of the immune response,[40] the efficacy of Id/IL-2 liposomal vaccine formulation[41] and the possibility of transferring Id-specific immunity from immunized allogeneic marrow donor to recipients[42] were predicted from preclinical studies in syngeneic murine B-cell tumor models. Therefore, the vaccine's initial design and its validation for future clinical trials are vigorously tested in murine models of B-cell malignancies of various genetic backgrounds. There are two ways to test vaccine formulation in mice - one way is to induce immunity prior to challenge with tumor cells (protection experiment), the other way is to treat non-immune naïve mice bearing tumor cells with vaccine (therapy/eradication experiment). Although the importance of therapy to eradicate an established tumor is often over emphasized since

it resembles the clinical situation, it is usually a challenging task by itself even if tumor cells grow slower in mice. For example, we reported for the first time that Id-vaccine can eradicate a slower growing established A20 lymphoma and induce antitumor immunity.[12] Furthermore, an established tumor may be eradicated by activation of such cells as NK without eliciting antitumor immunity. However, the majority of Id vaccine reports are obtained from tumor protection experiments. Several different transplantable murine B-cell tumors are used successfully to model immunotherapy strategies against human B-cell malignancies. Most murine B-cell tumors are usually weakly immunogenic and lethal in syngeneic mice, and they closely mimic different types of human B-cell malignancies from mature, non-secreting B-cell lymphomas to Ig-secreting myelomas. These include BCL1 and A31 B-cell splenic lymphomas, both of which express surface IgM, 5T33 myeloma, which predominantly secretes IgG2b, and MOPC-315 plasmacytoma, which secretes IgA.[5,43,44,45] Our experience is mostly with the A20 and 38C-13 lymphomas. A20 is a BALB/c B-cell lymphoma line derived from a spontaneous reticulum cell neoplasm, extensively used in various tumor studies. These cells are IgG+, Ig+ (with polyvalent anti-Ig), Ia+, Fc+, IgM–, IgA–, and complement receptor negative. A20 cells are tumorigenic in mice and have a generation time of 18 hours. When grown in Click's medium, these cells originally expressed very little surface IgG. T-cell factors and mitogens can induce these tumor cells to secrete IgG extracellularly.[46] 38C-13 is a carcinogen-induced lymphoid tumor, originally isolated from a T-cell-depleted mouse of the C3H/eB strain. 38C-13 cells have features of the transformed counterpart of small B lymphocytes and grow well in culture using RPMI with 10% FCS and 0.05 mM 2-ME. 38C-13 cells express surface IgMk (little to no secreted IgM), are Thy-1-, Ia-, Fc+, IgA–, and are complement receptor negative.[47]

Numerous strategies have been reported to reverse the weak immunogenicity of murine B-cell tumor-derived Ig and elicit anti-Id antibody (Ab) responses. High titers of anti-Id antibodies were elicited by Id immunizations together with a variety of immunologic adjuvants such as SAF, CFA, QS-21 or KLH.[48,49] Although induction of cell-mediated responses is considered to be more important for immunotherapy, some B-cell tumors, particularly those which express predominantly surface-bound Ig, can be eradicated by the induction of humoral responses against tumor Id, with no detectable role of cellular immunity. For example,

it was often observed that anti-Id antibodies could protect mice from the challenge with B-cell lymphomas expressing surface Ig.[43,50] In contrast, anti-tumor protection from other B-cell malignancies, such as A20, 5T33 and JLµs, which often preferentially excrete their Ig, was dependent mostly on induction of cellular Id-mediated anti-tumor responses.[7,12,51,52] However, there are only handful of examples which demonstrate a direct involvement of T-cells in Id-specific immunity, and traditional methods for assessment of cellular responses in Id vaccinated mice are inadequate because of low sensitivity.[53] T-cell epitopes have been found on only a few tumor-derived Id, such as CD4 epitopes in λ-chain of MOPC-315 tumor.[54] Moreover, no Id-specific CD8+-specific CTL epitopes have been identified, and no direct killing of tumor cells by CTL *in vitro* has been reported yet, although ample indirect evidence suggests that Id-vaccinations activated CD8+ effector cells.[11,12,53] Recently, use of more sensitive ELISPOT assay revealed that mice immunized with syngeneic 38C-13 tumor-derived Ig-KLH emulsified in CFA showed a significant increase in the frequency of Id-specific IFNγ-secreting T cells.[53]

Second generation of idiotypic vaccines: strategies and issues

Although proven effective in experimental models and in clinical trials, the traditional Id vaccine approach, which is based on the generation and culture of heterohybridomas, is complicated in view of clinical application by the need for large amounts of custom-made and individually tailored proteins that must be prepared and certified for each case within an appropriate time scale. Despite the fact that the prototypic Ig-KLH vaccine generates superb anti-Id Abs, this formulation does not induce efficient cell-mediated responses and protective antitumor immunity in every B-cell tumor model, as is the case for A20 lymphoma (Figure 2), which presumably is not affected by inhibitory effects of anti-Id Abs and ADCC (*Biragyn, unpublished data*). Therefore, new formulations of Id vaccines should be designed to streamline the production of these individualized vaccines and to optimally recruit appropriate effector cellular functions to elicit potent antitumor immunity. The second generation of Id-vaccines should induce both humoral and cellular arms of immune responses. Furthermore, a simpler vaccine production strategy would also enable to modify formulations at short notice to accommodate future treatments for vaccine-induced escape or mutant variants,
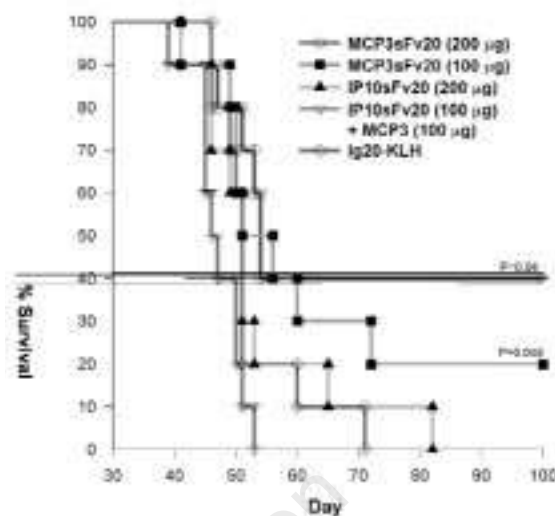

Figure 2. Proinflammatory chemokine-scFv fusion, but not Ig-KLH, protects mice from lethal syngeneic A20 tumor challenge. Ten syngeneic Balb/C mice per group were immunized intraperitoneally twice at 2 week intervals with 100-200 µg IP-10 or MCP-3 fused to scFv derived from A20 B cell lymphoma-specific Ig (Ig20), or with 50 µg Ig20-KLH. Two weeks after the last immunization, mice were challenged intraperitoneally with a lethal dose of A20 tumor cells. The data demonstrate that mice immunized with fusion proteins consisting of pro-inflammatory chemokine MCP-3 fused to scFv20 showed a dose-dependent antitumor protection. In contrast, no protection was observed when mice were immunized with prototypic vaccine Ig20-KLH. The chemokine vaccine required physical linkage between MCP-3 and scFv, since co-administration of unlinked active MCP-3 and non-immunogenic scFv20 did not elicit any immunity (IP10sFv20+MCP3), suggesting that the chemokine targeted Id-antigen to APC. *p*- values refer to comparison with Ig20-KLH.

observed in some murine B-cell lymphomas.[55]

The new generation of vaccines takes into account the fact that unique determinants of Ig are localized in two short regions designated as VH and VL. These fragments can be cloned from an individual malignant B-cell and expressed as scFv, which usually retains all the unique features of the parental tumor-derived Ig, not shared with other molecules.[56] This is a relatively simple and straightforward procedure, yet it is often limited by the extent of oligonucleotide primer mismatch. Recombinant scFv and its fusion proteins are produced successfully in almost every expression system including bacteria, yeast, plants and mammalian cells,[56,57] although various factors, such as proper protein folding and possible non-specific toxic effects on the host producer cells, may hamper a streamline production of the vaccine (*Biragyn, personal communication*). An alternative and much

simpler approach is currently being explored to avoid protein purification step, a task feasible for only well established laboratories and institutions. There has been an explosion of various Id-vaccine strategies since a pioneering report on the so-called *naked* DNA vaccination by Wolf and his colleagues, who expressed foreign genes *in vivo* by injection of myofiber cells with plasmid DNA.[58] Naked DNA vaccine requires only that the gene of interest is cloned under eukaryotic or viral regulatory elements (promoter) into an expression cassette, which is then either injected in solution via intramuscular or intradermal routes or delivered into the epidermis by particle-mediated bombardment of DNA-coated gold particles (gene gun approach).[59] Animal studies have shown that DNA vaccines can induce effective humoral and cellular immune responses to a wide variety of viral, bacterial and neoplastic antigens.[60] Despite earlier reports which suggested preferential induction of humoral vs. cellular responses depending on the route of immunization, it is becoming clear that optimal dose and immunization schedule more likely affect efficacy and immunogenicity of DNA vaccines.[61,62] Moreover, the amounts of DNA required vary drastically between vaccines delivered via different routes of immunization, as intramuscular immunization requires much higher amounts of DNA (50-200 μg), than gene gun vaccines (2 μg), which may in turn affect immunogenicity of the vaccine, for example, by immunostimulatory CpG motifs present on bacterial plasmids.[63,64]

The first anti-idiotypic responses using DNA immunization were induced in mice by direct injection of human V genes from B cell tumors.[65,66] However, scFv is weakly immunogenic in syngeneic mice, and it needs to be used together with a carrier protein or adjuvant to render it immunogenic. A traditional method such as cross-linking to KLH failed, possibly due to disruption of the fragile conformation of scFv.[11] Furthermore, DNA vaccinations alone could not reverse the weak immunogenicity of lymphoma-derived Id or scFv.[67] The immunogenicity of scFv was increased by immunizing mice with fusion proteins of scFv with various proinflammatory cytokines or their receptor binding domains, key players in adaptive immunity. Mice immunized with scFv fused with cytokines such as GM-CSF, IL-4 or IL-2 elicited high titers of anti-Id antibodies and showed comparable tumor protection.[6,68] The peptide sequence derived from human IL-1β, VQGEESNDK (163-171 amino acids) fused in tandem with a given immunogen maintains the immunostimulatory effect of the whole cytokine

and elicits an increased specific antibody response.[69,70] Thus, mice immunized with human IL-1β peptide fused with the lymphoma-derived scFv elicited anti-idiotypic antibody responses and protective antitumor immunity.[71] Alternatively, otherwise weakly immunogenic scFv was rendered immunogenic in two B-cell tumor models, 38C-13 and BCL1, by fusing it with foreign xenogeneic sequences, such as human Ig Fc region, although it is irrelevant to future human use.[6,72]

A growing number of reports support the hypothesis that activation of innate immunity through pattern recognition receptors of evolutionary distant pathogens is essential for initiation of adaptive immunity.[73,74] Moreover, optimal recognition of self-tumor antigens and induction of proinflammatory rather than tolerogenic responses may require activation of innate immunity by a *danger* signal.[75] Engagement of pattern recognition receptors induces up-regulation of CD80 and CD86 co-stimulatory molecules and production of various proinflammatory mediators, such as cytokines and chemokines, to enable more potent adaptive immune responses.[73,76-78] According to the *danger* model, vertebrates have evolved innate immune mechanisms, by which the immune system might distinguish dangerous, non-self antigens from non-dangerous, self antigens.[79,80] Bacterial cell-wall components, unmethylated DNA with CpG motifs, toxins, etc are major activators of innate immunity.[63,81] Consequently, an *alert* signal, fragment C of tetanus toxin (FrC), was successfully used to elicit Id-specific immunity.[7] It was reported in two murine B-cell tumor models, A31 lymphoma which expresses surface IgM and 5T33 myeloma which secretes IgG2b, that mice immunized with DNA construct expressing scFv fusion with FrC were protected from the challenge with syngeneic tumor.[7,44] The potency of the DNA vaccine was recently further improved by using xenogeneic self-aggregating protein from potato X virus. The resulting vaccine, a self-aggregated sFv fusion protein, elicited an effector CD4+ T-cell-dependent protective response superior to that elicited by scFv-FrC.[51] It is becoming apparent that effective antigen-specific immunity can be elicited by targeting antigens to antigen-presenting cells (APC) via ligands for cell surface receptors such as mannose receptor,[8] FcR[9] and DEC205,[10] and that this enables internalization and processing of the antigen. We have further developed this idea by utilizing chemokine receptors to target scFv to APC. The trafficking of DC is regulated by differential expression of heterotrimeric G-protein-coupled seven-transmem-

brane–domain chemokine receptors.[82] Immature DCs, which express high levels of endocytic receptors and exhibit potent antigen uptake capacity, express chemokine receptors such as CCR1, CCR2, CCR5, CCR6, CCR9 and CXCR4 to extravasate and enter peripheral sites.[83-85] Upon maturation of DC, expression of these receptors is down-regulated, while that of other receptors, such as CCR7 is up-regulated, enabling MIP3β (ELC) and SLC to recruit mature DC in lymph nodes.[84,86] Chemokines can be distinguished as inflammatory (inducible) or home-ostatic (constitutive), based on their pathophysiological activities.[87] Homeostatic chemokines are usually produced constitutively in discrete microenvironments and are involved in the maintenance of the physiological trafficking of immune cells.[87] In contrast, inflammatory chemokines are expressed during infection or tissue damage by resident and infiltrated leukocytes. Recently, it has been suggested that some antimicrobial peptides of innate immunity, such as human β-defensin 2, participate in adaptive immune responses by acting directly on immature DC via binding with CCR6.[88] Overall, defensins play a role in inflammation, wound repair, and regulation of specific immunity by inducing the expression of cytokines and chemokines, the production of histamine[89] and enhancing antibody responses.[90]

Therefore, we hypothesized that, perhaps, a general strategy for induction of effective adaptive immunity against weakly immunogenic tumor antigens may be to target *in vivo* the delivery of such antigens to receptors on professional APC such as DC. The proof of this concept has been reported recently using data obtained from two different B-cell lymphomas, 38C-13 and A20, which express surface IgM or secrete IgG2a, respectively.[11] Utilizing various chemokines and defensins, we were able to demonstrate that scFv acquired the ability to bind to chemokine receptors and induced chemotactic responses, both *in vitro* and *in vivo*, when it was fused with chemokine.[12] Moreover, mice immunized with scFv fused to chemokine (Figure 2) or defensin elicited anti-Id responses and protective antitumor immunity, and the vaccine did not require the use of any adjuvants (Figure 3). Our data also suggested that it was important that the vaccine targeted immature, but not mature DC. For example, constructs expressing scFv fusion proteins with SLC, a chemokine which binds to mature DC via CCR7, did not elicit any antitumor protection. In contrast, protective antitumor immunity was induced only in mice immunized with constructs expressing scFv fusion
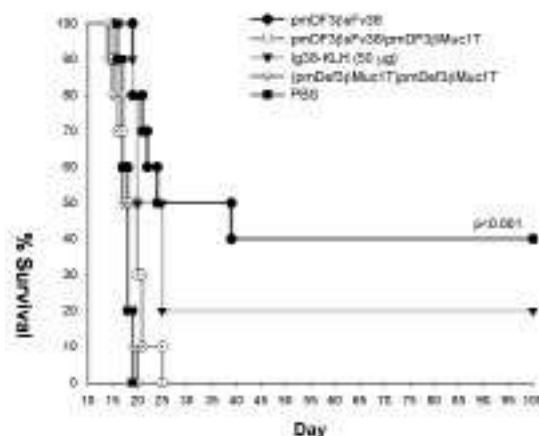

Figure 3. DNA vaccine fusion of scFv with innate immunity chemotactic factors such as β-defensin, which attracts immature DC, elicit potent antitumor immunity. Ten syngeneic C3H/NeN mice per group were immunized by gene gun with 1-2 μg plasmid DNA three times at 2 week intervals. Two weeks after the last immunization, mice were challenged intraperitoneally with 2×10³ 38c13 tumor cells and followed for survival. Mice immunized with fusion of murine defensin and scFv derived from 38c13 B-cell lymphoma-specific Ig (pmDef3βscFv38) elicited potent antitumor protection, comparable to the prototypic Ig38-KLH protein vaccine. No survival was observed in control mice immunized with PBS or DNA constructs encoding fusion of defensin to an irrelevant antigen (pmDef3βMUC1T). Co-administration of competing Def3βligand abrogates immune response, suggesting that scFv was required to be delivered via chemo-attractant receptor to elicit protective immunity (pmDef3βscFv38/pmDef3βMUC1T). *p* values refer to comparison with pmDef3βMUC1T.

with proinflammatory chemokines, such as MIP-3α or β-defensin 2, both ligands for CCR6 expressed on immature, but not mature, DC. While both humoral and cellular immune responses were required for rejection of the more aggressive 38C-13 tumor that expresses IgM primarily on its surface,[11,49] only cellular, but not humoral, immunity was needed for protection against A20 lymphoma, which largely secretes its idiotypic antigen.[12] Overall, protection was significant, and it was superior to that of the prototype Ig-KLH protein in both tumor models, particularly for the A20 tumor which can not be killed using Ig-KLH vaccine (Figure 2).[11] In fact, the breadth of this approach as a general strategy for vaccine development was also apparent from its ability to elicit for the first time effector CD4+ and CD8+ T-cell-dependent antitumor immunity and eradicate established A20 lym-

Table 3. Comparison of different Id vaccine formulations.

Formulation	Production time	Cost of production	Potency compared to Id-KLH + GM-CSF	Phase of clinical development	Ref.
Id-KLH + GM-CSF	3-6 months	Expensive	N.D.	III	(17)
Liposomal Id/IL-2	3-6 months	Expensive	>	I	(18)
Id-pulsed DCs	3-6 months	Expensive	N.A.	I/II	(27)
ScFv-chemokine DNA fusion	1-2 weeks	Cheap	≥	Preclinical	(11-12)

Cost of production is compared with traditional prototypic hybridoma-derived Id protein vaccine. Potency is in relationship to Ig-KLH vaccine as evaluated in syngeneic murine models of B-cell tumors. Abbreviations: N.A. = data not available; N.D = not applicable; > superior.

phoma.[11,12] The precise mechanism of the *carrier* activity of inflammatory chemokines and defensins remains to be elucidated. Delivery of chemokines to tumor cells, resulting in non-specific recruitment of effector cells, has been reported.[91] However, we favor an entirely novel mechanism of triggering anti-tumor immunity, namely, that the chemokine moiety targeted APC for efficient receptor-mediated uptake and processing of scFv, breaking tolerance. The targeted antigen would be efficiently endocytosed by DC, since CXC and CC- chemokine receptors are known to be internalized after binding to ligand.[92,93] In addition, we hypothesize that targeting with scFv fused to inflammatory chemokines induces activation of immature DC and production of a milieu of proinflammatory cytokines which directs adaptive immune responses. The most compelling data supporting this hypothesis are that mice immunized with control constructs expressing scFv fusion with a mutated form of MIP-3α, which carries a disrupted chemokine receptor binding site, and pro-defensin, the inactive form of defensin unable to chemo-attract immature DC via CCR6, were not protected. Similarly, no immunity was elicited when mice were immunized with DNA expressing a free unlinked mixture of scFv and chemo-attractant, suggesting that just cell infiltration to the site of vaccine without specific targeting was not sufficient to break unresponsiveness to scFv.

In summary, a successful antitumor immunity requires induction of both arms of the immune response, humoral and cellular. The extent and presence of T-cell epitopes on Id itself is still unclear, with a few exceptions such as CD4 epitope found on the light chain of Idλ2[315] MOPC-315 plasmacytoma.[94,95] Most idiotype protein and DNA vaccine formulations reported to date elicited exclusively antibody responses, with undetectable CD8+ T-cells.[53] Therefore, strategies which elicit enhanced protective antitumor immunity dependent upon effector CD4+ and CD8+ T-cells, for example co-administration of low doses of GM-CSF with Ig-KLH protein vaccine[40] and use of second generation vaccines based on the self-aggregating properties of potato X virus protein,[51] or on direct targeting of immature DC *in vivo* with fusion constructs with chemokines,[11,12] appear most promising for translation into clinical trials.

Conclusions

Substantial progress has been achieved in the development of cancer vaccines, particularly idiotypic vaccines. It is now clear that it is possible to elicit both humoral and cellular immune responses against a self- tumor antigen such as Id and induce antitumor immunity. These results have set the stage for a phase III clinical trial in FL already underway to answer the question of whether Id vaccines produce clinical benefit. Traditional formulations such as Ig-KLH + GM-CSF are becoming a standard against which new vaccine formulations for B-cell malignancies must be compared (Table 3). The second generation of Id vaccines take advantage of molecular cloning techniques and novel delivery systems, and tests in pre-clinical models demonstrate their superior potency over prototypic protein vaccine. The comparison, however, has yet to be performed in the clinical setting. Moreover, novel vaccine formulations are simpler to produce, particularly naked DNA vaccines, and they utilize fusions with various immunologic *danger* signals from xenogeneic foreign viral and bacterial antigens to activate DC –mediated primary immune responses to a weakly immunogenic Id. Methods which deliver/recruit *in vivo* Id-antigen directly to immature DC, such as via chemokine receptor engagement, may be an effective strategy for eliciting both CD4+ and CD8+ T-cell immune responses against self-tumor antigens.

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