Anti-Idiotypic Antibodies and "Tumor-Only" Antigens: An Update

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Abstract: The use of anti-idiotypic antibodies for cancer treatment continues to be a major field of investigation. One major challenge for tumor immunotherapy is the identification of antigens associated only or preferably with malignant cells. We summarize here some of the most recent preclinical and clinical advances using two targets that can be considered tumor-specific antigens: *N*-glycolyl (NeuGc)-gangliosides and the idiotype of B cell lymphomas. Recent developments with tumor-associated protein antigens and the role of anti-idiotypic antibodies in autoimmune diseases are also discussed.

Keywords: Anti-idiotypic antibodies, NeuGc, ganglioside, idiotype, B cell lymphoma.

INTRODUCTION

From the pioneer works by Oudin and Kunkel [1, 2], where the existence of individual antigenic determinants of the immunoglobulins (idiotopes) was demonstrated, to Jerne's Idiotypic Network Theory [3]; through Bona's "regulatory idiotope" [4] and Coutinho's "second generation" networks [5], the theory around anti-idiotypic antibodies has been extensively discussed and often poorly proved. In spite of this, the search for anti-idiotypic antibodies (or Ab2) that could serve as mimics of protein and non-protein antigens has been carried out along with the development of "classic" monoclonal antibodies (mAbs) directed against "nominal" antigens (Ab1).

The classification of anti-idiotypic antibodies is illustrated in Fig. (**1**). The antibody induced by a given antigen is represented by Ab1, which recognizes a defined region of the antigen (the epitope) through the two variable regions of the light (VL) and heavy (VH) chains that constitute the idiotype. The part of the antibody molecule directly involved in epitope recognition is operationally defined as the paratope. Since the antibodies themselves can also be antigens, antibodies directed against the Ab1 variable regions (anti-Ab1 or Ab2) are generally known as anti-idiotypic antibodies. However, there are different types of Ab2: (i) **Ab2α** recognizes an idiotope (i.e: an epitope on the idiotype) not structurally related to the paratope of the Ab1; (ii) in contrast, anti-idiotypic antibodies known as **Ab2β** bind to the paratope on Ab1 and mimic the original epitope recognized by the Ab1 on the nominal antigen, which means that when performing as antigens they induce an anti-Ab2 response (Ab3) similar to the Ab1 (called Ab1'), thus able to recognize the antigen; these **Ab2β** are said to carry the "internal image" of the antigen epitope; and (iii) **Ab2γ** recognizes an idiotope structurally associated to the paratope but not able to mimic the antigen epitope, therefore inducing Ab3 unable to recognize the antigen. The generation of anti-idiotypic antibodies against Ab2 (anti-Ab2 or Ab3) with the same specificity of the Ab1 (Ab1') can be impaired by the peripheral tolerance mechanisms of the organism even when the Ab2 carries the "internal image" of the antigen [6].

Anti-idiotypic antibodies have been widely used for cancer immunotherapy [7]. In this short review, we will discuss some of the most recent works published in two main fields: (i) the use of anti-idiotypic antibodies of the Ab2β type as antigens to mimic a particular kind of carbohydrate antigen (gangliosides) expressed on tumor cells; and (ii) idiotypic vaccines, using the idiotype of B cell lymphomas as antigens to induce anti-tumor idiotype-specific immune responses. Finally, we comment recent results on the mimic of tumor protein antigens and on the role of anti-idiotypic antibodies in preventing autoimmune diseases.

ANTI-IDIOTYPIC ANTIBODIES AND NON-PROTEIN ANTIGENS: THE GANGLIOSIDE CASE

The first evidences of the association between gangliosides, glycosphyngolipids with sialic acid residues in their structure, and cancer cells came from studies made in the seventies [8, 9]. The selection of gangliosides as potential targets for cancer therapy is still a current line of investigation [10].

Several well-characterized anti-idiotypic antibodies (Ab2β against anti-ganglioside Ab1) mimicking gangliosides have been tested as therapeutic molecules on some human cancers [11]. The most extensively studied is Bec2 mAb, which mimics the *N*-acetylated form of neuraminic acid (sialic acid) on ganglioside GD3 (NeuAc-GD3) [12]. This Ab2 has been tested in patients with tumors of neuroectodermal origin, as melanoma and small cell lung cancer (SCLC) [13]. However, a phase III clinical trial with Bec2/bacille Calmette-Guerin (BCG) in patients with SCLC failed to prolong overall survival, progression-free survival and quality of life. An antibody response against NeuAc-GD3 was observed only in one third of patients, but even within this group there was not a significant increase in survival [14].

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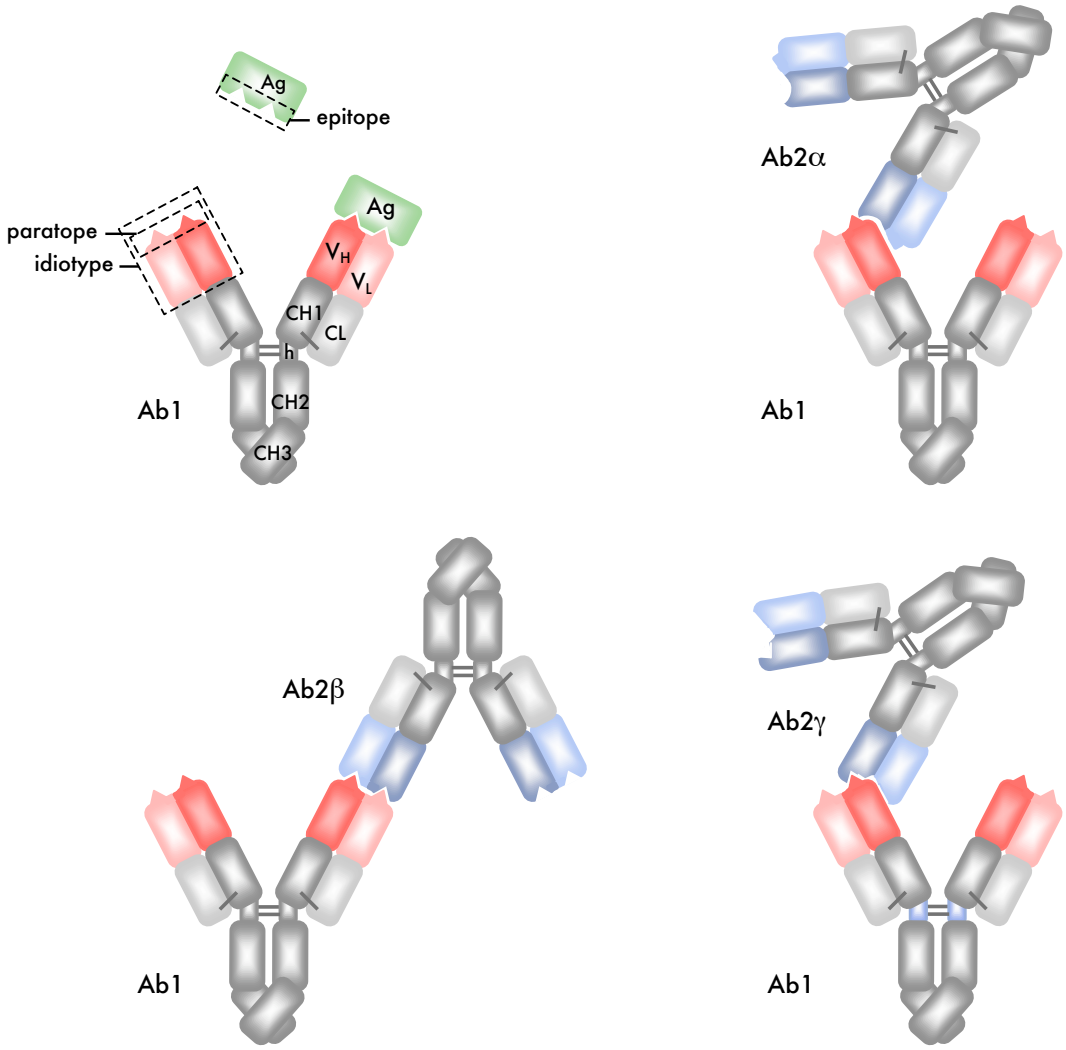

Fig. (1). Scheme of an antigen/antibody (Ab1, lgG class), interaction and the three possible anti-idiotypic Ab2 antibodies.

Recently, a new cancer vaccine candidate Ab2 was derived from a neuroblastoma patient [15] treated with an anti-NeuAc-GD2 ganglioside mAb (Ab1) [16]. B cells of the patient were used to isolate Ab2 antibodies through the construction of phage libraries. Two such antibodies, able to induce anti-ganglioside antibodies in vaccinated rabbits were identified. One of them (GK8) was proposed as a potential vaccine for NeuAc-GD2-expressing tumors, due to its ability to inhibit binding of Ab1 to the ganglioside as well as binding of Ab2s from the patient's serum to the Ab1 [15].

TUMOR-SPECIFIC *N*-GLYCOLYL-SIALIC ACID-CONTAINING GANGLIOSIDES

Glycoconjugates bearing the *N*-glycolylated variant of sialic acid (NeuGc) instead of the *N*-acetylated (NeuAc) are widely distributed in most mammals, while in human normal tissues are not present [17]. The enzymatic hydroxylation of the *N*-acetyl group in the sialic acid bound to CMP is the major mechanism for the biosynthesis of *N*-glycolyl-sialic acid [18]. In humans, the gene coding for the CMP-NeuAc hydroxylase has a deletion that renders it inactive [19-21]. However, a number of studies have reported the presence of these glycoconjugates as tumor-associated antigens in several human malignant diseases [22-24]. It has recently been reported the expression of small amounts of *N*-glycolyl-sialic acid in some human normal tissues that were attributed to dietary sources [25, 26]. The incorporation of the NeuGc residues can occur selectively in human tumor cells adapted to hypoxia, through an increased activity of a specific cellular transporter for sialic acid [27]. The NeuGc moiety is found for instance associated to GM3 forming NeuGc-GM3 ganglioside. Interestingly, the importance of NeuGc-GM3 in both, tumor-induced immunosuppresion and tumor development has recently been demonstrated [28-30].

P3 is a mAb specific for the NeuGc group, generated by immunizing mice with NeuGc-GM3 [31]. However, P3 can bind to different NeuGc-containing gangliosides [31, 32]. Several monoclonal Ab2s against P3 have been reported [33, 34]. The most studied one is 1E10 [33], which is the only anti-idiotypic mAb currently in clinical trials able to mimic NeuGc-gangliosides [35]. Interestingly, the ability of murine 1E10 to induce Ab1' antibodies (i.e.: able to bind to NeuGc) was demonstrated to be dependent on the nature of the antigen in the animal species tested. In other words, vaccination with 1E10 induces Ab3 antibodies able to bind NeuGc-gangliosides only in those species where these glycoconjugates are non-self

antigens, i.e., chickens and humans, whereas in mice and monkeys, which express NeuGc-gangliosides in their normal tissues, it induces antibodies that do not recognize these antigens [6, 33], suggesting a strong operating mechanism of tolerance.

The generation of both IgM and IgG antibodies against NeuGc-GM3, but not against its *N*-acetylated variant (NeuAc-GM3), through a vaccine consisting of mAb 1E10 in alum was originally demonstrated in patients with melanoma [36] and breast cancer [37], and more recently in phase I clinical trials of patients with breast cancer [38], SCLC [39] and non-small cell lung cancer (NSCLC) [40]. The existence of such antibodies has been confirmed by several methods, including ELISA, cytofluorimetry and high-performance thin layer chromatography [36-40].

1E10 mAb does not need adjuvants other than alum to induce Ab3 responses, including Ab1', in most of the vaccinated patients from the different trials mentioned above. The antibody response against the idiotype of 1E10 was significantly higher than the response against the isotype (i.e: human anti-mouse IgG) [36, 37, 40], with the obvious advantage of increasing the generation of the desired ganglioside-binding antibodies while diminishing the adverse effects often associated with the human anti-mouse isotype response. In addition, immunization with 1E10 induces, both in chickens and humans a subset of antibodies unable to recognize 1E10, yet with reactivity against NeuGc-GM3, which could be the result of the induction of anti-idiotypic cascades [6, 36, 37, 40].

Upon immunization with 1E10, in addition to the antibody response, a cellular specific response was demonstrated in some vaccinated breast cancer patients. In fact, interferon gamma (IFNγ)-secreting cells were detected when pheripheral blood mononuclear cells (PBMC) from such patients were incubated *in vitro* with dendritic cells pulsed with liposomes containing NeuGc-GM3 [38]. Moreover, antibodies from 1E10 vaccinated patients were able to induce complement-independent cell death in a NeuGc-GM3-expressing murine tumor cell line [40]. Interestingly, another mAb specific only for NeuGc-GM3, named 14F7 [41], exhibits a cytotoxic activity also independent of complement [42, 43]. The mechanism of cell death is different from apoptosis, dependent on the ganglioside and involves loss of membrane integrity [43].

The clinical outcome of the 1E10 vaccination trials on SCLC [39] and on stage IIIb/IV patients with NSCLC [44] showed a trend towards prolonged survival of vaccinated patients compared with historical controls. Even more encouraging, the development by NSCLC patients of IgM and/or IgG antibodies against NeuGc-GM3 was positively correlated with longer survival [40]. Phase II trials with the 1E10 vaccine in breast cancer, SCLC and NSCLC patients are currently ongoing to better define the clinical impact associated with the immunological response.

B CELL LYMPHOMAS: THE "MODEL" DISEASE FOR ANTI-IDIOTYPIC THERAPY

The idiotype of the membrane-bound immunoglobulin expressed on the surface of a cancer B cell is perhaps the only true tumor-specific antigen. The clonality of the events that lead to the malignant transformation of a B cell is a major challenge to immunotherapy and at the same time, a unique opportunity for the design of personalized vaccines. Indeed, the idiotype of the immunoglobulin expressed by the malignant cells is unique among all B cells and therefore can be used as an antigen to induce an anti-idiotype specific immune response. While the complete immunoglobulin of the lymphoma has been used as antigen, the idiotype is frequently engineered into the scFv format, which contains only the two variable regions linked through a flexible peptide linker (Fig. **2a**). In contrast to antigen-mimicking, the anti-idiotypic antibodies induced in this case, directly recognize the tumor antigen (i.e., the idiotype of the immunoglobulin) and mediate effector functions.

However, an important obstacle for achieving an effective immune response against this antigen is represented by the need to break the immunological tolerance against the self idiotype. In fact, despite the clonality and uniqueness of the sequences encoding the two variable regions that form the idiotype, most of the variable regions are constituted by highly conserved sequences (the so-called frameworks). Variable regions are the consequence of gene rearrangements in the bone marrow B cell precursors and of somatic hypermutations in activated mature B cells. Most amino acid differences between variable regions of different antibodies however, are found in the loops making contact with antigen (complementary-determining regions: CDRs), while the remaining constitute the more conserved frameworks regions (Fig. **2b**). Several strategies have been tested to achieve breakdown of tolerance, from the use of conventional immunostimulatory compounds, to vaccines based on dendritic cells or DNA [45-48].

As an alternative to constructing and expressing the idiotypic antigens for individual vaccines, which can be laborious and time-consuming, a vaccine formulation consisting in an extract of membrane proteins from tumor cells incorporated into proteoliposomes together with interleukin-2 (IL-2) has recently been proposed. This preparation was effective in inducing antitumor responses in mice at low doses of antigen [49]. In a pilot clinical trial on follicular lymphoma, tumor-specific responses were observed in some patients with advanced disease, although tumor progression was not affected. However, anti-idiotypic antibodies were not detected and an idiotypic-specific T cell response was demonstrated only in one out of four evaluated patients [50].

An advantage for the treatment of B cell lymphomas *via* induction of anti-idiotypic antibodies is represented by the fact that the expression and signaling of the membrane bound immunoglobulin constituting the B cell receptor (BCR), is critical for cell survival and proliferation [51]. Although idiotypic variants can arise due to genetic instability of tumor cells, patients vaccinated with their original own tumor-specific idiotype can develop an effective polyclonal response despite the heterogeneity of lymphoma cells [52]. In fact, in a follow-up study of average 100 months after vaccination, most of the patients that received their own tumor idiotypic protein (i.e: the secretory version of the membrane-associated immunoglobulin of the lymphoma) coupled to the carrier protein keyhole limpet haemocyanin (KLH) were in clinical remission, and some of them also in molecular remission [53]. Besides the anti-idiotypic antibody response, the clinical outcome depends on the Fc receptor genotype [54, 55] while chemotherapy prior to vaccination has been shown to be irrelevant [55]. Patients homozygous

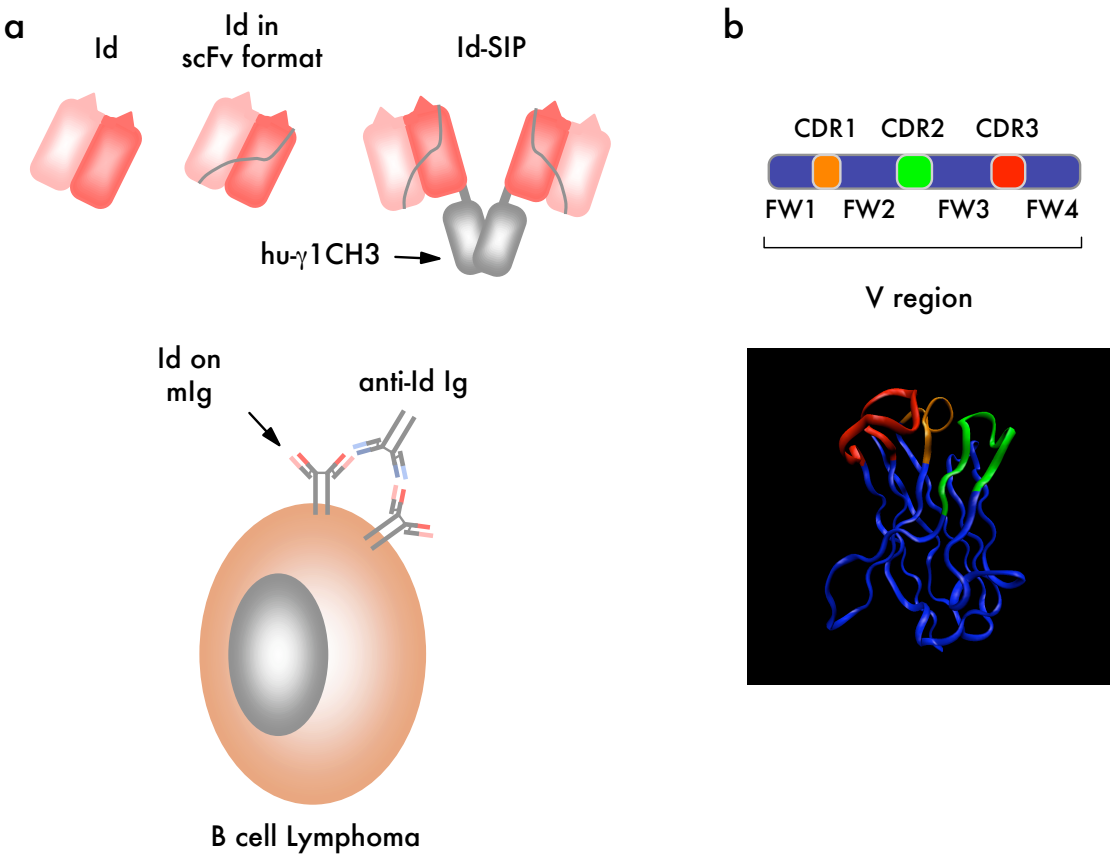

Fig. (2). (**a**) The idiotype (Id) and the scFv format in which the two variable regions are fused through a flexible peptide. A CH3 domain from a human IgG1 (hu-γ1CH3) was used as a T cell epitope carrier to render immunogenic the Id of a murine B cell lymphoma. SIP: small immune protein. (**b**) Representation of a variable (V) region showing the location of CDRs (orange, green and red) in the linear primary sequence and in the folded domain. All four framework regions (FW) are shown in blue.

for a particular allele of FcγRIIIa bearing a valine residue at position 158 showed longer progression free survival [54, 55]. The polymorphism of Fcγ receptors (FcγRs) can affect their affinity for the Fc of the antibodies, thus enhancing the efficiency of the antibody-dependent cellular cytotoxicity (ADCC) [56, 57] or the FcγR-mediated antigen delivery and activation of dendritic cells [58, 59]. Surprisingly, the anti-tumor effect of anti-idiotypic antibodies directed against cell surface-associated idiotypes has been also verified on plasmacytoma cells, which do not express membrane-bound immunoglobulins. Probably, a small amount of secreted antibodies retained in a non-specific way on the cell surface, or alternatively a minimal expression of membrane-bound immunoglobulin, is enough for tumor growth impairment by anti-idiotypic antibodies [60]. Additionally, vaccination with the patient's own tumor idiotype can induce not only humoral but also cellular responses [52]. Current works in B cell lymphoma immunotherapy include a vaccine consisting in the unique idiotype from the patient chemically conjugated to KLH and co-administered with granulocyte-macrophage colony-stimulating factor (GM-CSF) [61]. At present, this vaccine is being tested in a phase III clinical trial in follicular B cell non-Hodgkin's lymphoma [62].

There is, however, a current polemic about the role of cytotoxic T cells in B cell lymphoma progression [63, 64]. Some reports correlate the presence and activity of cytotoxic T cells with poor disease prognosis [65-67], while others state that infiltrates of both dendritic and T cells correspond to a favorable prognosis [68]. In the case of helper T cells, a recent report showed that infrequent Th2 cells specific for rare idiotopes could promote B cell lymphoma development, probably through the continuous induction of proliferation of idiotope-presenting B cell, thus augmenting the probability of oncogenic transformation [69].

GENETIC VACCINATION FOR B CELL LYMPHOMA TREATMENT

DNA vaccines are based on the administration of the gene encoding the antigen rather than the antigen itself. Antigen-specific antibodies and T cell responses can be induced by delivering the DNA in a number of ways promoting the expression of antigen in different cell types. An important aspect is constituted by the presence of immunostimulatory nucleotide sequences of bacterial origin [70, 71], which can induce inflammatory responses through activation of dendritic cells *via* Toll-like receptor 9 [72, 73]. We have previously demonstrated [74] that gene gun immunization is effective in inducing anti-idiotypic antibodies in the syngeneic model against a non-autoimmunogenic idiotype delivered as scFv fused to the murine γ1CH3 domain, which upon expression dimerizes as a SIP (small immune protein) (Fig. **2a**) [75]. The same DNA construct, however, when delivered by infection with a recombinant adeno-associated virus (rAAV, produced in mammalian cells), failed to generate a humoral

response against the idiotype [74]. In spite of this, a clear boosting effect on the anti-idiotypic antibody response and tumor rejection upon challenge was recently described for a rAAV encoding a lymphoma scFv, when administered after an initial priming with the same DNA construct by gene gun delivery, although in this case the scFv was fused to the xenogeneic human γ1CH3 domain as a carrier of T helper epitopes required to break tolerance [76]. DNA constructs for immunization can also include immunostimulatory molecules. A DNA vaccine consisting in the coinjection of two different plasmids encoding one, the tumor idiotype and the other the heat shock protein 70 from *Mycobacterium tuberculosis* was effective in prolonging survival of mice challenged with the 38C13 B cell lymphoma. However, no differences were observed with a construct in which the idiotype (in a scFv format) was fused to the GM-CSF sequence or even when the plasmid containing only the idiotype was used. Surprisingly, although all three groups of mice developed anti-idiotypic IgG2b antibodies, only in the two latter cases IgG1 and IgG2a antibodies were detected [77].

We have used DNA vaccination strategies in the murine BCL1 B cell lymphoma model [78]. Our results demonstrated that anti-idiotypic antibodies were responsible for protection against tumor challenge. In fact, vaccination with hybrid constructs consisting in the BCL1 VH or VL combined with irrelevant VL and VH, respectively, did not induce antibodies against the tumor-derived VL/VH combined idiotype [79, 80], and did not prevent tumor development, even when both hybrid constructs were co-administered. Moreover, *in vitro* the anti-idiotypic antibodies induced apoptosis and cell cycle arrest in idiotype-expressing cells, although they were not completely eradicated even in long-lasting surviving mice [80].

RECENT DEVELOPMENTS IN TUMOR PROTEIN ANTIGEN MIMICKRY WITH ANTI-IDIOTYPIC ANTIBODIES

The antigen-mimicking approach through the use of anti-idiotypic antibodies for tumor therapy is far to be discarded, despite some unsuccessful results. Several types of cancers are nowadays being treated with anti-idiotypic antibodies mimicking protein antigens.

A vaccine consisting in an anti-idiotypic antibody (105AD7) able to mimic a glycoprotein (CD55) overexpressed in colorectal cancer (CRC) cells induced cellular responses in patients against both the antigen and the Ab2. These responses were measured through T cell assays (IFNγ-ELISPOT and proliferation assay) and cytokine (tumor necrosis factor alpha, TNFα and GM-CSF) determination (Luminex). Interestingly, there was no correlation between the IFNγ-secreting cell numbers and the proliferation assays, indicating probably the measurements of different T cell populations [81]. This antibody has also been tested in patients with osteosarcoma [82]. As in the previous CRC trial [81], T cell responses against both the antibody and the antigen were generated. A positive correlation between GM-CSF and TNFα secretion (Luminex), but not between these molecules and IFNγ (ELISPOT), was observed [82].

The induction of antigen-specific T cell responses was also demonstrated in another study on CRC patients vaccinated with either a human Ab2 mimicking a tumor-associated glycoprotein (Ep-CAM; higher expression in malignant cells from epithelial origin), the antigen alone or a combination of both. It was found however that the two latter immunogens were more effective than the antibody alone in inducing a cellular response [83].

Anti-idiotypic vaccines targeting different antigens co-expressed in certain tumors have also been combined. A recent phase II clinical trial with anti-carcinoembryonic antigen (CEA) and anti-human milk fat globule (HMFG) anti-idiotypic antibodies (CeaVac and TriAb, respectively) after resection of CRC hepatic metastasis, failed to improve recurrence-free survival in comparison with surgery alone [84].

The association of the Her family of tyrosine kinase receptors with tumors has been extensively described. A humanized mAb targeting Her-2 extracellular domain, called Trastuzumab (Herceptin) [85], is one of the main current approaches for immunotherapy of Her-2-expressing tumors. An anti-idiotypic antibody (6D12) [86] against 4D5, the murine version of Trastuzumab [87], mimics a specific epitope of this receptor [86]. 6D12 induces in mice anti-anti-idiotypic antibodies (Ab3) able to inhibit *in vitro* proliferation of Her-2-positive tumor cells, and to exert ADCC [86, 88] and protection against challenge with these cells [88].

BEYOND MIMICKRY AND TUMOR IDIOTYPE BINDING

Anti-idiotypic antibodies have been implicated in several immune conditions, including sperm immunity in women [89]. However, although Jerne proposed anti-idiotypic interactions as a mechanism for immune response and memory regulation, still little is understood about how anti-idiotypic antibodies can modulate the immune system.

Intravenous immunoglobulins (IVIg) have been used for a wide range of immune-associated pathologies, including autoimmune diseases. A putative mechanism of action is the neutralization of pathologic autoantibodies by anti-idiotypic antibodies [90, 91]. According to Jerne's theory, self idiotypes can elicit a natural antibody response. In fact, it was recently demonstrated that antibodies against the self antigen glutamate decarboxylase 65 were present in patients with type 1 diabetes and in their first-degree relatives, as well as in healthy individuals in general. However, only patients with type 1 diabetes lacked a bound "inhibitor" that was present in the other two groups. The inhibitor was identified as anti-idiotypic antibodies [92]. Previously, it had been shown that when injected in non-obese diabetic (NOD) mice, a human anti-glutamate decarboxylase 65 antibody induced an anti-idiotypic antibody response able to block the antigen-antibody interaction, delaying the onset of the disease [93]. We have recently proposed that the existence in normal individuals of autoantibodies exhibiting high idiotypic immunogenicity could precisely be a mechanism of regulation of self-reactive antibodies [94]. The molecular basis for polyreactive anti-idiotypic antibodies with immunoglobulin cross-reactive activity have recently been postulated [95].

CONCLUDING REMARK

Although the existence of natural or induced idiotypic networks is still a matter of debate, anti-idiotypic antibodies continue to be a very interesting tool for the treatment of malignant or autoimmune diseases. One major technological

issue is augmenting the immunogenicity of anti-idiotypic antibody-based vaccines, if mimickry of the antigen or the anti-tumor idiotype response are the desired goals. Additionally, understanding how idiotypic interactions may play a role in the regulation of the immune system, would bring the possibility of manipulating the immune actors to achieve effective and long-lasting immunological responses or modulating immune-related pathological conditions.

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